

## Genetics

**G001. Genomic DNA Reference Panels for HLA Class I and II Loci: A GeT-RM Collaborative Project**

M.P. Bettinotti<sup>1</sup>, D. Ferriola<sup>2</sup>, J.L. Duke<sup>2</sup>, T.L. Mosbrugger<sup>2</sup>, N. Tairis<sup>2</sup>, L. Jennings<sup>3</sup>, D. Resquesens<sup>4</sup>, L.V. Kalman<sup>5</sup>, D. Monos<sup>2</sup>

<sup>1</sup>Johns Hopkins School of Medicine, Baltimore, MD; <sup>2</sup>The Children's Hospital of Philadelphia, Philadelphia, PA; <sup>3</sup>Ann and Robert H. Lurie Children's Hospital, Chicago, IL; <sup>4</sup>Coriell Institute for Medical Research, Camden, NJ; <sup>5</sup>Centers for Disease Control and Prevention, Atlanta, GA.

**Introduction:** The human Major Histocompatibility Complex (MHC) contains a large set of highly polymorphic genes located on chromosome 6 known as the human leukocyte antigen (HLA) loci. The proteins encoded by the HLA loci are responsible for displaying peptide antigens on the surface of cells and play a central role in the immune response. HLA genes and their products are divided into 2 groups, class I and class II. Molecular HLA typing is commonly used in clinical laboratories for several applications including matching patients and donors for transplantation, to stratify patients at risk of adverse drug reactions, and to assist the diagnosis of numerous autoimmune diseases. A publicly available and renewable source of well-characterized genomic DNA reference materials (RM) for molecular HLA typing is needed for assay development and validation, quality control and proficiency testing.

**Methods:** The Centers for Disease Control and Prevention's (CDC) Genetic Testing Reference Materials Coordination Program (GeT-RM) together with three clinical laboratories and the Coriell Institute, characterized genomic DNA from 108 cell lines from the National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPA1, and DPB1. DNA was isolated by Coriell and distributed to the 3 laboratories. Methodologies used were: PCR-SSO - LABType SSO (One Lambda, Canoga Park, CA), SSP - Olerup SSP (Stockholm, Sweden), SBT - AlleleSEQR HLA-SBT (Abbott Molecular, Des Plaines, IL) and NGS - HoloType HLA kits (Omixon Inc., Budapest, Hungary). Each of the 11 genes was tested by 2 or 3 methods. Consensus genotype was the NGS result, which provided the highest level of resolution, and was consistent with the results obtained by the parallel testing using other methods. **Results:** HLA typing results using SBT, SSP, SSO and NGS were 100% concordant. Five unique novel alleles with differences in exonic regions were identified and three were submitted to the World Health Organization (WHO) Nomenclature Committee. Thirty HLA-A, 54 -B, 30 -C, 36 -DRB1, 4 -DRB3, 4 -DRB4, 3 -DRB5, 19 -DQA1, 17 -DQB1, 10 -DPA1 and 29 -DPB1 alleles are represented in the panel. These alleles cover a high percentage of HLA specificities present in the USA population. Panels representing the most frequent alleles in five ancestry groups in the USA population have been selected and will be available through the Coriell Institute.

**Conclusions:** These characterized genomic DNA samples and the originating cell lines are publicly available from the NIGMS Repository at the Coriell Institute, and constitute a valuable resource to ensure the quality of HLA testing.

**G002. Frequency and Diagnostic Yield of Mosaic Variation Identified by Whole Exome Sequencing**

C.R. Miller<sup>1,2</sup>, E. Zmuda<sup>1</sup>, D.J. Cosmeier<sup>1</sup>, M. Fisher<sup>1</sup>, V. Magrini<sup>1</sup>, S.C. Reshmi<sup>1</sup>, J.M. Gastier-Foster<sup>1</sup>, C.E. Cottrell<sup>1</sup>

<sup>1</sup>Nationwide Children's Hospital, Columbus, OH; <sup>2</sup>The Ohio State University, Columbus, OH.

**Introduction:** Massively parallel sequencing, including whole exome sequencing (WES), has enhanced our ability to appreciate the contribution of somatic mosaicism in genetic disorders. Previous studies have found mosaicism in ~1% of pathogenic WES results (Yang, *et al.*, NEJM 2013; Retterer, *et al.*, Genet Med 2016). We reviewed 174 clinical WES cases submitted to our institution during 2017 to determine the frequency of mosaicism in variants likely causal for patient phenotype. **Methods:** DNA derived from peripheral blood was subject to target capture using Agilent SureSelect Human all Exon V6 followed by sequencing to 130x mean depth using Illumina instrumentation. Reads were aligned to the reference (GRCh37) with variants called by GenomeNext v1.1. GeneInsight

(Sunquest) was used for annotation and tertiary analysis filtering based on clinician-provided phenotypes. Variants were assessed according to ACMG/AMP consensus recommendations. Mosaic variants were confirmed by orthogonal methods. **Results:** We identified 5 variants with mosaicism. *De novo* causal mosaic variants were found in 3 probands, and 2 mosaic variants of uncertain significance were identified in parental samples due to non-mosaic heterozygous calls in the respective probands. Notably, 3 of the 5 variants have been reported in various cancers; however, the significance of mosaic variants to cancer predisposition in these individuals is unclear. Mosaicism represented 6.7% (3/45) of likely causal variants detected in our WES. A pathogenic variant at c.2879-1G>A in *ARID1A* with a variant allele frequency (VAF) of 19% was found in a child with global developmental delay, dysmorphic features, and agenesis of the corpus callosum. Loss of function mosaic variation in *ARID1A* is associated with Coffin-Siris syndrome. An X-linked likely pathogenic variant in *ARX*, c.1444G>A p.(Gly482Ser), was seen at 12% VAF in a male with early infantile encephalopathy. Finally, a pathogenic *TRIP12* c.1684C>T p.(Arg562Ter) variant with a 12% VAF was seen in a child with global developmental delay, seizures, hypotonia and dysmorphic features. Recently, *TRIP12* variants have been reported in patients with similar phenotypes. To our knowledge, this is the first report of a mosaic *TRIP12* variant. **Conclusions:** Mosaicism is an important contributor to genetic disease. By WES we detected 5 mosaic variants during analysis, including 3 causal alterations. Given adequate read depth, and appropriate bioinformatics processing parameters, WES can detect low VAFs. However, detection remains challenging due to issues such as false positive sequencing/alignment artifacts, false negatives due to differences in variant-calling algorithms, limitations in read depths, and the need to analyze disease-involved tissue.

**G003. WITHDRAWN****G004. Performance Evaluation of VCU Health NIPT, a Single-Nucleotide-Polymorphism (SNP) Based Non-invasive Prenatal Testing for Common Aneuploidy**

C. Yang, M.F. Sabato, V. Williamson, P. Morris, X. Yu, M. Chhoa, M. Sene, L.V. Nicely, S. Reihert, R.M. Lewandowsky, R.M. Ramus, A. Ferreira-Gonzalez

Virginia Commonwealth University Health System, Richmond, VA.

**Introduction:** Prenatal screening for chromosomal aneuploidy has made great progress with the effective clinical application of non-invasive prenatal testing (NIPT), which leads to less unnecessary invasive confirmatory procedures in both high-risk and low-risk populations due to NIPT's unprecedentedly high analytic sensitivity and specificity. In comparison to the whole genome counting methodology, SNP-based NIPT provides unique capability of identifying fetal triploidy and differentiating sex chromosomal aneuploidy between maternal and fetal origin. Here, we present the validation of VCU Health NIPT for common aneuploidy screening. **Methods:** We designed, developed, and validated a SNP-based NIPT to identify aneuploidy in chromosomes 13, 18, 21; and sex chromosomes. A total of 157 samples (44 blood samples from de-identified pregnant volunteers, 5 high-risk blood samples from a commercial resource, 3 synthetic control DNA samples from SeraCare, and 105 whole genome libraries from Natera's previously processed samples) were utilized in the validation. Plasma cell-free DNA (cfDNA) extracted from stabilized maternal blood was used to generate whole genome library, which was further amplified to target >13,000 SNPs sites on chromosomes 13, 18, 21, X and partially Y. The targeted libraries were barcoded and sequenced on Illumina NextSeq. Cloud-based Natera's Constellation software was used to facilitate the raw sequencing data analysis, SNP allele frequencies computation, and Bayesian probabilities calculation against copy number hypotheses. **Results:** The sensitivity of the test was 100% (16/16) for trisomy 21, 100% (7/7) for trisomy 18, 100% (4/4) for trisomy 13, 100% (5/5) for monosomy X, and 100% (13/13) for sex chromosome trisomy (4 XXX, 4 XXY, 5 XYY), based on the NIPT results from Natera Panorama test (39 samples), invasive diagnostic procedures (4 commercial samples), and synthetic design (2 SeraCare trisomy 21 samples). The "low risk" reports were 100% (103/103) concordant with expected results. There were a total of 6 samples with "No Call" results which provided a No Call rate of 3.8%, comparable to

Natera Panorama stated data. The lowest fetal cfDNA generating "low risk" report was 3.8%, and the lowest fetal cfDNA generating "high risk" report was 4.2%. All fetal fractions were called with highly correlated frequencies ( $r=0.995$ ) between VCU Health NIPT and Natera Panorama test. We obtained a 100% concordance for fetal sex.

**Conclusions:** Our validation data support the successful development of an assay having a high degree of reproducibility, and excellent concordance with both NIPT performed at Natera and known clinical outcomes.

#### G005. Development of a Clinical CD33 Genotyping Assay to Predict Response to Gemtuzumab

B.A. Barkoh, R. Kanagal-Shamanna, M. Konopleva, L. Medeiros, R. Luthra, A.E. Quesada, K.P. Patel

The University of Texas M.D. Anderson Cancer Center, Houston, TX.

**Introduction:** A single-nucleotide polymorphism (SNP), rs12459419 (c.41C>T; p.Ala14Val), in the transmembrane glycoprotein CD33 leads to alternative splicing and reduced expression of full length CD33. Detection of CD33 isoforms without the V-set domain is clinically important because this domain contains the immune-dominant epitope(s) recognized by most CD33 antibodies including gemtuzumab ozogamicin (GO). The CC genotype with full length protein appears to have a substantial response to GO. In comparison, CT or TT genotypes are associated with a suboptimal response to GO. We describe the validation of a CD33 SNP assay for the selection of patients with a higher likelihood to respond to GO. **Methods:** Real-time polymerase chain reaction analysis was performed on DNA to genotype rs12459419 (dbSNP) of the CD33 gene using the QuantStudio 7 Flex Fast Sequence Detection System (PE Applied Biosystems). The context sequence is AGGGG[C/T]CCTGG. The C and T alleles were discriminated using fluorescently labeled [VIC/FAM] allele specific oligomer (ASO) probes corresponding to [C/T], respectively (ThermoFisher). M13-tagged primers were designed to confirm ASO-PCR findings using traditional Sanger sequencing. We tested 102 unique samples by ASO-PCR; 93 patient samples and 9 cell lines (A375, H1650, H2122, HBL1, HL60, K1213 positive control, RET positive control and TIB180). The 93 patient samples included 53 bone marrow aspirates, 32 peripheral blood, 2 saliva, 2 buccal swabs and 3 from stock DNA used for controls. The H1650 and SW480 cell lines were derived from formalin-fixed, paraffin-embedded (FFPE) tissue. Of the total samples, we tested 26 by both ASO-PCR and Sanger sequencing. **Results:** ASO-PCR successfully allowed allelic calls for nearly all samples (99%) at 0.5ng or higher input. Of the 102 unique patient samples tested thus far, 51 (50%) were homozygous C/C, 36 (35%) were heterozygous C/T and 11 (11%) homozygous T/T by ASO-PCR. Thus far, confirmation with Sanger showed testing by ASO-PCR correctly identified genotype accurately in 25/26 (96%) samples. One sample (4%), the A375 cell line, was called homozygous T/T by ASO-PCR, but, was determined to be C/T by Sanger sequencing. **Conclusions:** We achieved a 96% concordance between ASO-PCR and Sanger sequencing for identification of the CD33 polymorphism rs12459419. The erroneous call for A375 cell line is likely due to a lack of a confirmed T/T control as reference for analysis, which is uncommon in the general population. Overall, this ASO-PCR assay was able to achieve a high concordance with Sanger sequencing over a broad spectrum of sample types and therefore may be helpful in the identification of possible candidates for GO targeted therapy.

#### G006. Characterization of Beta Hemoglobinopathy Results in a Large Population Referred for Carrier Testing

C.A. Holland, A. Kruger, J.P. Tazelaar, J. Zdrodowski, P.H. Fernandes Progenity, Inc., Ann Arbor, MI.

**Introduction:** Beta-Hemoglobinopathies are a group of hereditary blood disorders characterized by anomalies in the synthesis of the beta chains, resulting in beta thalassemia and/or abnormal structural hemoglobins. Transmission is mostly autosomal recessive with phenotypes ranging from severe anemia to clinically asymptomatic individuals. Beta thalassemia is one of the most common recessive disorders in the world with the incidence of symptomatic cases being approximately 1 in 100,000 individuals in the general population. **Methods:** We performed a retrospective analysis of 73,929 patients referred for carrier testing for  $\beta$ -globin (HBB) disorders. Testing included next generation sequencing

(NGS)-based analysis of 21 common beta-thalassemia mutations and mutations causing hemoglobins C, D, E, O and S. **Results:** Of the >73,000 patients, 1,471 (2.0%) were positive for one or more HBB mutation. Among those that were positive, 1,443 (98.7%) were heterozygotes for one mutation, 19 (1.3%) were compound heterozygotes, and 9 (0.6%) were homozygotes. Compound heterozygotes included 13 patients with both HbC and HbS, 4 with  $\beta$ -thalassemia and HbS, one with  $\beta$ -thalassemia and HbC, and one with HbD and HbE. Homozygosity was found for HbS in 4 patients and HbE in 5 patients. The African American population had the highest number of positive patients (12%), out of which, 8.6% were heterozygotes for HbS. Among the other ethnic populations, the highest positivity rates followed the order of Other/Mixed (2.0%), Asian (1.9%), Hispanic (1.0%), Native American (0.8%), Caucasian (0.3%) and Ashkenazi Jewish (0.2%). Among all the  $\beta$ -globin disorders, HbS accounted for the highest number of positives at 62.3% followed by HbC (16.5%),  $\beta$ -thal (14.9%), HbE (3.7%), HbD (2.6%) and HbO (0.2%). **Conclusions:** To our knowledge, this is the largest and most ethnically diverse population studied in relation to beta hemoglobinopathies carrier status. Testing for the common  $\beta$ -globin mutations was successful in identifying heterozygotes, compound heterozygotes, and homozygotes for  $\beta$ -globin disorders at an expected rate in high risk populations based on ethnicity. While this testing has historically been offered to individuals of specific ethnicities, these results show that all ethnicities are at risk. Given an increasing number of individuals of mixed ancestry and lack of accurate knowledge regarding family history, these results show the importance of pan-ethnic testing for  $\beta$ -hemoglobinopathies.

#### G007. Pro-fibrotic Cardiac Gene Activation in Diabetic Zucker Rat Model is Directly Associated to the Incremental Visceral Adiposity: The EPACs Proteins Signaling

M.M. Corsi Romanelli<sup>1,2</sup>, E. Dozio<sup>1,2</sup>, S. Brizzola<sup>1</sup>, F. Acocella<sup>1</sup>, L. Tacchini<sup>1</sup>, E. Vianello<sup>1</sup>

<sup>1</sup>Università degli Studi di Milano "LA STATALE" Milan, Italy; <sup>2</sup>IRCCS Policlinico San Donato, Milan, Italy.

**Introduction:** Diabetic cardiomyopathy is characterized by cardiac failure due to diastolic dysfunction and cardiac structural maladaptations, arising from cardiac cellular responses to the complex toxicity induced by increasing glucose and insulin concentrations. Elevated insulin and inflammatory cytokine levels can also increase in response to incremental visceral adiposity and may chronically activate signaling pathways regulating to cardiac growth and contractility especially associated to maladaptive heart remodeling and hypertrophy. The main regulator of these events in the heart is 3', 5'-cyclic adenosine monophosphate (cAMP) and its effectors the EPACs proteins. Our aim was to examine the effect of incremental visceral adiposity on cardiac EPACs expression and fibrotic gene activation in ZDF diabetic Zucker rat model. **Methods:** By using lean (n=5) and obese (n=5) ZDF diabetic Zucker rats (age: 20 weeks) (protocol number 325/2015PR - 2015/04/05, Italian Ministry), we tested the hypothesis that in case of incremental visceral adiposity, cardiac hypertrophy may be mediated by the deregulation of cAMP/EPACs signaling, causing an up-regulation of genes involved in collagen synthesis and deposition associated to cardiac fibrosis. Heart tissues were studied in terms of EPACs tissue expression, activation/modification of specific genes (n=84) involved in collagen synthesis and deposition (RT Profiler PCR Array, PARN-120Z, QIAGEN). The following methods were used: real time RT-PCR and Western blot. **Results:** We observed in obese ZDF diabetic Zucker rat heart biopsies a lower protein production of both EPAC1 and EPAC2 than age matched lean diabetic ZDF Zucker rats. Not differences were found in EPACs gene expression. Contrarily almost all 84 pro-fibrotic genes, normally inhibited by cAMP/EPAC signaling, resulted up-regulated in obese ZDF diabetic Zucker rats than ZDF control lean rats. In particular the main regulators of cardiac fibrosis including Endothelin-1 (ET-1) and TGF- $\beta$ 1 and their associated gene including  $\alpha$ SMA, MMPs family members and angiotensin II, presented a positive fold regulation in obese ZDF diabetic Zucker rats than ZDF control lean group.

**Conclusions:** Incremental visceral adiposity can promote cardiac fibrosis in diabetic ZDF Zucker rat model through the reduction of cardiac EPAC1

and EPAC2, suggesting their use as therapeutic targets in the prevention of cardiovascular disorders associated to type 2 diabetes.

**G008. Validation of a Targeted Variant Genotyping Assay for Personalized Antihypertensive and Chronic Kidney Disease Therapy**  
W.M. Stansberry, K.S. Collins, E.B. Medeiros, M. Swart, T.C. Skaar, B.S. Decker, M.T. Eadon, V.M. Pratt

Indiana University School of Medicine, Indianapolis, IN.

**Introduction:** Hypertension and chronic kidney disease are inextricably linked. Hypertension is a well-recognized contributor to chronic kidney disease progression and, in turn, renal disease potentiates hypertension. A generalized approach to drug selection and dosage has not proven effective in reducing the incidence of these conditions, in part because patients with heterogeneous kidney disease and hypertension etiologies are frequently grouped according to functional or severity classifications. **Methods:** We selected 72 genetic variants with corresponding gene-drug based recommendations and used Coriell DNAs (Camden, NJ) from the pharmacogenetics reference material projects (Pratt *et al.*, 2010 and 2016) for the analytical validity studies. DNA was amplified by real time PCR on the ThermoFisher QuantStudio 12K Flex (software v1.2.2; Waltham, MA) and subjected to Taqman allele discrimination using ThermoFisher (Waltham, MA) reagents and software (Genotyper software, v1.3) in a custom designed open array. **Results:** The analytical sensitivity was 100% for the detection of variant alleles, with no reported false negative results. The analytical specificity was 100% for detection of non-variant alleles, with no false positive results. DNA samples were also run for intra- and inter-assay variation. In all, 14 samples were included in intra-assay validation and 18 samples were included in the inter-assay validation. The intra- (within) assay variation studies showed that all three replicates of the 14 samples ran on the same plate, were concordant with expected results. The inter- (between) assay variation studies showed that the 18 samples consistently yielded the same result across three separate runs. Since there were no known reference materials for most variants, selected DNA samples were Sanger sequenced with custom designed primers (Integrated DNA Technologies, Coralville, IA) for 100% accuracy. **Conclusions:** Genetic testing may serve as an important tool for clinicians who embrace precision medicine. Increasing scientific evidence has supported the utilization of genomic information to select efficacious antihypertensive therapy and understand familial contributors to chronic kidney disease progression. Given the wide array of antihypertensive agents available and diversity of genetic renal disease predictors, a panel-based approach to genotyping may be an efficient and economic means of establishing an individualized blood pressure response profile for patients with various forms of chronic kidney disease and hypertension.

**G009. Analysis of ARID1A Mutations and Co-occurring Variants in Cancer Biopsies Reveals Significant Associations in Multiple Diseases.**

T. Buys, E.K. Sullivan, P. Kumar, D. Tefft, E. Keeton, E. Marchlik, B. Luke, E. Haynes, K. Lalowski, S.K. Elkin  
N-of-One, Inc., Concord, MA.

**Introduction:** ARID1A encodes Baf250a, a member of the SWI/SNF chromatin remodeling complex. Inactivation of ARID1A has been reported in many cancers, and functional studies have implicated it as a tumor suppressor. In this study, we interrogated the landscape of ARID1A alterations across cancer types and explored the variants that co-occurred significantly with ARID1A variants. **Methods:** The N-of-One, Inc. proprietary database was utilized to investigate the percentage of cancer patients harboring an alteration in ARID1A. Cases were further partitioned by cancer type and variant type. Cases known to be sequenced from circulating DNA were analyzed as an independent dataset. Cancer types in which ARID1A alterations were most prevalent were analyzed to identify genes in which variants co-occur most frequently with ARID1A variants. Chi-squared analysis ( $p \leq 0.05$ ) was performed to assess the significance of these interactions. Cramer's V was calculated to assess the strength of the interactions. R statistical software was used for all statistical analyses (<https://www.r-project.org>). **Results:** Of the ~60,000 patient cases tested for ARID1A alterations, 8.9% harbored an ARID1A variant; 3.9% of cases harbored an inactivating ARID1A variant. ARID1A alterations were detectable using both circulating DNA

sources and tissue sources, with inactivating ARID1A variants detected in 3.7% and 4.8% of cases, respectively. Inactivating ARID1A variants were detected most frequently in endometrial carcinoma (15.6%), followed by urothelial/bladder carcinoma (13.4%), gastric carcinoma (11%), and hepatocellular carcinoma (9.5%). Our analysis revealed that ARID1A variants co-occurred in a statistically significant manner with variants in multiple genes, including PTEN, PIK3CA, CTNNB1, and BRCA2. **Conclusions:** Our findings have revealed several significant associations and suggest that further research into the effects of co-occurring ARID1A variants is warranted. Studies have shown that in a transgenic murine model with PIK3CA mutation and conditional ARID1A inactivation, PI3K inhibition resulted in prolonged survival, and that depletion of ARID1A in tissue culture cells and a xenograft tumor model led to enhanced sensitivity to PI3K/Akt pathway inhibition. Taken together with our data, these findings suggest that, for the significant subset of patients who harbor both types of alterations, targeting the PI3K pathway may be an effective therapeutic approach. BRCA2 alterations were also found to co-occur with ARID1A alterations. Alteration of both genes may have a synergistic effect on PARP inhibitor sensitivity. Together, these findings suggest several pathways for which targeting may be enhanced in the presence of concomitant ARID1A alteration.

**G010. Validation of an Economical, Real Time PCR Genotyping Assay for Detection of ACMG/ACOG Recommended Mutations in the CFTR Gene for General Population Screening**

S. Beqay, A. Goldman, S.R. Opie  
Universal Diagnostic Laboratory, Irvine, CA.

**Introduction:** Cystic fibrosis testing has become more complex and expensive with increasingly larger panels and/or next generation sequencing even though the American College of Medical Genetics (ACMG) and the American College of Obstetricians and Gynecologists (ACOG) recommend against comprehensive testing. Furthermore, reimbursement is decreasing and not linked to the number of variants tested or technology employed. Following Clinical Laboratory Improvement Amendments (CLIA) regulations and College of American Pathologists (CAP) guidelines, we validated a robust and inexpensive quantitative polymerase chain reaction (qPCR) assay to detect 23 ACMG/ACOG recommended CFTR mutations, reflex assays for the 5T/7T/9T alleles at the polymorphic poly(T) tract in intron 8, and other non-CF causing variants I507C, I506V and F508C. **Methods:** One hundred twenty six DNA samples were used: 96 patient samples composed of 76 blood samples and 20 buccal swabs previously genotyped in a CLIA/CAP accredited laboratory (Pathology Inc., CA), and 30 well characterized gDNAs (Coriell Institute for Medical Research Institute, NJ). All DNA samples were amplified using Taqman chemistry on a QuantStudio 12K Flex, (ThermoFisher, MA), and analyzed by referring to a mutation database which flows into automated reporting through our LIS. Importantly, each genotyping assay is internally replicated to increase call confidence. All studies were performed by two technicians on at least 5 different days over a 30-day time period and contained multiple replicates. **Results:** Consolidated data across all assays and all specimen types demonstrated: true positives, 968; true negatives, 20,054; false positives, 5; false negatives, 0; total 21,027. Quality metrics were calculated: accuracy, 99.98%; analytical specificity, 99.98%; analytical sensitivity, 100%; positive predictive value, 99.49%; negative predictive value, 100%; inter-assay precision, 100%; intra-assay precision, 100%, analytical limit of detection, 1ng/uL. I507C, I506V, and F508C were shown not to interfere with assay performance and are always included for all samples and results reported as a reflex to  $\Delta$ F508 mutation. **Conclusions:** These results validate for clinical use a rapid, laboratory developed, qPCR test for 23 CFTR mutations in one reaction that provides high sensitivity and specificity. This assay follows recommendations for general population screening by ACMG and ACOG, is validated for both blood and buccal swab specimens, with significantly less expense compared to commercially available tests.



### G011. Cre Recombinase-mediated Circularization for Custom Mate Pair Library Preparation

E. Zimmerman Zuckerman, J.H. Blommel, S.H. Johnson, G. Vasmatzis, M.J. Ferber, L.B. Baughn  
Mayo Clinic, Rochester, MN.

**Introduction:** Mate Pair Sequencing (MPSeq) is used to characterize chromosomal rearrangements in order to identify clinically significant genes at breakpoints that may help determine diagnosis, prognosis, and in some cases, therapeutic options. The Illumina Nextera Mate Pair library preparation (NXT-LP) protocol takes two days to complete, primarily due to a 12-16 hour circularization incubation. Establishment of a one day protocol would allow for faster turn-around time of results. **Methods:** To improve NXT-LP, we replaced the Nextera tagmentation reaction with Covaris G-Tube fragmentation and KAPA HTP library prep using biotinylated LoxP adapters. We also replaced the Nextera Circularization Ligase and its 12-16-hour incubation with a 50-minute Cre recombinase incubation. Linear DNA was digested with Plasmid-Safe ATP-Dependent DNase (Lucigen) and Exonuclease I (NEB). The rest of the protocol resembles post-circularization NXT-LP with these changes: washes were done with only resuspension buffer and 12 cycles of PCR were run with KAPA HotStart ReadyMix. Qubit quantitation was done after strand displacement (SD), shearing, capture, and on final libraries to assess DNA yield. Fragment sizes of post-SD and final libraries were determined by an Agilent Bioanalyzer. Illumina HiSeq Rapid 2x101bp sequencing was run on the final libraries. Data analysis focused on quality metrics including Mate Pair to Paired End (MP/PE) ratio, replication rate, and bridged coverage. **Results:** Our custom protocol yielded post-SD libraries similar to those made by NXT-LP (300-700ng/6-7kbp, 300-800ng/5.5-6.5kbp). Samples processed through circularization with the Nextera protocol and then captured and processed with the custom protocol yielded final libraries comparable to NXT-LP libraries (17-23ng/ $\mu$ L/0.9kbp, 4-25ng/ $\mu$ L/0.8-1.1kbp). Using positive and negative LoxP controls, we found that Cre circularization is 20-30% efficient. The custom protocol yielded final libraries containing 1-2 ng/ $\mu$ L of DNA between 1 and 1.2kbp in size, which is less DNA than we usually see with NXT-LP (4-25 ng/ $\mu$ L, 0.8-1.1kbp). Sequencing results compared to NXT-LP results showed high replication rate (27-63% vs <10%), low MP/PE ratio (0.04 vs >2.5), and low bridged coverage (3x vs >30x). **Conclusions:** Our protocol succeeded in generating mate pair libraries, though they were of lower quality than NXT-LP libraries. This can be attributed to low circularization efficiency. We found that only 12.5% of post-SD material is circularized, compared to ~40% in NXT-LP, due to the combined inefficiencies of ligation and Cre. Groups aiming to use Cre-Lox for MPSeq should be aware of this limitation. Regardless, these results indicate that a one day protocol is theoretically possible, but will take more optimization.

### G012. Clinical Laboratory Experience with Different Carrier Screen Panels

H. Zhu, J. Teicher, N. Leach, L.S. Rosenblum, Z. Zhou, J. Rafalko, B. Dyr, R. Heim  
Integrated Genetics, Westborough, MA.

**Introduction:** Carrier screening is typically performed in the preconception or prenatal period to assist couples in making informed reproductive decisions. A next generation sequencing 141-gene carrier screen panel developed in our clinical laboratory includes disorders that meet eligibility criteria suggested for expanded carrier screens by the American College of Medical Genetics and Genomics (ACMG)/American College of Obstetricians and Gynecologists (ACOG). A subset of the 141 genes is included in a 38-gene panel relevant to the Ashkenazi Jewish (AJ) population, and a smaller subset is included in a 12-gene panel of conditions for which ACMG/ACOG have made specific recommendations. To provide a snapshot of the uptake of these panels, a retrospective pilot analysis of a cohort of patients tested consecutively will be presented. **Methods:** Data from 6,186 consecutive patients were analyzed. Carrier detection (positive) rates were calculated as the percentage of individuals heterozygous for a pathogenic or likely pathogenic variant in at least one gene, of the total number of individuals tested. Uptake and carrier detection were assessed based on self-identified patient ethnicity when possible, although ethnic information was not received for 69% of patients. **Results:** Fifty-one percent of patients

were received for the 141-gene panel, 8% for the 38-gene AJ panel, and 41% for the 12-gene panel. Overall positive rates were 36%, 33%, and 10%, respectively. The majority of African American patients were received for the 141-gene panel (63%), followed by the 12-gene panel (34%), with positive rates of 24% for both panels. Most Ashkenazi Jewish patients were received for the 141-gene panel (54%) and the AJ panel (45%), with positive rates of 63% and 48%, respectively. 89% of Asian patients were received for the 141-gene panel, with a positive rate of 22%. Most Hispanic patients were received for the 141-gene panel (64%) and 12-gene panel (34%), with positive rates of 26% and 6%, respectively. Caucasian patients were received for the 141-gene panel (77%) and for the AJ panel (19%), with positive rates of 41% and 25%, respectively. **Conclusions:** Although individually rare, the recessive conditions included in the 141-gene panel are collectively common, shown by a 36% carrier detection rate. This supports the clinical utility of an expanded carrier screen panel, as improved carrier detection allows for more informed reproductive decision making. These data suggest that a patient's ethnicity may influence the decisions patients and providers make regarding choice of panel. A limitation of this analysis is the unknown influence of insurance coverage on these decisions. These data may be useful both to laboratories constructing panels and providers ordering panels.

### G013. Rapid Molecular Haplotyping of Thiopurine Methyltransferase \*3A, \*3B, and \*3C

M. Leong<sup>1</sup>, P. Huan<sup>1</sup>, H. Lee<sup>1</sup>, L. Chiu<sup>1</sup>, T. Loh<sup>1</sup>, E. Koay<sup>1,2</sup>, S. Vasoo<sup>1,2</sup>, B. Yan<sup>1</sup>, K. Tan<sup>1</sup>, C. Lee<sup>1</sup>

<sup>1</sup>National University Health System, Singapore; <sup>2</sup>National University of Singapore, Singapore.

**Introduction:** Thiopurine methyltransferase (TPMT) genotyping is an advancement in the practice of precision medicine. According to the Clinical Pharmacogenomics Implementation Consortium guidelines, in patients with an intermediate metabolizer TPMT genotype, the initial dose of azathioprine (AZA) or 6-mercaptopurine (6-MP) should be reduced by 30-70%, however, in patients with poor metabolizer TPMT genotype, the initial dose of AZA or 6-MP should be reduced by 10-fold and the dosing frequency extended from daily to three times weekly, or an alternative drug should be selected. Conventional assays, i.e., Sanger sequencing and polymerase chain reaction (PCR), cannot discriminate between \*1/\*3A (intermediate metabolizer) and \*3B/\*3C (poor metabolizer), owing to the inability to conduct linkage analysis between rs1800460 and rs1142345 which are >8 kilo base pairs (kbp) apart in the genome. To overcome this limitation, transcript sequencing was performed using Illumina short-read paired-end high-throughput sequencing (SR-PE-HTS) to phase rs1800460 and rs1142345 which are <300 bp apart in the TPMT messenger RNA. **Methods:** The SR-PE-HTS was performed on the MiSeq instrument for a patient with inconclusive TPMT status (\*1/\*3A or \*3B/\*3C) when analyzed using a Sanger sequencing method. After sequencing, the FASTQ reads were processed by an in-house computational pipeline on an Ubuntu Linux machine to extract PE reads that covered rs1800460 and rs1142345, map the extracted reads with Burrows-Wheeler Aligner PE mode, and manipulate the alignments with SAMTools for visualization on the Integrative Genomics Viewer. With SR-PE-HTS, the PE reads generated from the ends of a clonally amplified DNA fragment possess a substantial advantage in haplotyping over single-end short-read sequencing. If rs1800460 and rs1142345 are found on the same read pair, the variants are on the same chromosome (in *cis*) which defines a \*1/\*3A genotype. Otherwise, the variants are on different chromosomes (in *trans*), defining a \*3B/\*3C genotype. **Results:** The sequencing experiment yielded a total of 141,033 PE reads, of which 3,358 PE reads (2.38%) were found to cover rs1800460 and rs1142345. Among the reads, 42.88% (1,440/3,358) were classified as \*1 and 57.12% (1,918/3,358) were classified as \*3A. **Conclusions:** The patient with inconclusive TPMT status was successfully characterized to be \*1/\*3A by SR-PE-HTS on the TPMT transcripts. This method is fast and relatively straightforward. Previously, haplotypes were further investigated by either pedigree analysis or clone-based sequencing of long-range PCR amplicons. Pedigree analysis may not be possible in all circumstances while clone-based sequencing is laborious and the development of a long-range PCR assay is challenging.

#### G014. Pathogenic Variants in MID1 in Patients with X-linked Opitz G/BBB Syndrome Type 1

L. Fan, F. Xu, D. Allingham-Hawkins, P. Bergmann, J. Weber  
Prevention Genetics, Marshfield, WI.

**Introduction:** Opitz G/BBB syndrome type 1 is an X-linked recessive congenital disorder characterized by multiple midline structure malformations including facial, laryngo-tracheo-esophageal and genitourinary abnormalities, cardiac defects, developmental delay and intellectual disability. *MID1*, encoding Midline 1, is currently the only gene causative for X-linked Opitz G/BBB syndrome. Here we present the results of *MID1* sequencing tests performed at Prevention Genetics since 2016 to provide new information for the variant spectrum. **Methods:** For Sanger sequencing, PCR was used to amplify each coding exon plus flanking non-coding sequences of *MID1*. Sequencing was performed using the ABI Big Dye Terminator v.3.1 kit and ABI 3730xl capillary sequencer. For gene-centric array comparative genomic hybridization (aCGH), equal amounts of genomic DNA from the patient and a sex matched reference sample were analyzed using a custom Agilent microarray with X probes across the entire *MID1* gene spanning ChrX:10413349-10851809. **Results:** Forty male patients with suspected X-linked Opitz G/BBB Syndrome Type 1 were tested by Sanger sequencing with reflex to aCGH in negative sequencing cases. In total, 11 of 40 patients (28%) were found to harbor a pathogenic or likely pathogenic variant in *MID1*. One other patient had a suspected pathogenic variant. By sequencing, 9 patients were found to have a pathogenic variant in *MID1*. Four variants were novel: c.622C>T (p.Gln208\*); c.709\_713dupCTGGA (p.Glu238Aspfs\*27), c.1447+1G>A, and c.1483\_1516dup (p.Val506Alafs\*8). Two variants (c.1444\_1447dupAACA (p.Ser483Lysfs\*6), c.1483C>T (p.Arg495\*)) were previously reported. In reflex array CGH analysis, 31 cases with negative sequencing results were tested. Of these 2 patients, whose sample was failed in PCR amplification in certain exons by sequencing, were found to be hemizygous for large deletions, encompassing exon 10 and exons 4 to 7. Of note, one patient was found to have a suspected pathogenic insertion of 1.29 kilobases (kb) in intron 1 (c.-56-9\_c.-56-8). This 1.29 kb DNA fragment was comprised of an inverted *OARD1* gene which encodes O-acyl-ADP-ribose-deacylase 1. **Conclusions:** This study expanded the spectrum of variants known to be causative for X-linked Opitz G/BBB syndrome type 1. Whereas current variant spectrum in *MID1* includes many missense variants, all of the pathogenic variants detected in patients in the current study were truncating variants. Only one missense variant of uncertain significance was observed in a patient in the study. Further investigations in variant segregation in families could help to improve our knowledge of the relationship between genotype and phenotype underlying this syndrome.

#### G015. Clinical Evaluation of the Luminex ARIES System for Single Nucleotide Polymorphism Analysis of rs6025 (Factor V Leiden), rs1801133 (Methylenetetrahydrofolate Reductase), and rs1799963 (Prothrombin)

M. Leong, C. Chai, P. Tan, K. Poon, H. Lee, L. Chiu, B. Yan, K. Tan, C. Lee  
National University Health System and National University of Singapore, Singapore.

**Introduction:** The Factor V Leiden (F5) G1691A and the Prothrombin (F2) G20210A variants increase the risk of venous thrombosis. Homozygous methylenetetrahydrofolate reductase (*MTHFR*) C677T variant, in conjunction with elevated homocysteine, increases the risk of venous thrombosis and recurrent pregnancy loss. Here, we evaluated the performance of the Luminex ARIES System, a fully integrated sample-to-result diagnostic platform for performing single nucleotide polymorphism analysis of these variants when compared to an existing method used in our laboratory, the LightScanner 32 high resolution melting system. **Methods:** To evaluate the performance of the Luminex ARIES System, we tested 93, 83, and 19 whole blood samples for which we had previously determined the genotypes for F5 (rs6025: G1691A), *MTHFR* (rs1801133: C677T), and F2 (rs1799963: G20210A) variants using the LightScanner, respectively. Because of the low prevalence of F5 and F2 variants in the recruited blood samples, 35 DNA samples from the College of American Pathologists (n=24), Mayo Medical

Laboratories (n=7), and Addenbrooke's Hospital (n=4) with known F5 or F2 genotype status, or both, were included in the evaluation. Reproducibility of the ARIES System (intra- and inter-run variation between runs, modules, days and operators) was evaluated using a panel comprising of nine blood samples. The panel was tested in replicates, using one lot of ARIES extraction cassettes, MultiCode DNA Ready Mix and primer/probe mix, by four operators on separate days across different ARIES modules. **Results:** We have tested 195 whole blood and 35 DNA samples on the ARIES System, of which 104 were tested for F5 variant (heterozygous G1691A: 7.7%; homozygous G1691A: 7.7%), 83 were tested for *MTHFR* variant (heterozygous C677T: 36.1%; homozygous C677T: 10.8%), and 47 were tested for F2 variant (heterozygous G20210A: 18.2%; homozygous G20210A: 9.1%). The ARIES System showed complete agreement when compared against the intended result with a Cohen's unweighted  $\kappa$  of 1.00. Excellent reproducibility (100%) was observed when the same samples were tested by four operators on separate days with different modules on the ARIES System.

**Conclusions:** The ARIES System has the lower hands-on time and faster assay time (2 hours for up to 12 samples, including test setup and sample preparation) than the LightScanner (7 hours for up to 25 samples). The results are also accurate and reproducible. It is worth noting that among the four operators, two had limited hands-on experience running the ARIES System during the multi-operator study. Overall, our study shows that the Luminex ARIES System appears to be a practical and suitable sample-to-result solution for polymorphism testing of rs6025, rs1801133, and rs1799963.

#### G016. How to consistently determine if a Variant is a Polymorphism?

D. Qin

Moffitt Cancer Center, Tampa, FL.

**Introduction:** To determine if a certain variant is a polymorphism, multiple parameters are usually considered, e.g. variant allelic frequency (VAF), minor allele frequency (MAF), and biological impact. A polymorphism usually has a VAF of about 50% or 100%. However, not all variants with a VAF of about 50% or 100% are polymorphisms. When the MAF of a variant is > 1%, it is considered as polymorphism. However, a MAF of < 1% cannot rule out the possibility of polymorphism. Since multiple parameters are involved, sometimes this seemingly simple question may get different answers. Such inconsistency poses a problem for issuing consistent variant interpretation. Such problems could also be prominent in a clinical trial. If polymorphisms cannot be determined consistently among the patients enrolled in a trial, the patient stratification will be affected, which may mislead the data analysis. **Methods:** To facilitate a consistent determination of a polymorphism, I propose a score system shown in the table below. In this score system, the significance of total score is defined as following. If the total score is  $\geq 10$ , the variant is considered as a polymorphism. If the total score is >7 and <10, the variant is considered as a possible polymorphism. If the total score is  $\leq 7$ , the variant is not considered as a polymorphism. **Results:** Are tabulated and will be outlined at the meeting presentation. **Conclusion:** It is understood that such score distribution is somewhat arbitrary and germ line testing can further help in determining a polymorphism. However this score system will improve the consistency in determining a polymorphism in practice.

#### G017. High-throughput Approach for Multi-omic Testing for Prostate Cancer Research

M. Shannon, F. Le, A. Shi, D. de Castro, N. Elder, N. Fantin, L. Tracy  
Thermo Fisher Scientific, South San Francisco, CA.

**Introduction:** The proliferation of genetic testing technologies and genome-scale studies has significantly increased our understanding of the genetic basis of complex diseases. However, genetic information alone tells an incomplete story of the underlying biology. Integrative approaches that combine data from multiple sources, such as the genome, transcriptome and/or proteome, can provide a more comprehensive and multi-dimensional model of complex diseases. Similarly, the integration of multiple data types in disease screening can improve our understanding of disease in populations. Prostate Cancer is an illustrative example where population screening could be significantly improved with a multi-omic

approach rather than the traditional single Prostate Specific Antigen (PSA) protein marker test. **Methods:** Many multi-omic approaches use different platforms for nucleic acid and protein analysis, which adds a layer of complexity to data collection and interpretation. In order to simplify and streamline these processes, we have developed a workflow that enables high-throughput testing of both genetic markers and protein markers on the same real-time PCR system. As proof of concept, we have applied our technology to a Prostate Cancer prediction method developed by the Karolinska Institute called the "Stockholm3" model. Our workflow leverages the Applied Biosystem QuantStudio 12k Flex as well as TaqMan SNP Genotyping Assay and TaqMan Protein Assay technologies. **Results:** The chemistry is optimized to maximize sensitivity and specificity and requires only a blood draw sample. Results from development show >99% call rate and >99.9% accuracy for genetic markers and  $\geq 4$  log dynamic range of protein concentration measurement. The workflow is automated to support over 100 samples per 24-hr period with minimal hands-on-time and minimal manual intervention. **Conclusions:** This workflow could enable Prostate Cancer researchers to perform both large-scale studies and routine testing of genetic and protein markers on a single system.

#### G018. Clinically Integrated Molecular Diagnostics in Adenoid Cystic Carcinoma

J.C. Thierauf<sup>1,5</sup>, N. Ramamurthy<sup>1</sup>, V.Y. Jo<sup>2</sup>, H. Robinson<sup>1</sup>, R.P. Frazier<sup>1</sup>, M. Pacula<sup>3</sup>, E. Dominquez-Meneses<sup>3</sup>, V. Nose<sup>3</sup>, V. Nardi<sup>1</sup>, D. Dias-Santagata<sup>1</sup>, L.P. Le<sup>1</sup>, L.J. Wirth<sup>3</sup>, J. Hess<sup>4</sup>, A. Iafrate<sup>1</sup>, J.K. Lennerz<sup>1</sup>

<sup>1</sup>Massachusetts General Hospital/Harvard Medical School, Boston MA;

<sup>2</sup>Brigham and Women's Hospital/Harvard Medical School, Boston MA;

<sup>3</sup>Massachusetts General Hospital Boston, Boston MA; <sup>4</sup>Heidelberg University Hospital, German Cancer Research Center, Heidelberg, Germany;

<sup>5</sup>Heidelberg University Hospital, Heidelberg, Germany.

**Introduction:** Adenoid cystic carcinoma (ACC) is an aggressive, metastasizing salivary gland malignancy without effective systemic therapies. Aggressive phenotypes associated with *NOTCH1* mutations and *MYB* fusions carry prognostic and potentially therapeutic relevance; however, the prevalence and clinical utility of delineating these aberrations in routine practice have—to our knowledge—not been assessed. **Methods:** Workup in 20 routine ACC samples included targeted next-generation sequencing (NGS) for the detection of point and insertion-deletion mutations, copy-number changes, and gene fusions. We employed MYB break-apart fluorescent *in situ* hybridization (FISH) for validation of fusions and assessed MYB expression by immunohistochemistry (IHC). Utility was defined as the fraction of patients potentially amenable to targeted therapies; for outcome assessment, we included n=201 patients from publicly available databases. **Results:** In our clinical practice, 87.5% of patients had locally advanced and 21% metastatic disease. Genotyping identified n=5/20 (25%) *NOTCH1* aberrations, n=6/20 (30%) *MYB-NFIB*, and n=2/20 (10%) *MYBL1-NFIB* fusions; all MYB fusions were confirmed by FISH. With one exception (*NOTCH1/MYB*), these alterations were mutually exclusive and overall identified 65% of patients. Anatomic location and/or IHC, positive in 90% (n=10/11), cannot serve as surrogates for fusion identification and tumors without *MYB/MYBL1/NOTCH1* aberrations showed no additional mutations or fusions. In 75% (n=6/8) of our patients with non-resectable tumors, we identified potentially actionable alterations whereas the tumors with *MYB/MYBL1/NOTCH1* alterations had a more aggressive course with significantly shorter progression free survival (P=0.04 log-rank).

**Conclusions:** Clinically integrated workup of ACC identifies a significant subset of potentially actionable molecular alterations in non-resectable tumors and serves as a prognostication tool for progression free survival in initially surgically curable patients.

#### G019. Germline BRCA Mutation Studies in a Select Indian Cohort Using Next-generation Sequencing (NGS)

J.C. Vyas, M. Pusalkar, R. Kittu, S. Limaye, A. Gomes, R. Tiberwala, S. Khaimar, S. Vora, N. Londhe, M. Hastak, B. Kulkarni, N. Athikari, S. Goyle, M. Nadkarni, Y. Kulkarni, A. Shetty, I. Shaikh  
Kokilaben Dhirubhai Ambani Hospital, Mumbai, India.

**Introduction:** Testing of *BRCA* germline mutations has assumed predictive and therapeutic significance in Hereditary Breast & Ovarian Cancer Syndrome (HBOC) recently. Next generation sequencing (NGS) has empowered us in screening large genes like *BRCA* for multiple patients at affordable costs. In this paper, we present our data in a select cohort of 96 patients with breast/ ovarian cancer and/ or with relevant family history. **Methods:** Of 96 patients, 68 had breast cancer (BC), 11 had ovarian (OC), 1 had both breast & ovarian and 2 had prostate or pancreatic cancers. Fourteen cases had family history of other cancers. Informed consent was taken from the patient during pre-test counseling. Targeted *BRCA1* & *BRCA2* libraries were prepared from peripheral blood genomic DNA, using the TruSeq Custom Amplicon kit and were sequenced on the Illumina MiSeq. The data analysis was performed using MiSeq reporter and in-house Linux based pipeline. Reads (Q30) were mapped against HG19 and variants were called using GATK. The variants were annotated using Variant studio as well as using ClinVar, dbSNP, BIC database. Annotated variants were filtered, interpreted using available literature and reported as per CAP/ACMG recommendations. Results were explained during post-test counseling. **Results:** Breast & Ovarian cancer (BOC) was observed in 43% cases with age below 45yrs. Incidence of pathogenic variants was found higher in patients with history of BOC (27%) compared to those without (18%). Incidence of variants of unknown significance (VUS) was however not different within these groups (9% versus 14%). In patients with BC, frequency of pathogenic mutations was 19% and VUS was 13%. In OC patients, pathogenic variants were observed in 73% cases whereas no VUS were observed. Frequency of pathogenic mutations in *BRCA1* (71%) was higher than *BRCA2* mutations (29%), whereas frequency of VUS was higher in *BRCA2* (64%) than *BRCA1* (36%). Amongst cases with triple negative BOCs, pathogenic variants and VUS were found in 20 & 11% cases respectively. Patients who did not show presence of *BRCA* mutations despite family history of BOCs (64%) were recommended to be evaluated for *BRCA* deletion duplication analysis and other hereditary panels. **Conclusions:** Our data from Indian cohort showed increased (43%) occurrence of BOC at early age (<45yrs) along with increased frequency of pathogenic *BRCA* variants in patients with family history of BOCs. Incidence of pathogenic mutations in *BRCA1* was higher compared to *BRCA2* and our data was in concordance with western and Indian literature. OC cases showed high prevalence of *BRCA* mutations (35%) irrespective of their family history. The study emphasizes the importance of *BRCA* testing for predictive & therapeutic management in HBOC.

#### G020. Phylogenetic Analysis of Duffy, Kidd, and Lewis Allele

M. Kim, D. Ko, S. Hwang, H. Oh  
Ulsan University, Asian Medical Center, Seoul, South Korea.

**Introduction:** The diversity among the blood group antigens is a result of sequence variations in genes that directly or indirectly affect expression or epitopic nature of the antigens on the surface of erythrocytes. We built the phylogenetic trees of the sequence and the phenotypic data of the three blood groups, Duffy (*DARC*), Kidd (*SLC14A1*), and Lewis (*FUT3*) type with important clinically relevant antigen. The aim of this study is to compare the data between alleles and phenotype of the minor blood group. **Methods:** The sequence and phenotype data were assembled in the Blood Group Antigen Gene Mutation Database ([www.bioc.aecom.yu.edu/bgmutil/index.htm](http://www.bioc.aecom.yu.edu/bgmutil/index.htm)). The phylogenetic trees for 9 Duffy alleles, 22 Kidd alleles, and 13 Lewis alleles examined in this study were constructed. The phylogenetic analysis was performed according to the neighbor-joining method using Molecular Evolutionary Genetics Analysis (MEGA) 7 program. **Results:** In the phylogenetic analysis of three blood group alleles, *DARC* was divided into two big clusters. The one big cluster with two small clusters was composed of the same or similar phenotypes. But the other small cluster consisted of the two phenotypes; The one phenotype was FY(a-b+) of FY\*01 allele and the other phenotype was null phenotype of FY\*02N.03 allele. *SLC14A1* also



was divided into two big clusters containing three small clusters. Most Kidd alleles showed JK(a-b-) phenotype. The two alleles with the same phenotype, JK(a+b-) were included in two different clusters. The phylogenetic tree for *FUT3* showed a sequential evolutionary pattern. The one small cluster had the reference sequence without phenotype and Le 59G, 1067A (le2, le3) allele with negative phenotype. **Conclusions:** The phylogenetic analysis of Duffy, Kidd, and Lewis revealed the relationship between alleles and phenotypes. Because the alleles with the same phenotype may belong to different clusters, it is important to distinguish the phenotype in the new allele. The phylogenetic tree based on blood group alleles of each country could be constructed using this data. This is the first phylogenetic analysis of the minor blood group-specific sequences.

#### G021. Brazilian Panorama of Whole Exome: Details of 315 Cases

R.M. Minillo<sup>1</sup>, N.H. Muto<sup>1</sup>, P.M. Oliveira<sup>1</sup>, R. Sitnik<sup>1</sup>, M.C. Cervato<sup>1</sup>, G. Carvalho<sup>2</sup>, M. Caraciolo<sup>2</sup>, W. Galvão<sup>2</sup>, C.L. Manguiera<sup>1</sup>, J.B. Oliveira Filho<sup>2</sup>

<sup>1</sup>Hospital Israelita Albert Einstein, São Paulo SP, Brazil; <sup>2</sup>Genomika, Recife PE, Brazil.

**Introduction:** Whole Exome Sequencing (WES) test is currently one of the most effective diagnostic tools in molecular investigation of genetic diseases and its analysis is entirely dependent on the clinical information provided by the requesting physician. **Methods:** We describe demographics and genetic results from 315 individuals who were referred for WES test from January, 2015 to February, 2018 in a Brazilian reference laboratory. **Results:** The positivity rate was 30.2% among the 315 cases analyzed, and negativity rate was 30.3% (36.8% of cases with no variant reported and 63.2% with a pathogenic variant in heterozygosis in a recessive gene). Inconclusive results were seen in 39.4% of the cases (only with variants of uncertain significance (VUS), with the mean number of variants reported per case of 1.08 - ranging from 1 to 7). Among positive cases, the most frequent inheritance was autosomal dominant (55.5% of cases), followed by autosomal recessive (17.8% of cases, 37.5% of them with homozygous variants), autosomal dominant and autosomal recessive inheritance in the same gene (17.8% of cases), X-linked inheritance (6.7% of cases) and mitochondrial (2.2% of cases). The mean age of the patients was 5.1 years (ranging from less than one year to 73 years). Considering the clinical indications, delayed neuropsychomotor development was the most common (25%), with a frequent association with dysmorphism and malformations (58% of cases, 5.7% of them positive), followed by seizures (12%, with 22.5% positivity), immunodeficiency (10%, with 37.5% positive cases), autism (8.5%, with 29.6% positive cases) and suspected specific syndromes with genetic heterogeneity (5.1% of cases, with 38.5% positivity). Complex phenotypes comprised 6.3% of the cases (40% positivity rate, 30% partially explaining the phenotype.) Incidental findings were rare and were found in 0.95% of the cases. **Conclusion:** Our results are consistent with the literature, with the exception of the positivity rate of complex phenotypes. We believe that one of the reasons that can contribute to this finding is that 25% of these cases were requested by medical geneticists, who tend to better compose the constitutive parts of the patient's phenotype.

#### G022. Clinical Validation of a Multi-gene Panel on Myeloid Malignancies by Next Generation Sequencing

G. Liu, B. Austin, M. Donovan, H. Gu, E. Bauman, J. Orton, C. Diedrich, L. Guidot, X. Li

Sonora Quest Laboratories, Tempe, AZ.

**Introduction:** Current analysis of myeloid malignancies involves multiple sequential tests and laborious workflows. Adoption of next generation sequencing (NGS) methods into clinical practice has created an unprecedented opportunity to profile the multiple relevant driver genes in myeloid malignancies and has been instrumental in facilitating the innovation of cancer research toward precision medicine. To enable this advancement, our lab has implemented the Ion Torrent OncoPrint Myeloid Assay which can detect variants in 74 clinically actionable genes for myeloid malignancies. **Methods:** The assay allows concurrent analysis of DNA and RNA to simultaneously detect variants including single nucleotide variants (SNVs), insertion/deletions (indels), gene fusions and expression in a single workflow. A total of 67 samples were used in the

validation including 56 patient samples (20 DNAs and 36 RNAs) and 11 controls (1 DNA Mix, 1 RNA Mix and 9 DNA cell lines). The cancer types from these patient samples include acute myeloid leukemia, myelodysplastic syndrome and myeloproliferative neoplasm, etc. These samples contain variants previously detected by orthogonal methods, i.e. real-time PCR, NGS, cytogenetics and fluorescence *in situ* hybridization (FISH), from our and other CLIA labs. In total, 108 libraries (54 DNAs + 54 RNAs) were prepared by automation using Chef Ready Kit and templates were prepared using AmpliSeq™ Technology on the Ion Chef System, and sequenced on the Ion Torrent S5 platform. **Results:** Analytical performance of the assay revealed 95.5% accuracy, 100% specificity and precision. The analytical sensitivity achieved 2.5% limit of detection for mutations and fusions. **Conclusions:** Our results demonstrate that NGS is a robust and powerful tool for detecting genetic alterations; in particular, for identifying the mutational patterns that can further improve diagnostics, risk stratification and treatment in myeloid malignancies.

#### G023. A Recurrent Heterozygous *RPL21* Mutation Responsible for Hereditary Hypotrichosis Simplex in a Chinese Family

Z. Xu<sup>1</sup>, Y. Tang<sup>2</sup>

<sup>1</sup>Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing, China; <sup>2</sup>Memorial Sloan-Kettering Cancer Center, New York, NY.

**Introduction:** Hereditary hypotrichosis (HH) is a heterogeneous group of inherited hair loss disorders. After mapping the locus to 13q12.12-12.3 in a Chinese family with a generalized variant of autosomal dominant HHS (ADHHS), a heterozygous missense mutation has been identified in the *RPL21* gene, which encodes a ribosomal protein. Here, we report a recurrent missense mutation c.95G>A in the *RPL21* gene in a Chinese family with HHS. **Methods:** Our study consisted of two affected individuals, one unaffected relatives from a Chinese family clinically characterized as HHS. The proband was a 4-year-old boy born at 39 weeks of gestation undergo clinical and mutation diagnosis in China. The affected boy and his mother (32-year-old) had normal scalp hair density at birth. At approximately 4 months of age hair loss began and progressed gradually with age until near complete loss of scalp hair. The length of the remaining hairs could reach the length of normal hair, but grew slowly, and were thin, sparse, dry, and fragile with the phenotype of hypotrichosis. Eyebrows, eyelashes and body hair were also sparse, or missing. A skin biopsy from the scalp of affected boy revealed hair follicles significantly decreased in number and size. We analyzed three genes (*CDSN*, *APCDD1* and *RPL21*) from this family by direct sequencing using primers and reaction conditions as previously described. **Results:** Mutation was identified by comparing with the reported cDNA reference sequence (Gen Bank accession number: 153000.4). **Results:** DNA obtained from the boy and his affected mother had the same recurrent p.Leu9Arg mutation of the *RPL21* gene while that was negative in his unaffected aunt. Sequence analysis of two other genes, *CDSN* and *APCDD1*, failed to detect sequence variants in either affected or unaffected individuals of the family. **Conclusions:** We sequenced the *RPL21* gene in a Chinese family and found the recurrent variant. However, the mechanism by which this ribosomal protein mutation causes such a specific disruption of hair growth is unknown. Functional study of this gene will provide important insights into the molecular and cellular basis of hair growth.

#### G024. Confirmation of Cis Inheritance of Variants in *ABCB1*, *SHROOM3*, and *SLC28A3* During the Validation of a Targeted Genotyping Assay

W.M. Stansberry, K.S. Collins, E.B. Medeiros, M. Swart, T.C. Skaar, B.S. Decker, M.T. Eadon, V.M. Pratt

Indiana University School of Medicine, Indianapolis, IN.

**Introduction:** A generalized approach to drug selection and dosage has not proven effective in treating kidney disease and various cancers. We validated a targeted panel based on pharmacogenomic implications linking genetic variation to drug efficacy and toxicity. During the validation process, we noted that several DNA samples obtained from the Coriell Cell Repository (Camden, NJ) were homozygous positive for one variant and heterozygous positive for a second variant in the same gene. Samples containing three variant alleles suggest that the two variants are

in *cis* on the chromosome in some individuals. **Methods:** We used existing trio (mother, father, and child) DNA samples in the laboratory and de-identified them. The DNA was amplified by real time PCR on the ThermoFisher QuantStudio 12K Flex (software v1.2.2; Waltham, MA) and subjected to Taqman allele discrimination using ThermoFisher (Waltham, MA) reagents and software (Genotyper software, v1.3) in a custom designed open array. **Results:** Using trio studies, we were able to confirm that variants in the following genes were in *cis*: *ABCB1* (c.1236T>C, c.2677T>G, c.3435T>C), *SHROOM3* (c.168+54767G>A, c.168+11474G>A), and *SLC28A3* (c.1381C>T, c.862-360C>T).

**Conclusions:** Genetic testing may serve as an important tool for clinicians who embrace precision medicine. Increasing scientific evidence has supported the utilization of genomic information to select efficacious therapies for kidney disease and cancers. Understanding the phasing of genetic variants allows for the improved definition of risk assessment as well as understanding of inheritance.

#### G025. Verification of Very Small Copy Number Variants (Micro CNVs) Detected on Whole Genome CMA Analysis and Implications for Clinical Reporting

U.P. Kappes, G. Scharer, E. Virlee, N. Zemlicka, P. Aggarwal, R. Lorier, U. Broeckel

Medical College of Wisconsin, Milwaukee, WI.

**Introduction:** Chromosomal microarray (CMA) analysis has become the accepted clinical standard for genetic testing of individuals with unexplained developmental delay/intellectual disability (DD/ID), autism spectrum disorders (ASD), or multiple congenital anomalies (MCA). While large (>1Mb) copy number variants (CNVs) are no longer verified with an orthogonal method, smaller CNVs are often followed by FISH (fluorescence *in-situ* hybridization) analysis for variant confirmation and determination of parental origin. However, FISH technology has its own limitations, e.g. probe size, and conversely, molecular methods such as quantitative PCR (qPCR) are being used to confirm results from FISH analysis. With the evolution of array platforms the resolution has increased significantly allowing for the detection of CNVs <100kb. Sound thresholds for reporting of micro CNVs detected by microarray, and for the need of confirmatory analysis are still under discussion. **Methods:** In the context of our annual laboratory QA/QI process, we retrospectively reviewed the results of >500 clinical de-identified CytoScanHD microarray assays that were performed in 2017. All results that had been reported as indeterminate (deletion <200kb and duplication <500kb) were selected for analysis. CMA results were compared to the results of the confirmatory qPCR (RealTime-PCR). **Results:** Fifty-six very small copy number variations were observed on CytoScanHD microarray and reported as indeterminate. We observed recurrent micro CNVs, including several intragenic CNVs which are likely of clinical significance. 41 of these cases were subsequently tested by qPCR. In 38 of these cases (92.7%) CMA results were confirmed by qPCR. In 3 cases (7.3%) results did not confirm even after a second qPCR with alternate Taqman probe. Only in one case qPCR identified a discordant copy number when compared to the microarray result. Overall, the positive predictive value for the detection of very small copy number variations by CMA was 93%.

**Conclusions:** While current CMA analysis is able to detect micro CNVs, false positive calls cannot be completely excluded. The discordance rate of 7.3% between results from microarray and qPCR warrants further investigation. There was no correlation between the size of the micro CNV and result concordance. The thresholds for reporting micro CNVs detected by microarray alone depend on the resolution of the array platform and its probe coverage for the genomic region in question.

#### G026. Two-site Evaluation of a One-tube PCR/CE Assay that Resolves CAG Length Polymorphisms in Exon 1 of the *HTT* Gene

S.N. Statt<sup>1</sup>, E. Suh<sup>2</sup>, J.R. Thibert<sup>1</sup>, A.D. Bossler<sup>3</sup>, V.M. Van Deerlin<sup>2</sup>, G.J. Latham<sup>1</sup>

<sup>1</sup>Asuragen, Austin, TX; <sup>2</sup>Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA; <sup>3</sup>University of Iowa Hospitals and Clinics, Iowa City, IA.

**Introduction:** Huntington disease (HD) is a progressive brain disorder caused by expansion of an unstable, trinucleotide CAG or poly-Q repeat in exon 1 of the huntingtin (*HTT*) gene. Accurate determination of CAG repeats is critical for molecular diagnosis of HD. HD CAG expansion is typically identified using PCR-based methods. Polymorphisms near the CAG tract can lead to mis-priming and inaccuracies in allele sizing. Here we describe a robust, rapid and accurate PCR assay evaluated at two sites that can resolve *HTT* zygosity and enable accurate repeat quantification even in the presence of known gene polymorphisms. **Methods:** A prototype assay was evaluated at Asuragen (Site 1) and the University of Pennsylvania (Site 2). Both sites evaluated a common set of cell-line samples and NIST controls that covered a range from 15 to 250 CAGs. Each site also assessed peripheral blood samples independently. Additionally, Ultramer DNA Oligonucleotides (IDT) were synthesized as controls containing well-characterized *HTT* SNPs between the polymorphic CAG and CCG regions associated with allele dropout in other PCR-based detection methods. Sample gDNA and/or Ultramers were PCR amplified using AmpliPhiX reagent technology and amplicons were resolved by capillary electrophoresis (CE) on either a 3130xl or 3500xL Genetic Analyzer (Thermo Fisher). Genotypes were determined from the mobility of target peaks relative to a calibration curve. **Results:** *HTT* PCR amplicons from Coriell and NIST reference standards generated at the two sites produced CAG genotypes that were in good agreement across both sites. Ultramers containing *HTT* polymorphisms produced robust repeat-primed peaks and no allele dropouts even in the presence of multiple regions of sequence variability. Comparison of a subset of the same samples at Site 2 generated concordant genotypes on CE instruments. Inputs as low as 5 ng produced consistent genotypes in normal and expanded *HTT* samples. Assay performance fell well within recommended precision limits of CAG repeat sizing accuracy and achieved allelic resolution with accurate and unambiguous CAG repeat determination for >100 repeats. Expansions with >200 repeats were reliably flagged by the assay. **Conclusions:** Our results indicate that the described PCR/CE technology can accommodate known polymorphisms and resolve zygosity in unexpanded homozygous and expanded heterozygous samples via a robust repeat peak pattern. By effectively combining two complementary amplification reactions in a single-tube, this assay has the potential to streamline clinical research free from the concern of false-negative results surrounding current *HTT* technologies.

#### G027. A Streamlined, Single-Tube PCR Assay that Quantifies *SMN1* and *SMN2* Copy Numbers Using Capillary Electrophoresis

W. Laosinchai-Wolf, S. Filipovic-Sadic, L. Chen, S. Gokul, V. Le, M. Church, M. Dodge, J. Milligan, G. Latham

Asuragen, Austin, TX.

**Introduction:** Spinal Muscular Atrophy (SMA), an autosomal recessive neuromuscular disease caused by a loss of *SMN1* gene function, is the primary genetic cause of infant death. The copy number of the highly similar *SMN2* gene is an important predictor of the severity of SMA, as the *SMN2* gene can produce some functional SMN protein that partially restores biological function. The antisense oligonucleotide nusinersen (marketed as SPINRAZA) promotes *SMN2* alternative splicing to enhance the effectiveness of *SMN2* functional replacement of *SMN1*. Early detection of SMA, along with knowledge of *SMN2* copy number, is critical for effective medical management. Herein we report the performance of a prototype AmpliPhiX PCR/CE *SMN1/2* kit, a single-tube PCR assay that quantifies *SMN1* and *SMN2* copy number using capillary electrophoresis (CE). **Methods:** A multiplexed PCR was developed that simultaneously amplifies *SMN1*, *SMN2* and an endogenous control (EC) in a single well. The PCR products were separated and quantified via CE (3500 Genetic Analyzer, Thermo Fisher). The copy number of *SMN1* or *SMN2* was calculated as the peak area ratio of target gene and EC normalized to a calibrator. Coriell DNA samples with 0 to 3 of *SMN1* copies and 0 to



5SMN2 copies were used to assess DNA input range. SMN1 or SMN2 synthetic templates diluted in Coriell DNA were generated for specificity and linearity studies. To date, 63 genomic DNA samples isolated from human blood and 26 cell-line genomic DNA samples have been analyzed using this assay, including 15 Coriell cell-line samples. To evaluate accuracy, a subset of samples was also characterized using orthogonal assays. **Results:** Preliminary data demonstrated that the single-tube PCR assay accommodated DNA inputs ranging from 20 to 80 ng per reaction. The linear range for both SMN1 and SMN2 copy numbers was estimated to be 1 to  $\geq 6$  using blood, cell-line, or contrived synthetic samples. SMN2 was not detected in SMN2-negative, SMN1-positive samples, or vice versa, demonstrating high assay specificity. In accuracy studies, the overall percent agreement between the prototype reagents and the comparator method for 63 clinical samples was 98% for SMN2 and 100% for SMN1. **Conclusions:** Prototype AmpliX SMN1/2 reagents accurately quantified both SMN1 and SMN2 copy numbers in one PCR reaction. The streamlined workflow is both fast (<6 hours) and straightforward, with minimal sample handling and pipetting steps. This single-tube method has the potential to significantly reduce the complexity and time-to-result for SMN1/2 testing compared to existing methods using multiplex ligation-dependent probe amplification (MLPA) or next-generation sequencing (NGS), and provide a unified workflow compared to gene-specific qPCR methods.

#### G028. New Variant-centric XML for ClinVar Data

M. Landrum, D.R. Maglott, C. Chen, S. Chitipiralla, M. Benson, G. Brown, B. Gu, J. Hart, D. Hoffman, W. Jang, C. Liu, Z. Maddipati, R. Maiti, G. Riley, G. Zhou, K. McDaniel, A. Glodok  
National Institutes of Health, Bethesda, MD.

**Introduction:** ClinVar is a public archive of submitted records interpreting human genetic variants relative to health and disease. The database holds more than 600,000 submitted records for more than 400,000 unique variants. ClinVar aggregates submitted data by variant-disease pair so that users can compare interpretations and evidence from different submitters. The full set of aggregated data is available for download on the ClinVar FTP site (<ftp.ncbi.nlm.nih.gov/pub/clinvar/xml/>). Users of ClinVar's XML noted that in some cases, aggregation by variant only (ClinVar's Variation ID), not the variant-disease pair, would be beneficial, e.g. a BRCA1 variant reported for its relationship to several different related concepts (familial breast cancer, breast-ovarian cancer, etc). Variant-centric XML would be consistent with ClinVar's web pages and VCF files, and would reduce redundancy in records for a variant. To address this need for variant-centric XML files, we investigated methods to aggregate and export data by the variant only. **Methods:** A new XML schema definition (XSD) was developed to represent data in ClinVar organized by the Variation ID. Database processes were updated to aggregate submitted data by the Variation ID. A process to emit the variant-aggregated data in XML format to the ClinVar ftp site was added. Users were given an opportunity to review the output and provide feedback. **Results:** An initial version of variant-centric XML, named ClinVarVariationRelease, was published on the ClinVar FTP site in July 2017; it is now updated every two weeks. All aggregated and submitted data for a variant or set of variants that was interpreted are reported under a single element called InterpretedRecord. Elements were added to indicate whether the interpretation is for a single variant (SimpleAllele), a set of variants in cis (Haplotype), or two sets of variants in trans (Genotype). The variant-centric XML also has explicit representation of variants that are only reported in ClinVar because they are included in a haplotype or genotype that was interpreted ("included variants"). These are represented in an element called IncludedRecord. **Conclusions:** The variant-centric XML file ClinVarVariationRelease is available for download and testing, and feedback is encouraged. The file makes it easier for those parsing the XML to identify all data for a variant, e.g. all diseases reported for a variant. ClinVarVariationRelease also provides more explicit representation for haplotypes and genotypes, as well as "included variants". The XML continues to be updated biweekly in a beta release. Production release is expected in late 2018. This presentation will highlight the features of the new XML file and explore examples for using it in clinical genetic testing.

#### G029. Measuring the Economic Value of Sequencing: Why is it Important, Why is it Challenging, and What are Solutions

K.A. Phillips<sup>1</sup>, P.A. Deverka<sup>2</sup>, D.A. Marshall<sup>3</sup>, S. Wordsworth<sup>4</sup>, D.A. Regier<sup>5</sup>, K.D. Christensen<sup>6</sup>, J. Buchanan<sup>4</sup>, M. Douglas<sup>1</sup>

<sup>1</sup>University of California, San Francisco, San Francisco, CA, <sup>2</sup>American Institutes for Research, Chapel Hill, NC, <sup>3</sup>University of Calgary, Alberta, Canada; <sup>4</sup>University of Oxford, Oxford, United Kingdom;

<sup>5</sup>University of British Columbia, Vancouver, Canada; <sup>6</sup>Harvard University and Brigham and Women's Hospital, Boston, MA.

**Introduction:** It's been said that the greatest challenges facing the appropriate use of precision medicine are economic, not scientific. These challenges are particularly acute for assessing the value of next-generation sequencing tests (NGS) due to their complexity. Although economic value (costs compared to outcomes as measured by cost-effectiveness analysis or other methods) is just one factor in determining the appropriate use of NGS, it is integral to designing an efficient and effective health care system. Economic analyses can help inform decision-making and indicate where and when technologies will be of most value. Our objective was to synthesize the current state of the literature to identify challenges in measuring the economic value of NGS and possible solutions. **Methods:** We conducted a structured literature synthesis of peer-reviewed publications (15 cost-effectiveness analyses of NGS and 11 studies of patient valuation of outcomes), assessed case studies (the MedSeq trial and large national initiatives, e.g., 100K Genomes Project, All of Us Program), and convened an Expert Working Group to review the findings. **Results:** We found that 3 of the methodological challenges to appropriately measuring the value of NGS are of particularly high priority to address: 1) choosing the appropriate type of analysis and comparator(s) to NGS, 2) addressing the complexity of adequately modeling tests that provide multiple results, and 3) accounting for costs and benefits including those that may occur long after initial testing. We also found that incorporating the value of non-health and process outcomes important to patients needs to be considered. Large national sequencing initiatives can address some of these challenges but raise other challenges in data collection, management, and analysis of these "Big Data" that have to be addressed to apply these data to economic analyses. **Conclusions:** There are many challenges to measuring the economic value of NGS and some solutions are starting to emerge such as new approaches to modeling tests with multiple results and obtaining patient preferences for NGS findings. Further work is necessary to further refine and implement solutions including obtaining stakeholder input and developing consensus guidelines on additional solutions, evidence-based case studies of successful solutions, developing standardized methods and common data elements that appropriately measure the value of NGS, and registries that compile economic evidence so that such evidence can inform decision-making.

#### G030. Newborn Screening, Diagnosis, and Carrier Testing for Spinal Muscular Atrophy by Multiplex Droplet Digital PCR

N. Vidal-Folch, D. Gavrilov, K. Raymond, P. Rinaldo, S. Tortorelli, D. Matern, D. Oglesbee

Mayo Clinic College of Medicine, Rochester, MN.

**Introduction:** Spinal muscular atrophy (SMA) is a neuromuscular disorder characterized by motor neuron degeneration leading to muscular atrophy. SMA is frequently caused by a homozygous SMN1 exon 7 deletion. SMA severity is related to the copy number of a nearby paralog, SMN2. With the advent of targeted therapy, SMA is a novel newborn screening (NBS) target. Multiple Ligation Probe Amplification (MLPA) and real-time PCR (qPCR) can be used to detect SMA. However, qPCR is susceptible to PCR inhibitors and MLPA requires DNA concentrations above what is obtainable from dried blood spots (DBS). We developed a multiplex droplet digital PCR (ddPCR) method for SMN1 exon 7 and SMN2 exon 7 quantification for DBS, whole blood, and cultured cells. Additionally, we developed a ddPCR assay for the quantification of SMN1 rs143838139 (g.271134T>G), a SNP that is linked to SMA silent [2+0] carriers. Lastly, we developed a long-range PCR assay for SMN1 gene sequencing to complete a suite of SMA testing. **Methods:** For the SMA Del/Dup assay, SMN1, SMN2 and RPP30 copies were simultaneously measured, and SMN1 and SMN2 copy numbers reported. 1530 donor, 12 SMA

positive and 9 proficiency specimens were tested. For the SMA Residual Risk Assay, *SMN1* T allele and *SMN1* G allele were simultaneously measured, and percent fractional abundance of the *SMN1* G allele calculated. 51 samples received for SMA carrier testing were evaluated for g.27134T>G. Recovery studies with g-block fragments were performed to assess cross-reactivity between *SMN1* T allele and *SMN1* G allele. Accuracy was evaluated by method comparison with Sanger sequencing (n=10). For the *SMN1* gene sequencing, 12 samples were tested and concordance with next generation sequencing (NGS) data was reported. **Results:** For the SMA Del/Dup assay, donor specimens showed 1 to 5 *SMN1* exon 7 copies. SMA patients showed 0 copies of *SMN1*. Intra-assay and inter-assay imprecision was <7.1 %CV for individuals with 1 to 4 copies of *SMN1*. Testing 12 SMA confirmed-positive specimens resulted in 100% sensitivity and specificity. For the SMA Residual Risk Assay, 4.2% of SMA carriers with 2 copies of *SMN1* were positive for g.27134T>G ([2+0] silent carriers), which were confirmed by Sanger sequencing. *SMN1* long-range PCR is able to discriminate between *SMN1* and *SMN2*, except for exon 1 variants. **Conclusions:** Our multiplex ddPCR method for SMA is sensitive, specific, and applicable to newborn screening, diagnostic, and carrier testing. We also propose an SMA NBS algorithm that includes the simultaneous detection of *SMN2* copy number, *SMN1* silent carriers and *SMN1* mutations.

### G031. What's in a VUS Rate? Simulated VUS Rate Calculations for Hereditary Cancer Genes Using Population Frequency Data and ClinVar Submissions

K.E. Kasenit<sup>1</sup>, M. Judkins<sup>2</sup>, R. Rebecca Mar-Heyming<sup>3</sup>, H. Kang<sup>1</sup>, E.A. Evans<sup>1</sup>, K. Ready<sup>4</sup>, D. Muzzey<sup>1</sup>

<sup>1</sup>Counsyl, South San Francisco, CA; <sup>2</sup>Counsyl, Salt Lake City, UT; <sup>3</sup>Counsyl, Sacramento, CA; <sup>4</sup>Counsyl, Austin, TX.

**Introduction:** Hereditary cancer panel tests are often compared by the likelihood of reporting a variant of uncertain significance ("VUS"), yet this "VUS rate" has questionable value because multiple factors influence it (e.g., the patient population). By permuting various parameters, we explore the extent to which the reported VUS rate can vary even if the underlying characteristics of variants are the same. **Methods:** We simulated hypothetical laboratories by sourcing assertions from ClinVar and allele frequencies (AF) from gnomAD. We permuted 6 parameters: (1) the AF above which variants were automatically classified as benign; (2) the number of exon-padding intronic bases sequenced; (3) the cohort's ethnic composition; (4) whether to include patients with both VUS and pathogenic variants in the rate; (5) how variants with multiple ClinVar assertions were interpreted, and (6) the genes on the test's panel. Unless in ClinVar, low-AF variants were classified using Variant Effect Predictor (HIGH/LOW impacts only). Variance-based sensitivity analysis was used to assess the relative importance of parameters. **Results:** The main determinants of the VUS rate were panel composition (VUS rate increased with more genes), the ethnicity composition of the patient cohort (an African/African American cohort had the highest VUS rate at 88.1% on average for the largest panel, and a European cohort had the lowest, 70.5%), and the method used to reconcile variants with multiple ClinVar assertions (a simple majority rule had a smaller VUS rate than a full consensus rule). The other three parameters had smaller effects. Across all simulations, the VUS rate varied from 1.5% to 96.5%. The Ashkenazi Jewish cohort had the smallest ratio of the number of unique unknown variants to unique deleterious variants (13 VUSs per deleterious variant), followed by the European cohort (15 VUS/deleterious). The East Asian cohort and African/African American cohorts had the largest VUS/deleterious ratios with 25 and 33 unknown variants per deleterious variant, respectively. **Conclusions:** VUS rates can vary widely, in part due to variables not under laboratory control. As ethnicity was a key VUS-rate determinant and the ratio of unique unknown variants to deleterious variants was high in several minority populations, further research of the genetic variants in these populations is warranted. Our results indicate that the VUS rate in isolation is not a reliable measurement of quality, suggesting that multiple criteria should be considered when evaluating which genetic tests to offer to patients.

### G032. What Can We Learn From Oncologists? A Survey of Molecular Testing Patterns

J.S. Menezes, V.E. Joy, A.A. Vora

Thermo Fisher Scientific, Waltham, MA.

**Introduction:** Oncologists are increasingly incorporating next generation sequencing (NGS) testing to guide targeted and immuno-oncology therapies. Most testing is confined to large academic institutions and reference labs, despite the fact that most cancer patients are treated in the community settings. We sought to examine molecular testing selection patterns directly from oncologists to better identify perceived gaps in testing and treatment paradigms. **Methods:** A population-based survey of 100 practicing oncologists was conducted across various practice types in the US (80%) and (19%) Europe, to assess oncology needs in NGS testing for clinical therapy guidance. Relevant topics regarding NGS testing queried in this survey included: use of local versus commercial labs, turn-around time (TAT), report information, reportable results, and actionable variants. **Results:** One hundred oncologists completed the survey with an average of 16 (±7) years of post-residency experience, managing an average of 451 (±514) patients. The respondents included a cross section of academic (48%), private practice (25%), community hospital (20%), and non-academic (7%) oncologists. Overall, 98% of the oncologists surveyed used NGS testing and most indicated that the test was either extremely (36%) or moderately (51%) important to their practice, with actionable mutation identification, successful result rate, and TAT being the top three requirements. Of the oncologists surveyed, 81% used external NGS testing (exclusively or in combination with in-house testing). Of these, approximately 1/4, despite having in-house NGS testing, reported that the local labs were unable to meet their clinical needs (e.g., actionability, interpretable reports, TAT, etc.). Nearly half of the physicians sending samples out (49%) did not have institutional NGS testing. The majority of oncologists are supportive of their local pathologists, though approximately 1/4 of those sending samples to external labs stated that their local pathology labs do not meet critical variables. Additionally, the majority of those surveyed (68%) indicated that improvement in clinical criteria (i.e., TAT) would directly lead to improved patient care. **Conclusions:** Overall, the survey results highlight crucial issues regarding NGS testing by oncologists. Clinical alignment is crucial for optimal patient care. In this study, four out of five oncologists sent out their NGS testing, yet most oncologists (72%) would switch to in-house testing if it was available and met key clinical criteria. By directly addressing Oncology/Pathology gaps (both real and perceived), local pathology labs can play a pivotal role in improving patient care and increasing the number of cancer patients receiving next generation treatment modalities within their communities.

### G033. A Data-Driven Approach to Determine Disease Content in Expanded Carrier Screening Panels

R. Ben-Shachar, A. Svenson, D. Muzzey

Counsyl, South San Francisco, CA.

**Introduction:** The American College of Obstetricians and Gynecologists (ACOG) suggests that diseases on expanded carrier screening (ECS) panels meet several of the proposed guidelines for inclusion criteria, including that each screened condition should have a carrier frequency greater than 1 in 100, cause a severe phenotype, and be amenable to prenatal diagnosis. The impact of these criteria on the ability of ECS to detect couples at-risk for conceptuses affected with serious diseases needs to be evaluated. **Methods:** De-identified data from 44,483 patients who underwent ECS on a 176-condition panel were analyzed to assess how exclusion of diseases that did not meet all clinical guidelines impacted the rates of detection of both individual carriers and at-risk couples. Postulating that a disease is worthy of screening only if the clinical sensitivity is high, we developed a statistical framework that estimates the achievable detection rate of carriers for rare diseases by modeling if sufficient reported cases in the literature exist to interpret the pathogenicity of observed variants. **Results:** One hundred-seventy (97%) of the 176 conditions met at least six of the ACOG criteria, including those relating to disease severity and availability of prenatal diagnosis. Limiting an ECS panel to conditions meeting these criteria would reduce identification of at-risk couples by 3%. One criterion, which an ECS panel disease must have a carrier frequency above 1 in 100, can be interpreted

in different ways that have important consequences, as carrier frequencies vary widely by ethnicity. Depending on the definition used, the criterion is satisfied by between 12 (7%) and 75 (43%) conditions, and if met, would reduce identification of at-risk couples by between 5% and 51% and carriers by between 20% and 83%, respectively. If the carrier-frequency guideline were interpreted as requiring a 1-in-100 frequency in all ethnicities, even common diseases such as cystic fibrosis and 21-OH-deficient congenital adrenal hyperplasia would not meet these criteria. Our modeling suggests that a disease with a carrier frequency as low as 1 in 4,000 could achieve a clinical sensitivity of 50%. **Conclusions:** In order for an ECS panel to be clinically meaningful to patients, disease-inclusion criteria are needed. However, by analyzing data from a large patient cohort, we demonstrate that the 1 in 100 carrier-frequency threshold suggested by ACOG is unclear and arbitrary. When strictly enforced, it limits at-risk couple detection without yielding a substantial reduction in carrier detection, suggesting that this threshold should be reconsidered. To that end, we propose an alternative model that can identify when a disease is too rare to include in an ECS panel based on its estimated clinical sensitivity.

#### G034. Clinical Impact and Cost Effectiveness of a 176 Condition Expanded Carrier Screen

K.A. Beauchamp, K.K. Wong, K.J. Taber, G. Lazarin, E.A. Evans, J.D. Goldberg, D. Muzzey  
Counsyl, Inc., South San Francisco, CA.

**Introduction:** Carrier screening identifies couples at high risk for conceiving offspring affected with severe heritable conditions. Minimal screening guidelines mandate testing for two diseases (cystic fibrosis and spinal muscular atrophy), but expanded carrier screening (ECS) assesses reproductive risk for hundreds of conditions simultaneously. Although key medical societies consider ECS an acceptable practice, and hundreds of thousands of patients have undergone ECS testing, the health economics of ECS remain incompletely resolved. **Methods:** The clinical impact and cost-effectiveness of a 176-condition ECS panel were investigated using a decision-tree model comparing minimal screening and ECS. Carrier rates from >40,000 ECS patients informed disease-incidence estimates, while cost and life-years-lost data were aggregated from the literature and a cost-of-care database. Model robustness was evaluated using one-way and probabilistic sensitivity analyses. **Results:** For every 100,000 pregnancies, approximately 300 are predicted to be affected by conditions on the ECS panel, which individually incur \$1,000,000 in lifetime costs and increase mortality by 30 undiscounted life years on average. Relative to minimal screening, ECS reduces the affected-birth rate by 66% and is cost-effective (i.e., <\$50,000 incremental cost per life year), findings that are robust to reasonable model-parameter perturbation. **Conclusions:** ECS is predicted to substantially reduce the population burden of Mendelian disease in a manner that is cost-effective compared to other common medical interventions.

#### G035. Detection of Copy-Number Variants in Expanded Carrier Screening Maximizes Identification of Cystic Fibrosis Carriers

D. Muzzey, P.V. Grauman, L. Spurka, K.A. Beauchamp  
Counsyl, Inc., South San Francisco, CA.

**Introduction:** Cystic fibrosis is one of the most common autosomal recessive conditions. Medical-society guidelines recommend routine carrier screening for cystic fibrosis via targeted genotyping of 23 frequent single-nucleotide variants (SNVs) and short insertions or deletions (indels) in the *CFTR* gene. Screening for copy-number variants (CNVs) is recommended only when a reproductive partner is a known carrier. Here we assess the performance and clinical impact of routinely screening for SNVs, indels, and CNVs in a next-generation sequencing (NGS)-based expanded carrier screen (ECS). **Methods:** Pathogenic variants in *CFTR* from 103,718 patients were discovered via a validated NGS-based ECS. A custom algorithm identified CNVs via relative deviations in NGS read depth: downward depth deflections signified deletions and upward deflections indicated duplications. Approximate CNV breakpoints were inferred from the NGS-depth profile. Positive CNVs were orthogonally assessed via multiplex ligation-dependent probe amplification (MLPA). *CFTR* CNV sensitivity was explored across a range of length scales via in silico simulations. **Results:** Among carriers of cystic fibrosis,

98.7% had pathogenic SNVs or indels in the *CFTR* gene (79% had one of the 23 common variants), and the remaining 1.3% harbored a pathogenic CNV spanning at least one exon. In total, we observed 25 unique CNVs with a diversity of breakpoints, suggesting that algorithms must be configured to detect novel CNVs. Further, single-exon deletions were observed for 7 different *CFTR* exons; analysis of confidence scores for these empirical deletions—coupled with extensive simulations—demonstrated that the bioinformatics pipeline was both accurate and robust, even for short CNVs. Simulations further show that CNV calling maintains high accuracy amid large deviations in read depth and/or in the number of background samples used for normalization. Additional analysis revealed that NGS-based CNV detection has expected accuracy comparable to MLPA. **Conclusion:** CNV detection maximizes identification of cystic fibrosis carriers and can be applied to all patients undergoing an NGS-based ECS test validated to detect these complicated variants. Clinical guidelines recommending screening of only the 23 most frequent variants miss critical identification of carriers and should be revisited.

#### G036. The Algorithm for Estimation of Human T-cell Receptor Repertoire with Single Cell RNA Sequencing

Y. Cho<sup>1,2</sup>, S. Yun<sup>2</sup>, M. Han<sup>2</sup>, M. Lee<sup>1</sup>, J. Seok<sup>1</sup>

<sup>1</sup>Korea University College of Medicine, Seoul, Korea; <sup>2</sup>Korea University Hospital, Seoul, Korea.

**Introduction:** For the clinical use of T-cell receptor (TCR) repertoire analysis, such as a marker for the immune status of cancers or infectious diseases, the magnitude and distribution characteristics of TCR repertoire in T cell subsets should be first elucidated. So far, the TCR repertoire in human was reported approximately as  $2.5 \times 10^7$ , and that of memory T cells was around  $1 \sim 2 \times 10^5$  when  $\alpha\beta$  TCRs were amplified and sequenced separately. Simultaneous TCR RNA sequencing of  $\alpha\beta$  chains in single cells has been recently available, so we analyzed TCR repertoire using single cell TCR RNA sequencing and set up a model algorithm for the number of unique clonotypes in CD4+ and CD8+ T cells, respectively. **Methods:** Twenty mL of peripheral blood was drawn from a healthy donor and T-cells were enriched using EasySep human T cell isolation kit (Stemcell Technologies, Vancouver, Canada). CD4+ T cells and CD8+ T cells were then separated using MACS memory CD4+ T cell and CD8+ memory isolation kit (Miltenyi Biotec Inc., Auburn, USA), respectively. The isolated cells were divided into seven channels for CD4+ and eight for CD8+ cells, and run on Chromium controller with the single cell immune profiling solution (10x genomics, Pleasanton, USA). Raw sequencing data obtained from HiSeq 4000 (Illumina, San Diego, USA) were processed using 10x Genomics' Cell Ranger 2.1 software. Statistics and algorithm modeling was performed using R programming language. **Results:** Mean estimated number of clonotypes for CD8+ and CD4+ T-cells were 4,095 and 7,456 with no significant difference in the clonotype frequency among eight channels ( $p > 0.05$ ). Only  $1\alpha$  and  $1\beta$  TCR variable chain pairs, comprising 75% and 79% of CD8+ and CD4+ T-cells, respectively, were used for further analysis. 6.1% and 0.5% of specific TRA/TRB v gene pairs and specific TRA/TRB cdr3 pairs of CD4+ cells were shared with CD8+ cells, respectively. 9.4% and 1.4% of specific TRA/TRB v gene pairs and specific TRA/TRB cdr3 pairs of CD8+ cells were shared with CD4+ cells, respectively. Only the non-shared cdr3 clonotypes were included in algorithm modeling and amino acid (aa) number analysis. The calculated exponential regression models that fit the number of observed unique clonotypes for CD8+ and CD4+ T-cells will be outlined at the meeting presentation, but indicated that the number of aa of TRA cdr3 in CD8+ cells is lower than that of CD4+ cells, leading to a lower aa number of TRA/TRB cdr3 in CD8+ cells compared to that of CD4+ cells (12 & 15,  $p < 0.5$ ). The other aa numbers didn't contribute the length of aa of TCR cdr3 pairs. **Conclusions:** We established an algorithm model to estimate the TCR repertoire in human and found the contributing factor in the TCR cdr3 aa length.

#### G037. Leveraging Unique Molecular Indices to Detect Chimeric Gene Fusions

B. Vilhjalmsdottir<sup>1</sup>, F. Lescai<sup>1</sup>, T. Ras<sup>1</sup>, Z. Etches<sup>1</sup>, V. Gregersen<sup>1</sup>, R.G. Madsen<sup>1</sup>, S. Tian<sup>2</sup>, D. Kamaev<sup>1</sup>, M.M. Johansen<sup>1</sup>, L. Husted<sup>1</sup>, J. Caffrey<sup>2</sup>, A. Hein<sup>1</sup>, E. Lader<sup>2</sup>, L. Schausser<sup>1</sup>, M. Simonsen<sup>1</sup>



<sup>1</sup>QIAGEN Aarhus, Aarhus, Denmark; <sup>2</sup>QIAGEN Sciences, Frederick, MD.

**Introduction:** Fusion genes are driver mutations in several diseases, particularly cancer. Because they produce chimeric proteins, fusion genes are useful biomarkers for diagnosis, prognosis and treatment in numerous types of neoplasia. Detection of certain fusion genes in a cancer patient therefore becomes an important task in bioinformatics analyses. For this reason, there are many fusion gene detection methods available. However, most of them are not compatible with reads tagged with Unique Molecular Indices (UMIs), and thus unable to make use of their advantages for estimating expression levels and detecting variants (Xu *et al.*, BMC Genomics 2017). **Methods:** To address this, we developed a novel fusion detection method that leverages UMIs to identify gene fusions. We developed a workflow for analysing datasets obtained with the QIAseq Targeted RNAseq panels. The method first identifies fusion candidates by re-mapping unaligned ends of reads that map partially to one gene to all gene models of the genome, taking broken paired-end read information into account. In addition, the method considers exon boundaries, fusion redundancy, and other features when aggregating the evidence for the candidate fusion(s). It then re-maps all of the reads to a transcript reference database that includes the identified candidate fusions using the QIAGEN CLC RNA-seq aligner. The method along with user-friendly analysis workflow is implemented in the QIAseq Targeted Panel plugin v. 1.1 or higher, which is available in the CLC Biomedical Genomics Workbench. **Results:** We benchmarked the workflow by applying it to 7 test datasets, and demonstrate that the method is able to detect gene fusions with high accuracy. Indeed, out of 38 fusions we detected 36 and only two false positives. In addition, the method successfully identified an exon skipping event. **Conclusions:** Fusion genes play a significant role in tumorigenesis, and some are already used as biomarkers for specific cancer types or subtypes in clinical settings. Using QIAseq Targeted RNAseq panels with UMIs, together with a dedicated bioinformatics pipeline, we observe increased accuracy for detecting fusions.

#### G038. Pharmacogenomics: *VKORC1* + *CYP2C9* and *TPMT*: Two New, Ready-to-use Real-time PCR Assays

M. Gramegna, L. Bavagnoli, M. Ballarín, G. Ferri, M. Incandela, D. Rigamonti, L. Turner, L. Spinelli  
Sentinel CH SPA, Milan, Italy.

**Introduction:** Pharmacogenomics is the study of the role of the genome in drug response. It analyzes how the genetic composition of an individual affects his/her response to drugs. Warfarin is an anticoagulant that acts by reducing the activity of vitamin K-dependent clotting factors. It is used in the prevention and treatment of thrombotic disorders. Warfarin dosing is associated with highly variable responses between individuals and challenges achieving and maintaining levels within the narrow therapeutic range that can lead to adverse drug events. The dose of warfarin must be tailored for each patient according to the patient's INR response and the condition being treated. The *VKORC1* gene encodes the vitamin K epoxide reductase enzyme, the target of warfarin. Patients who carry the -1639G>A polymorphism in the promoter region of the *VKORC1* gene are more sensitive to warfarin and require lower doses. Thiopurine S-methyltransferase (TPMT) is a key enzyme that deactivates thiopurines drugs used to treat acute lymphoblastic leukemia (LDT), autoimmune diseases and recipients of transplanted organs, into their inactive metabolite 6-methylmercaptopurine. Intermediate and low TPMT activity may lead to leukopenia following thiopurine treatment. In this context, genetic testing is a useful tool to allow the prediction of the response to the initial dose during therapy. The aim of this study was the development of Real-Time PCR tests for the detection and genotyping of the polymorphisms of *VKORC1* and *TPMT*. **Methods:** A novel Real-time PCR-based assay was developed as a ready-to-use test with a specific set of primers and probe able to identify of the following polymorphisms: *VKORC1* (G1639A), *CYP2C9* \* 2 (430 C> T), *CYP2C9* \* 3 (1075 A> C) and the following polymorphisms: *TPMT* \* 2 (G238C), *TPMT* \* 3B (G460A), *TPMT* \* 3C (A719G) within human genome. The sets were combined in a lyophilized freeze-dried ready-to-use mix and both targets were co-amplified and detected using different Real-time PCR instruments. In the present study, several samples extracted from whole blood obtained from San Raffaele Hospital in Milan and previously

genotyped with a LDT were investigated. **Results:** This new freeze-dried ready-to-use assay showed to be specific and sensitive for every polymorphism tested. All the tests performed with these assay confirmed the results obtained at San Raffaele Hospital and allowed the discrimination and genotyping of each sample. **Conclusions:** These novel Real-time PCR assays proved their effectiveness for the detection and genotyping of DNA in clinical samples. Its high-sensitivity and specificity, associated with the ready-to-use feature and room temperature storage, would easily improve the early and correct management of patients avoiding ADR's events.

#### G039. Interpretation of Microdeletion Variants Aided by Population Analysis of Copy-number Variation

K.E. Kaseniit, G. Hogan, K. D'Auria, C. Haverly, D. Muzzey  
Counsyl, Inc., South San Francisco, CA.

**Introduction:** While non-invasive prenatal screening (NIPS) has largely focused on detecting fetal trisomies of chromosomes 13, 18, and 21 due to their well-established clinical consequences, novel fetal microdeletions can also be identified by NIPS. However, these variants are challenging to interpret clinically and are often reported as findings without specific interpretations. We investigated whether the prevalence of copy-number variants (CNVs) in an ostensibly healthy population could aid the interpretation of novel microdeletions. **Methods:** We identified maternal copy-number variants (mCNVs) spanning at least 200kb in 87,255 NIPS samples. We calculated the proportion of each autosomal, 10Mb sliding window that was covered by at least three observed deletions in our mCNV dataset, termed the "deletion span." The corresponding duplication span served as a proxy to control for CNV propensity in the region. The ratio of the two spans ("dup:del ratio") and the gene density was evaluated for known pathogenic microdeletions, as well as 10Mb windows tiling the genome. **Results:** The deletion and duplication span measurements were significantly correlated (Pearson  $r = 0.73$ ), consistent with there being an intrinsic positional-dependent propensity for CNVs. We postulated that microdeletions would be pathogenic if they had high dup:del ratio and/or high gene density. Three of five commonly tested pathogenic microdeletions (1p36, 4p16, 15q11) had high gene density and elevated dup:del ratio. The 22q11.21 region had a nearly 1:1 dup:del ratio (10th percentile), but high gene density. The 5p13 region had the opposite: an elevated dup:del ratio ( $\geq 99$ th percentile) but near-average gene density. Most additional expert-curated microdeletions of at least 1Mb were also outliers in one or both metrics, with two exceptions that had low span values ( $\leq 10\%$ ). These findings suggest that general CNV-intolerance can be an additional metric to consider when interpreting novel microdeletions. Large mCNVs observed in our ostensibly healthy cohort were evaluated using the dup:del ratio and gene density metrics, and all but one had low or average values, consistent with these variants being benign as expected. The one outlier with high dup:del ratio (13q34 terminal deletion) was indeed reported as pathogenic in the literature. **Conclusions:** Novel microdeletions could be evaluated for their gene density and duplication:deletion ratio to determine possible pathogenicity. Either metric in isolation might fail to identify certain pathogenic variants.

#### G040. Frequency of Deletion 13q Associated with other Abnormalities Detected by Fluorescence *in situ* Hybridization (FISH) in Multiple Myeloma Patients – an Experience from a Referral High-end Diagnostic Centre

M. Kumar, B. Salona, L. Kini, S. Mohanty  
CORE Diagnostics, Gurgaon, India.

**Introduction:** Plasma cell myeloma (PCM) has known to exhibit cytogenetic abnormality, which helps in risk stratification and therapy selection. Fluorescence *in situ* hybridization (FISH) assay following PE is a relatively novel technique to increase the cellular yield for detecting abnormalities in PCM. However, there is no such data available from India. Therefore, we sought to evaluate the potential utility of FISH assay following PE for detection of genetic abnormalities in PCM. **Methods:** FISH testing for del13q(RB1), del17p(TP53), rearrangement of IGH locus t(4;14) IGH/FGFR3, t(11;14) IGH/CCND1, t(14;16) IGH/MAF, and t(14;20) IGH/MAFB were performed in 145 bone marrow samples obtained for Multiple myeloma patients over a one year period from December 2014 to December 2015. **Results:** Out

of 145 cases, 55(37.9%) were observed for of del13q (Age mean-61.04, Median-62, Range-35-90 years). Male female ratio was 1.11:1. Interstitial deletions were observed 10(18.18%). Male female ratio was 9:1 for the cases of deletion of interstitial cases. Terminal deletions were observed in 44 cases (80%). Male to female ratio was 0.83:1 for the cases of deletion of terminal cases. 27 cases (49.09%) of del13q were observed with other cytogenetic abnormalities. However, 4 cases (14.8%) from interstitial deletions and 23 cases (85.18%) were observed from Terminal deletion. 42 cases (76.36%) were found in the age group > 55 years and 13 cases (23.64%) found in the < 55 year age group. **Conclusions:** Deletion 13q is the most frequent chromosomal abnormality in the form of terminal deletion found in cases of multiple myeloma patient ( $p < 0.0001$ ). Interstitial deletions were commonly observed in male patients. This may be an incidental finding and further studies with large patient cohorts and clinical outcomes are needed to definitively determine the predictive value of this cytogenetic finding. Interestingly, no molecular abnormality was detected in patients less than 35 years old. Further studies in a larger cohort of young patients are needed to understand the biology of PCM in young individuals. These terminal deletions have paved the way for further research at the molecular level, to find out the effects on prognosis. Furthermore, cytogenetic studies are warranted in larger groups of multiple myeloma cases to identify different types of del 13q that may aid in cloning new genes involved in the prognosis of patients, ultimately helping in the development of targeted therapeutic drugs.

#### G041. Molecular Diagnosis of Graft-versus-Host Disease after Liver Transplantation: an Institutional Experience

S.M. Hosseini, J. Saab, V.M. Van Deerlin, M. Haldar  
University of Pennsylvania, Philadelphia, PA.

**Introduction:** Graft-versus-host disease (GVHD) is a serious multi-organ immune mediated complication of hematopoietic stem cell and solid organ transplants and an underdiagnosed cause of mortality in liver transplantation. Diagnosis of GVHD after liver transplant is challenging as it is rare (incidence 0.5-2%) and typically presents with a constellation of non-specific clinical and histologic signs. The identification of significant lymphoid macrochimerism (> 1% donor cells) can be used to confirm GVHD. Chimerism analysis using short tandem repeat (STR) PCR has been successfully used for this purpose. Here, we present our institutional experience with molecular identification of GVHD in liver transplant recipients. **Methods:** Recipient and donor STR alleles were genotyped using DNA extracted from paraffin blocks of explanted liver and donor gallbladder, respectively. When possible, DNA from buccal swabs and/or pre-transplant peripheral blood was used to confirm the recipient genotype. Donor chimerism in post-transplant peripheral blood, CD3+ T-cell subset, or bone marrow was determined by PCR amplification of up to 15 STR markers and comparison of donor and recipient allele peak areas using either manual calculations or ChimerMarker software. Chimerism data were systematically extracted from reports of testing in cases of suspected GVHD. Descriptive statistics were summarized with R.

**Results:** Among 1393 liver transplantations performed at our institution between 2007 and 2017, 17 patients were referred for testing, 6 (35%) of them showing macrochimerism, with percent donor DNA between 6% and 95% in peripheral blood, consistent with GVHD (period prevalence = 0.0043). The primary test was requested 45 (range 22-892) days post-transplant with cytopenia (53%) and rash (35%) as the most common presenting symptoms. Seven patients had follow-up testing (1 to 5 follow-up tests), which were all consistent with the primary test results (6 positive and 1 negative). Median report time was 6 days (range 0-14). All patients (6 of 6) with confirmed GVHD died 32 (range: 6-64) days after diagnosis due to related causes, versus 5 of 11 (45%) patients without macrochimerism ( $p = 0.02$ ). There was no correlation between the quantitative level of chimerism and time to death. **Conclusions:** Molecular testing is efficient and reliable for diagnosis of GVHD after liver transplantation. The complexity of obtaining pre-implant specimens is the main contributing factor to turnaround time, which could be reduced by solutions such as banking donor and recipient DNA in advance. Overall, molecular testing could improve timely diagnosis of GVHD that is important in optimizing therapy and increasing survival in these patients. Further scrutiny of risk factors was precluded by rarity of cases.

#### G042. Analytical Performance of the Oncomine BRCA1/2 Assay on the Ion Torrent S5

D. Saxena<sup>1</sup>, A. Set<sup>1,2</sup>

<sup>1</sup>Lincoln Diagnostics, Linden, NJ; <sup>2</sup>Accureference Medical Laboratory, Linden, NJ

**Introduction:** Germline mutations in tumor suppressor *BRCA1* and *BRCA2* genes predispose women to greater risk of hereditary breast and/or ovarian cancer. Next-generation sequencing is advantageous over traditional sequencing methods to enable simultaneous detection of a variety of pathogenic variants in these genes. These include single nucleotide variants (SNVs), small insertion/deletions (indels) and large exon level copy number variants (CNV). The aim of this study was to evaluate the performance characteristics of the Oncomine *BRCA1/2* panel on the Ion Torrent S5 platform at Lincoln Diagnostics, NJ. **Methods:** A total of 33 specimens (reference standards from Horizon and Coriell  $n = 18$ , and patient specimens  $n = 14$ ) with previously determined mutation status were used for validation. DNA extraction from blood and buccal swabs was validated on the QIAGEN EZ-1 Advanced XL. Library preparation and templating was performed on Ion CHEF followed by sequencing on the Ion Torrent S5 sequencer. Sequencing data was analyzed using the Ion Reporter Software bioinformatics pipeline. **Results:** Using the Oncomine *BRCA1/2* panel, 23 SNVs, 19 insertions, deletions or frameshift mutations, and 4 CNVs were identified in the validation specimens. The sequencing performance showed the average total number of reads per run to be 5 million ( $SD \pm 0.5M$ ). Average output was 560M bases and out of those, an average of 514M bases met a quality score of Q20. Average mean read length obtained was 109 bases. The depth of coverage obtained ranged between 1463X to 6391X. Uniformity of coverage averaged about 98.5% across the samples. Accuracy of *BRCA1/2* mutation detection using specimens with known *BRCA1/2* mutations was 100%. All SNVs, indels and CNVs were correctly identified. Analytical sensitivity was assessed using reference standard HD 795 harboring wild-type or mutated *BRCA1/2* at a pre-verified allelic frequency for each mutated allele. Allelic frequency of variants in HD795 using the Oncomine *BRCA1/2* were detected with high level of accuracy and concordance (Correlation Coefficient  $R^2 = 0.99$ ). To assess the reproducibility of variant calling, representative specimens having either SNV, indel or CNV were correctly called in both inter ( $SD \pm 0.7$  to  $\pm 1.5$ ) and intra-run assays ( $SD \pm 2.1$ ). The Ion Reporter Software bioinformatics pipeline was able to annotate all variants except one 40bp deletion which was detected using custom analysis in Torrent suite software.

**Conclusions:** The Oncomine *BRCA1/2* assay showed excellent performance characteristics in our lab. The panel is very useful for identification of single nucleotide variants, small indels as well as large copy number variations in a single assay and is a viable alternative to multiple conventional technologies.

#### G043. A Novel Custom Panel Target Sequencing with Molecular Tags for 0.1% Allelic Frequency Detection

X. Peng, D. Mazur, G. Luo, S. Chen, K. Park, K. Heinemann, A. Kraltcheva, B. Ding, A. Hatch, Y. Fu, M. Andersen  
Thermo Fisher Scientific, Carlsbad, CA.

**Introduction:** Liquid biopsy Next-Generation Sequencing (NGS) assays for non-invasive cancer mutation detection using only cell-free DNA (cfDNA) from plasma samples have gained momentum in recent years. Comparing to solid tumor NGS assay, mutant detection in liquid biopsy samples is very challenging due to low level (less than 1%) of cancer cell derived DNA presence in samples on the background of nonmalignant cells germline origin DNA. To achieve relevant sensitivity and specificity for liquid biopsy NGS assay, a new approach using molecular tags has been introduced to link the sequencing reads with their corresponding origin cfDNA molecules for ultrasensitive and reliable variant detection. However, there is an unmet need in current available technology for customers to design and order their own target gene panels and to use directly as custom panel NGS assays. **Methods:** We developed a novel Ampliseq™ HD featuring custom panel target sequencing with molecular tags for ultrasensitive cancer mutations detection in liquid biopsy and FFPE samples. **Results:** We have

demonstrated down to 0.1% allelic frequency detection with up to 90% sensitivity and up to 100% specificity. We have also achieved up to 100% sensitivity and up to 100% specificity for RNA fusion detection. This novel chemistry has been successfully tested for a wide range of custom panels with complexity up to 1000 Amplicons. **Conclusions:** We have developed a novel custom panel target sequencing with molecular tags method for 0.1% allelic frequency detection.

#### G044. Designing and Implementing NGS Tests for Inherited Disorders – a Practical Framework with Step-by-step Guidance for Clinical Laboratories

A.B. Santani<sup>1,9</sup>, B. Simen<sup>2,9</sup>, M. Briggs<sup>3</sup>, M. Lebo<sup>4</sup>, J. Merker<sup>5</sup>, M. Nikiforova<sup>6</sup>, K. Voelkerding<sup>7</sup>, J. Pfeifer<sup>8</sup>, B. Funke<sup>2</sup>

<sup>1</sup>Children's Hospital of Philadelphia, Philadelphia PA; <sup>2</sup>Veritas Genetics, Boston, MA; <sup>3</sup>College of American Pathologists, Chicago, IL; <sup>4</sup>Harvard Medical School/Brigham and Women's Hospital, Boston, MA; <sup>5</sup>University of North Carolina School of Medicine, Chapel Hill, NC; <sup>6</sup>University of Pittsburgh Medical Center, Pittsburgh PA; <sup>7</sup>University of Utah School of Medicine/ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT; <sup>8</sup>Washington University School of Medicine, St Louis, MO; <sup>9</sup>University of Pennsylvania, Philadelphia, PA.

**Introduction:** Comprehensive next generation sequencing (NGS) tests are increasingly used as first-line tests in the evaluation of patients with suspected heritable disease. Despite major technical simplifications, these assays still pose significant challenges for molecular testing laboratories. Existing professional guidelines and recommendations provide a framework for laboratories implementing such tests, but in-depth, concrete guidance is generally not provided. Consequently, there is variability in how laboratories interpret and subsequently implement these regulatory frameworks. To address the need for more detailed guidance, the College of American Pathologists (CAP) with representation from the Association for Molecular Pathologists (AMP) assembled a working group to create a practical resource for clinical laboratories. **Methods:** The working group consisted of experts in NGS, bioinformatics, quality management and accreditation requirements from academic and commercial entities. Members included clinical laboratory directors, bioinformaticians, clinicians and individuals engaged in developing CAP's accreditation guidelines. Regular meetings were held by phone to review and iteratively improve the worksheets. **Results:** The enormous diversity of clinical testing scenarios and technology platforms makes all-encompassing guidance challenging. The working group therefore focused on major concepts and the most frequently used NGS selection methods (hybridization capture and amplicon sequencing). This resource consists of a set of practical, ready-to-use worksheets that were structured to cover each step in the lifecycle of an NGS test and expand the scope significantly compared with existing guidance documents, which are traditionally less focused on test design considerations. The worksheets cover test design, optimization, validation, and quality management. Bioinformatics aspects, which are integral components of every step in the lifecycle of an NGS based laboratory developed test (LDT), are addressed within each document as well as a separate bioinformatics guidance worksheet. To limit complexity, this initial set of worksheets was designed to address NGS testing for germline disorders as practices and requirements for somatic testing have significant differences. **Conclusions:** This resource is designed to be publicly available and will be updated with user and expert feedback as the wet bench and bioinformatic landscapes continue to evolve. It is intended to facilitate the standardization of NGS testing across laboratories and therefore to improve patient care.

#### G045. SMA Complete: Addressing SMN Copy Number and Silent Carrier Status with a Single Complete Multi-plex qPCR Assay

R. Daber<sup>1</sup>, R. Schaller<sup>2</sup>, A. Lee<sup>2</sup>, J. Ching<sup>2</sup>

<sup>1</sup>Gnosity Consults, Farmingdale, NJ; <sup>2</sup>Coyote Bioscience USA, Inc., Campbell, CA.

**Introduction:** Spinal Muscular Atrophy (SMA) is an autosomal recessive neuromuscular disorder with severe manifestations characterized by muscle weakness and atrophy. It is caused by deletions in the *SMN1* gene or by conversion of the *SMN1* gene to its non-functional homolog *SMN2*. With an incidence of approximately 1 in 10,000 livebirths, SMA is

one of the most common autosomal recessive diseases, resulting in a carrier frequency as high as 1 in 40 depending on ethnicity. Routine carrier screening addresses *SMN1* loss by interrogating copy number status. In some ethnicities, individuals may present with a *SMN1* copy number status of 2, while still being at risk of passing a null allele to their offspring due to the presence of a 2-copy allele on a single chromosome. Routine screening to identify individuals who possess this 'silent' allele is generally not performed due to the lack of method to do so. Here we describe a highly multiplexed qPCR assay that leverages Locked Nucleic Acid (LNA) probes for increased affinity to capture *SMN* copy number status and genotype status for the 2-0 allele in a single reaction.

**Methods:** *SMN1* and *SMN2* differ in genomic sequence in only several locations. One of these locations was selected to molecularly discriminate between the two genes through highly selective LNA probes. Multiplex PCR primers were designed for 3 genomic positions: 1) the RPP30 copy number reference loci, 2) c.840 and 3) g.22706\_22707 within the *SMN1* gene. Due to sequence homology, the primers for amplicon 2 and 3 simultaneously amplify *SMN1* and *SMN2* genomic DNA. LNA probes were designed to differentiate between *SMN1* and *SMN2*, while additional LNA probes were utilized to genotype the 2-0 silent allele within the *SMN1* gene. The 5-color multiplex assay was optimized on multiple different qPCR platforms including the QuantStudio 7. **Results:** Across both cell line controls and DNA derived from tissue specimens, the multiplex assay was able to correctly identify *SMN1* deletion carriers and silent allele carriers. Additional testing also demonstrated that the assay could be utilized to detect complete loss of *SMN1* which results in various degrees of clinical presentation as well as homozygotes for the 2-copy haplotype. The LNA probes showed 100% specificity when tested on homozygotes for the alternative allele demonstrating the robust nature of the molecular approach on highly homologous genes. **Conclusions:** We developed a robust easy to use lyophilized, complete single-tube, 5-color, multi-plex qPCR assay that successfully determines *SMN* copy number status and detects the presence of the silent allele haplotype within a single reaction. This novel assay improves overall sensitivity compared to traditional *SMN1* copy number assays.

#### G046. Double Splicing Variants in BCR-ABL are Associated with Tyrosine Kinase Inhibitor (TKI) Resistance in Chronic Myelogenous Leukemia (CML)

D. Dash, V. Trapp-Stamborski, L.D. Wang  
Bloodcenter of Wisconsin, Milwaukee, WI.

**Introduction:** More than 90% of chronic myelogenous leukemia (CML), and 25 to 30% of adult and 2 to 10% of childhood acute lymphoblastic leukemia (ALL) have a Philadelphia chromosome with the t(9;22) translocation. This translocation generates an oncogenic *BCR-ABL* fusion gene which results in constitutive tyrosine kinase activity and unregulated cell division. Although targeted tyrosine kinase inhibitors (TKIs) to *BCR-ABL* fusion protein have demonstrated significant antileukemic activity, many CML patients ultimately develop clinical resistance to TKIs after an initial obvious response. Convincing evidences demonstrate that the majority of relapsed patients carry mutations within the *BCR-ABL* kinase domain. **Methods:** The kinase domain of *BCR-ABL* fusion gene was analyzed by RT-PCR followed by DNA sequence analysis covering amino acids 236-486. This region covers all the reported kinase domain mutations. **Results:** Double splicing variants in *BCR-ABL* were detected in two patients with resistance to TKIs. A 35INS variant results in a 35-bp insertion at the junction of exons 8 and 9; a 84INS variant results in an 84-nucleotide insertion at the junction of exons 7 and 8. No other known TKIs-related mutations were identified in *BCR-ABL* kinase domain of either of the two patients. **Conclusions:** It has been well studied that missense *BCR-ABL* mutations lead to resistance by impairing TKI binding to the *BCR-ABL* kinase domain. The finding of double variants of 35INS and 84INS in this study indicates that splicing variants may also be associated with TKIs resistance in CML patients, and suggests a potential alternate mechanism of TKIs resistance.



#### G047. Validation of a Neuro-Oncology Next-generation Sequencing 219-Gene Panel

C. Zysk, E. Barr Fritcher, J. Voss, J. Balan, J. Balcom, X. Wu, L. Peterson, K. Thomas, A. Raghunathan, C. Giannini, E. Klee, B. Kipp, R. Jenkins, C. Ida

Mayo Clinic, Rochester, MN.

**Introduction:** The WHO classification scheme of central nervous system (CNS) tumors has incorporated molecular parameters. Thus, genetic testing became central to yield an integrated histological-molecular classification of CNS tumors in addition to identifying potential therapeutically relevant alterations. This validation study describes the performance characteristics of a next generation sequencing (NGS) panel that interrogates 219 genes associated with adult and pediatric CNS tumors. This assay includes DNA (150 genes) and RNA (80 genes) subpanels (11 genes are included in both panels) and was developed to replace our current NGS neuro-oncology 50-gene panel. **Methods:** DNA and/or RNA were extracted from 104 formalin-fixed paraffin embedded (FFPE) samples predominantly consisting of adult and pediatric CNS tumors. NGS library preparation was performed using custom DNA and RNA panels with the QIAseq targeted chemistry (Qiagen Inc., Germantown, MD), which utilizes molecular bar code technology to trace PCR artifacts/duplicates. Final PCR product from the DNA and RNA panels were respectively sequenced on HiSeq and MiSeq instruments (Illumina, Inc., San Diego, CA). Sequencing data were processed through custom bioinformatics pipelines. Detected DNA sequencing variants were compared to previous NGS neuro-oncology 50-gene results or confirmed with an alternative NGS assay. Detected fusions and transcript variants were confirmed using reverse transcription PCR or chromosomal microarray. **Results:** Variant/fusion transcript detection accuracy and success rate were 99% (481/488) and 97% (64/66) for the DNA panel, and 98% (48/49) and 95% (59/62) for the RNA panel, respectively. Inter and intra-assay reproducibility was 100% for both panels. Analytical sensitivity levels were estimated as 8.5 ng input DNA and 15% mutant allele frequency with a minimum coverage of 100X in a sample with  $\geq 30\%$  tumor content for the DNA subpanel; and 10 ng input RNA, a minimum of 5 unique fusion molecules and 10 targeted fusion reads in a sample with  $\geq 10\%$  tumor content for the RNA panel. Analytical specificity was high, with per base DNA sequencing false positive rate  $< 0.4\%$  and absent fusion transcript detection in non-neoplastic samples. **Conclusions:** We verified the analytical validity of a custom 219-gene neuro-oncology NGS clinical test for FFPE specimens, including biopsies with small amounts of tissue and low tumor content. This clinical test can be used to assist in the diagnosis/classification and therapeutic management of adult and pediatric patients with CNS tumors, and has the potential to lead to the discovery of novel genomic abnormalities that may expand the understanding of the molecular biology of these tumors.

#### G048. IGF1 Proteomic Variant Confirmation using Genotyping Assay

A.D. Maus, J.V. Kemp, R.J. Singh, D. Milosevic, S.K. Grebe

Mayo Clinic and Foundation, Rochester, MN.

**Introduction:** Insulin-like growth factor 1 (IGF1) is the main mediator of the effect of growth hormone (GH). Serum concentrations of IGF1 are used as to diagnose growth related disorders. Recently, IGF1 immunoassay was replaced with high-resolution accurate mass LC-MS assay (HRAM LC-MS) in order to minimize reagent lot-to-lot inconsistencies that bedevil all IGF1 immunoassays. During assay validation we noticed that the LC-MS assay gave lower results than the immunoassay in about 0.6% of patients, if a narrow data acquisition window was used. Widening the window we found that these cases contained single amino acid variants. The two most common variants observed were normal sequence variants, but several other variants of either unknown significance or known pathogenicity were also seen. Some of these potentially damaging variants were very close in their mass charge ratios to the two common normal sequence variants, but could still be separated by the high instrument resolutions when using a center of mass (COM) algorithm, followed by confirmation with MS/MS. In order to validate the accuracy of this approach, we developed a method to retrieve, and sequence, cell free DNA from residual serum samples that had been found by HRAM LC-MS to contain IGF1 variants. **Methods:** Residual patient serum samples (N=177) with single

amino acid variants, which had been detected by HRAM LC-MS, were selected for this study. Cell free DNA was extracted from these samples (~200ul) with the Qiagen Circulating Nucleic Acid kit (Qiagen, Valencia, CA). Two targeted PCR reactions were performed to amplify the entire exonic and flanking intronic genomic sequence corresponding to the mature IGF1 protein (70aa). The PCR reaction products were then sequenced using a universal primer protocol and BigDye Terminator chemistry (ABI BigDye Terminator TM v1.1). The sequencing traces were analyzed with Mutation Surveyor software. **Results:** IGF1 sequencing showed a 100% concordance with COM and MS/MS data. We were able to identify A67T (n=94), A70T (n=69), A67T/A70T (n=1), A67V (n=1). A pathogenic V44M variant (n=2) and a novel A38V variant (n=1) that has likelihood of being pathogenic using prediction software (PolyPhen2). Nine DNA samples had low sequence quality and will need repeating. **Conclusions:** HRAM LC-MS has the potential to discover protein sequence variants. Cell free DNA can be easily used to confirm the proteomic results in most cases, with sequencing failures or disagreements with proteomic data being uncommon. This approach could potentially be extended from IGF1 to other proteins.

#### G049. Adaptation and Validation of a Pan-cancer Somatic Next Generation Sequencing Assay for Detection of Germline Hereditary Cancer Predisposition Variants

D.K. Manning<sup>1</sup>, E.P. Garcia<sup>1</sup>, P.K. Davineni<sup>1</sup>, L. Bialic<sup>1</sup>, M.D. Ducar<sup>2</sup>, P. Shivadasani<sup>1</sup>, A.J. Church<sup>3</sup>, D.P. Simmons<sup>1</sup>, Y.Y. Li<sup>2</sup>, R.K. Jacobs<sup>2</sup>, M. Baltay<sup>1</sup>, J. Sotelo<sup>2</sup>, L.E. MacConaill<sup>2</sup>, N.I. Lindeman<sup>1</sup>, J.A. Nowak<sup>1</sup>

<sup>1</sup>Brigham and Women's Hospital, Boston, MA; <sup>2</sup>Dana Farber Cancer Institute, Boston, MA; <sup>3</sup>Boston Children's Hospital, Boston, MA.

**Introduction:** Between 10-15% of cancer patients harbor germline alterations associated with cancer predisposition. While somatic-only tumor sequencing has become commonplace, this approach cannot reliably distinguish between somatic and germline alterations. Our institution has performed somatic-only sequencing for over 30,000 cancers using a next-generation sequencing assay (OncoPanel) that currently tests 447 genes for single nucleotide variants (SNVs), insertions/deletions (indels), copy number variants (CNVs), and select rearrangements. We have now adapted and validated OncoPanel to detect germline alterations in peripheral blood samples across 147 genes associated with hereditary cancer risk. **Methods:** To enable analysis of somatic and germline specimens in the same sequencing run, a 48-plex plate configuration containing 22 somatic samples, 16 germline samples, and controls was developed in which somatic and germline libraries were combined at a 3:1 ratio for hybrid capture. Somatic validation samples (n=88) were chosen based upon prior OncoPanel or other clinical results. Germline validation samples (n=64) with known pathogenic or likely pathogenic (P/LP) SNVs, small insertion/deletions (indels), and CNVs were selected based upon prior whole exome sequencing or commercial genetic test results. A standalone germline bioinformatic pipeline utilizing the GATK HaplotypeCaller, or the internally developed tools RobustCNV and Breakmer, was implemented to detect SNVs, indels, and CNVs. Limit of detection and reproducibility studies were used to determine variant calling thresholds, and concordance analyses were performed to calculate clinical sensitivity and specificity for each variant type. **Results:** Germline SNV/indel detection exhibited 95% sensitivity and 98% specificity, with 100% reproducibility at a 15X or greater variant coverage depth. Germline CNV detection demonstrated 94% sensitivity, 99% specificity and 97% reproducibility for variants involving two or more exons of a gene. CNV detection for single exon variants demonstrated limited sensitivity and specificity. Novel variants were identified in previously untested genes for some patients. Modification of the assay to simultaneously sequence germline and somatic specimens did not compromise somatic variant detection; all prior established metrics were maintained. **Conclusions:** The OncoPanel germline assay successfully identified a broad array of known P/LP germline variants in validation samples and led to the discovery of new alterations. Germline variant detection across SNVs, indels, and copy number alterations involving at least two exons was highly concordant and reproducible. Germline samples were successfully sequenced in the same run as somatic samples without loss of somatic sequencing performance.

### G050. Expression Analysis of Telomere-related Genes in Solid and Hematologic Tumors Using RNA-Seq

M.A. Atiq, J.I. Davila, A.A. Nair, N.M. Fadra, R.N. Wehrs, R.A. Jackson, J.S. Voss, K.C. Halling  
Mayo Clinic, Rochester, MN.

**Introduction:** A telomere maintenance mechanism (TMM) is considered fundamental in conferring cancer cells with unlimited replicative potential. In the majority of cancers, this involves the upregulation of telomerase. We used RNA-seq to quantify the abundance of telomere-related transcripts in 69 tumors. **Methods:** TruSeq RNA-seq was performed on 69 tumor samples (31 carcinomas, 22 sarcomas, 11 leukemias, and 5 lymphomas) and 16 normal samples. All samples were derived from fresh frozen tissue or cell cultures. Whole transcriptome analysis was performed using the MAP-Seq bioinformatics pipeline and normalized RPKM expression values were obtained for 4 genes known to be involved in telomere maintenance. These included the genes encoding subunits of the telomerase enzyme complex (*TERT* and *TERC*) and surrogate genes of the 'Alternative Lengthening of Telomeres' (ALT) pathway of TMM (*ATRX* and *DAXX*). Using the two-tailed un-paired student's t-test, the tumor samples were compared to normal samples for assessment of differential expression. **Results:** There was no significant difference in expression of *TERT* or *TERC* in any of the 4 cancer types compared to normal. Individual samples exhibiting *TERT* over-expression (defined by a Z-score greater than 3 with respect to the normal samples) included 3 leukemia and 3 carcinoma samples, and 1 lymphoma and 1 sarcoma sample. *TERC* over-expression was found in 1 leukemia, 2 carcinomas, and 3 sarcoma samples. A significant upregulation of *ATRX* was found in carcinomas when compared to normal tissues (Mean RPKM: 5.6 vs 3.5, p: 0.03). Similarly, *DAXX* was significantly upregulated in the lymphomas compared to normal (Mean RPKM: 29.2 vs 14.9, p: 0.01) as well as in leukemias (Mean RPKM: 21.4 vs 14.9, p: 0.04). Six carcinoma samples (prostate adenocarcinoma, SCLC, NSCLC, bladder TCC, breast adenocarcinoma, and colon adenocarcinoma) demonstrated *ATRX* over-expression while 1 B-cell lymphoma sample and 1 leukemia sample (AML) showed *DAXX* over-expression. **Conclusions:** This study demonstrates the relative absence of *TERT* and *TERC* expression in a cohort of 69 tumor samples. This finding is largely consistent with data from cBioPortal where *TERT* and *TERC* over-expression is only observed in a small fraction of tumors. *TERT/TERC* over-expression and, consequently, increased telomerase activity may not be the dominant mechanism of telomere maintenance in a high proportion of tumors. Our data also suggests that the ALT pathway is not activated in most of these tumors either. Our findings suggest the involvement of additional, as yet unrecognized, genes/pathways in telomere maintenance in cancer cells. Furthermore, this study emphasizes the role of RNA-Seq in elucidating such pathways.

### G051. Meta-analysis of AKT1 rs2494732 Genotype and the Risk of Psychotic Adverse Effects by Cannabis Use

M. Nakano, T. Nappi, Z. Hsu, C. Sailey  
Molecular Testing Labs, Vancouver, WA.

**Introduction:** Transient anxiety and paranoia effects by  $\Delta$ -9 tetrahydrocannabinol (THC) were commonly observed adverse effects for many cannabis users. Beyond that, some users developed schizophrenia-like psychotic episodes. The variability of why some had experienced worse psychotic effects than others were not well understood, yet gene-cannabis interactions have been studied. The *AKT1* gene has been identified as a potential candidate because of its association with an increased risk of schizophrenia. Various *in vitro* and *in vivo* studies support this association: AKT serine/threonine kinase coded by the *AKT1* gene is activated by THC, and this kinase is involved in the striatal dopamine receptor signaling cascade. Additionally, multiple studies indicate that *AKT1* rs2494732 C/C genotype is significantly associated with risks of psychosis in cannabis users. Our company has interpreted individual's genomic DNA to assess cannabis use-related risks, and the *AKT1* variant is included in the testing panel. Therefore, the objectives of this study were to determine the odds ratio of rs2494732 genotype and self-reported anxiety and paranoia effects from those who participated in follow-up questionnaires and to perform a meta-analysis for the overall association of the rs2494732 variant and psychotic adverse effects of

cannabis. **Methods:** De-identified data were queried for the *AKT1* rs2494732 genotype and correlated to questionnaire answers inquiring if participants experienced adverse effects, anxiety and/or paranoia. For the meta-analysis, the NIH PubMed library was systematically researched for studies evaluating the association of *AKT1* polymorphisms and psychotic adverse effects. The odds ratios of the CC genotype were utilized for the study. **Results:** Of 178 genomic DNA analyzed, one hundred twenty one entries of self-claimed adverse effects paired with *AKT1* genotype data were utilized for the odds ratio analysis. Although multiple studies proved the association of rs2494732 variant and psychosis-like effects of cannabis use, the odds ratio was not statistically significant on either CC vs. TT genotype or C vs. T allele comparisons. The meta-analysis of odds ratios from our study and retrieved from 4 studies in publications indicated that the *AKT1* variant was significantly associated with psychosis-like effects with the averaged odds ratio of 2.27 (p<0.001). **Conclusions:** The association of the *AKT1* variant and psychotic effects were not reproduced with our study. However, the sample sizes and the self-reporting questionnaire system were the limiting factors of our study, and further investigation is warranted. The meta-analysis results confirmed that the *AKT1* variant was a potential genomic marker of psychotic adverse effects of cannabis.

### G052. In cis Heterozygous BRCA2 Pathogenic Mutations in a Jordanian Family: Case Report

L. Abu Jamous, W.M. Naser, R. Abu-Obeid, O. Alsmadi, N. Abdullah, F. Alul, H. Abdel-Razek, A. Tbakhi  
King Hussein Cancer Center, Amman, Jordan.

**Introduction:** Five to ten percent of breast cancers are thought to arise from genetic predisposition of highly penetrant breast cancer associated genes, *BRCA1* and *BRCA2*. The vast majority of patients with *BRCA1* and *BRCA2* mutations showed single and heterozygous variant. Several forms of Fanconi Anemia, characterized by bone marrow failure and malignancy, can be a consequence of biallelic mutations in *BRCA2* or biallelic mutations in genes encoding *BRCA1/2* associated proteins such as *PaLB2* and *BRIP1*. Biallelic *BRCA1* mutations have not been detected because one wild-type *BRCA1* allele is required during embryogenesis. Here we report a case of a 49 year-old lady with breast invasive ductal carcinoma, grade 3, ER positive, PR negative, HER negative. She was found to have two deleterious mutations in *BRCA2*, c.2254\_2257delGACT and c.5351dupA. Due to limited availability of family members, we did predictive test analysis by Sanger sequencing and fragment analysis for the patient's offspring to determine the phase of mutations (*i.e.* in *cis* or in *trans*). **Methods:** Two pathogenic mutations in the *BRCA2* gene, exon 11, were detected in the patient's blood by next generation sequencing (NGS) method, including [NM\_000059.3:c.2254\_2257delGACT; NP\_000050.2:p.Asp752Phefs] (rs80359326) and [NM\_000059.3:c.5351dupA; NP\_000050.2:p.Asn1784Lysfs] (rs80359508). Beside the index case, 4 family members including 3 daughters and 1 son were assessed for the presence of the same variants using flanking primers followed by PCR amplification. The amplified targets were sequenced and sized using 3130 Genetic Analyzer following standard procedures. Sequence traces and fragment analysis results were analyzed using Mutation Surveyor and GeneMarker (SoftGenetics, USA), respectively. **Results:** One of the 3 daughters showed *BRCA2* heterozygous pathogenic mutations (c.2254\_2257delGACT and c.5351dupA). The other 3 children were free of both mutations. These results confirm the *cis* phase of *BRCA2* mutations in the index patient. **Conclusion:** To our knowledge, this is the first report of a patient with a *BRCA2* compound heterozygous deleterious mutations in *cis*. This result highlights the uniqueness of breast cancer genetic predisposition in the Jordanian population with implications on genetic counseling.

### G053. Characterization of Novel Aneuploidy Reference Materials for NGS-based Non Invasive Prenatal Screening (NIPT)

F. Sabato<sup>1</sup>, V. Williamson<sup>1</sup>, C. Yan<sup>1</sup>, Y. Konigshofer<sup>2</sup>, R. Garlick<sup>2</sup>, A. Ferreira-Gonzalez<sup>1</sup>

<sup>1</sup>VCU Health, Richmond, VA; <sup>2</sup>SeraCare, Gaithersburg, MD.

**Introduction:** Widespread clinical implementation of next-generation sequencing (NGS)-based non-invasive prenatal screening (NIPT) highlighted the urgency to establish reference materials which could provide quality control of the process from testing to report generation. However, limited number of quality control and other reference materials are currently available to support this clinical testing. A challenging aspect of cell-free DNA (cfDNA) testing is the need to develop a reliable external quality assessment to compare the performance of different methods or algorithms for analysis of cfDNA in maternal plasma. Here, we evaluated the performance of reference materials in collaboration with SeraCare, using the VCU Health SNP-based NIPT test. **Methods:** We evaluated 2 NIPT reference materials, SeraCare1 (SC1) and SeraCare4 (SC4) derived from pregnant volunteers. In order to obtain relatively high fetal fractions, plasma samples were obtained on an IRB approved protocol from two pregnant donors in their third trimester (SC1, 2 days; SC4<1 day antepartum). cfDNA was isolated and reference materials prepared using a proprietary cfDNA amplification method that generated micrograms of output from nanograms of input. They were tested with the VCU Health NIPT SNP-based assay to generate whole genome library, which was further amplified to target >13,000 SNPs sites on chromosomes 13, 18, 21, X and partially Y. The targeted libraries were barcoded and sequenced on Illumina NextSeq500. Cloud-based Natera's Constellation software was used to facilitate the raw sequencing data analysis, SNP allele frequencies computation, and Bayesian probabilities calculation against copy number hypotheses. These samples were evaluated in duplicate or triplicate over 5 different runs performed on different days. **Results:** Evaluation of SC1 and SC4 by the VCU Health NIPT test provided the correct risk assessment of High Risk for trisomy 21 for all measurements performed on the same day and between days. Consistent and accurate high levels of estimated fetal fraction were obtained across replicates and across runs with a mean fetal fraction of 38.75% (SD:0.17) for SC1 and 19.03% (SD: 0.05) for SC4. Both SeraCare samples showed comparable performance to in-house samples for the following QC metrics: 1) total reads mapped, 2) on-target reads, 3) mean read depth per chromosome, 4) error rate for transitions and transversions, 5) mean number of heterozygotes and homozygotes reported. SeraCare samples do demonstrate elevated values for Uniformity 95% (UNI95%) and Chi Square Score (CHI). **Conclusion:** Our results demonstrate that these reference materials performed as expected and they would be crucial for quality control of NGS-based NIPT in clinical practice.

### G054. The SureMASTR BRCA Screen Assay Combined with MASTR Reporter Analysis is an Accurate and Precise Workflow for SNV, Indel and CNV Detection in Blood- and FFPE-derived DNA

A. Rothier, K. Bettens, B. Slabbinck, N. Remmerie, J. Vermeire, M. Vyverman, B. Tegenbos, L. Claes, L. Heyrman, H. Swennen, O. Herd, E. De Schutter, C. Marques, J. De Schrijver, D. Goossens, J. Crappé, J. Del Favero

Agilent Technologies, Niel, Belgium.

**Introduction:** Targeted next-generation sequencing has tremendous potential to steer targeted therapy. Inhibitors of poly-(ADP-ribose) polymerase (PARPi) have emerged as a new class of targeted anti-cancer drugs, specifically for tumors showing homologous recombination repair deficiency in ovarian and breast cancers. We are developing a new solution for BRCA testing with an improved, customer-oriented workflow and a tailored data analysis software. **Methods:** The SureMASTR BRCA Screen workflow comprises a sample to result solution. The library preparation is a single plex assay enabling mutation analysis of all coding regions of *BRCA1*, *BRCA2*, *PALB2* and *CHEK2*. The assay comprises 355 amplicons ranging in size from 80 to 224 bp. Data quality control as well as variant calling of SNPs, insertion/deletions (indels) and CNVs are performed using MASTR Reporter. The SureMASTR BRCA Screen workflow was extensively examined in house and by 3 external clinical labs using both blood- and formalin-fixed, paraffin-embedded (FFPE)-

derived DNA. **Results:** In total, 105 clinical samples were analyzed by three external clinical sites for germline mutation detection during a beta testing study. These samples harbored 114 annotated, mostly pathogenic variants, of which 8 in *CHEK2*, 14 in *PALB2*, 49 in *BRCA1* and 43 in *BRCA2*. The 114 variants included 8 CNV events and 49 indels up to 13 basepairs. All indels and single-nucleotide variants (SNVs) were correctly called with the BRCA Screen workflow. In addition, all CNV events were correctly observed. The assay is optimized for cost-efficient use of the sequencing capacity obtained by (i) > 99% of the reads mapping to the target region and (ii) > 99.4% of the amplicons having a coverage within 20% of the mean coverage. Testing was performed on Illumina's MiSeq sequencer, and compatibility with Illumina's NextSeq and MiniSeq was demonstrated. Currently, the scope of the study is being extended to FFPE derived DNA as input material. Two FFPE-derived reference samples of Horizon were first assessed for the quality of the DNA with the QC Plex assay. Uniformity of amplification remained high, with on average 99.1% of amplicons reaching a coverage of 20% of the mean coverage. Variant analysis is currently ongoing and promising, as coverage of the amplicons is uniform and the quality of the bases is high. A study with clinical FFPE derived samples is initiated to confirm these data. **Conclusions:** The SureMASTR BRCA Screen assay is a single-plex assay which significantly reduces the hands-on time, DNA input, required sequencing capacity and in combination with the MASTR Reporter results in an accurate and precise workflow. Using the SureMASTR BRCA Screen workflow, we demonstrated that it can be routinely applied with a LOD down to 5% to detect somatic or germline *BRCA1*, *BRCA2*, *PALB2* and *CHEK2* mutations.

### G055. CNV Contribution to Pathogenic Alleles within a Healthy Population: Results from Expanded Carrier Screening of 137,000 Individuals

S.G. Cox, E. Kaseniit, R. Mar-Heyming, P. Kang, S. Guha Counsyl, Inc., South San Francisco, CA.

**Introduction:** Copy number variants (CNVs) are large, exon-level deletions or duplications that require special handling to detect with high accuracy. In conventional expanded carrier screening, specific testing for CNVs is restricted to a subset of genes where a frequent contribution to disease has been established (e.g., *DMD*) or a founder variant is known. However, gene selection varies between laboratories, and the contribution of CNVs has not been determined for many diseases; thus, an unknown proportion of carriers may be missed in the absence of CNV calling. Here we report findings from an expanded carrier screen incorporating panel-wide CNV calling. **Methods:** We explored a next generation sequencing (NGS)-based expanded carrier screen for 176 recessive diseases that includes panel-wide screening for CNVs. Here, we present results from CNV screening of 136,946 individuals for 165 of the panel's diseases; eleven conditions were excluded from this study due to specialized assay design or frequent involvement in contiguous gene deletion. CNVs were determined using custom software that leverages NGS read-depth values. ACMG-based variant classification was employed for all variants, including CNVs. Pathogenic classifications relied heavily on published cases and/or a predicted high-impact deleterious effect on gene function. The proportion of detected known versus novel pathogenic CNVs, as well as diversity of pathogenic CNVs in the population, were investigated. **Results:** Pathogenic CNVs accounted for 1 in 30 detected carriers across the 165 disease genes. Furthermore, pathogenic CNVs were detected in 84% (139/165) of genes. Among these, CNVs accounted for a highly variable share of disease carriers, surpassing 50% for a handful of genes, e.g., *CLN3*, *DMD*, *CTNS*. In addition, CNVs accounted for a significant share (>5%) of carriers for a number of diseases (36). Among CNV carriers, 19% were found to harbor novel pathogenic variants, (~300 distinct CNVs previously unseen in the literature), with some observed in multiple patients. The number of distinct CNVs contributing to carrier burden varied widely between disease genes. For certain genes, e.g., *CLN3*, *CTNS*, *GALC*, *HEXB*, over 90% of CNV carriers were attributed to a single known founder pathogenic variant. At the opposite extreme, over 30 distinct CNVs were found to contribute to disease carriers for *DMD*, *FANCA* and *USH2A*. **Conclusions:** CNVs make a considerable and widespread contribution to population carrier burden for serious and clinically actionable Mendelian diseases. Inclusion



of panel-wide CNV calling in an expanded carrier screen will, therefore, serve to maximize the detection rate for patients.

#### G056. Use of Molecular Identifiers and Targeted NGS to Enable Variant Detection Below 1% Allele Frequencies in Circulating Cell-free DNA

L. Kurihara, A.J. Wood, S. Sandhu, J. RoseFigura, V. Makarov  
Swift Biosciences, Ann Arbor, MI.

**Introduction:** The growing use of circulating cell-free (cfDNA) necessitates accurate variant detection at less than 1% allele frequencies due to a low population of DNA of interest within total cfDNA. This level of detection is critical for a variety of cfDNA applications including detection of circulating tumor DNA (ctDNA), detection of graft-derived cfDNA (GcfDNA) within normal plasma as a marker of graft integrity after organ transplantation, and for non-invasive prenatal testing (NIPT) where fetal cfDNA is detected at low frequencies in maternal plasma. However, reliable low-frequency variant detection by next-generation sequencing (NGS) is challenging due to background noise from PCR and sequencing errors. Additionally, while NGS is well suited for high throughput analysis, whole-genome sequencing can be cost prohibitive. To overcome these challenges, we employed a targeted NGS assay that uses molecular identifiers (MIDs) to uniquely label individual DNA molecules prior to amplification. MID families consisting of PCR duplicates can then be used to distinguish true variants from PCR and sequencing errors and an error-corrected consensus sequence can be generated. **Methods:** We incorporated MIDs into a 17 amplicon EGFR pathway panel that can be used for ctDNA detection in lung cancer and a 101 amplicon SNP panel that contains 92 amplicons targeting SNPs with high minor allele frequency and 9 amplicons that identify gender ideal for GcfDNA or fetal cfDNA detection. These assays generate an NGS library using a single-tube multiplex PCR that is compatible with cfDNA. To test assay sensitivity, we performed low frequency spike-in experiments with control DNA or patient derived cfDNA that had previously been analyzed for SNP content. We prepared MID libraries from 10-50ng of input DNA and deep sequencing to >20,000x was done to maximize MID family size (number of PCR duplicates) and optimize generation of a consensus sequence. **Results:** This analysis accurately and reproducibly identified known variants as low as 0.1%. Importantly, the use of MIDs drastically reduced the number of false positive variants called at less than 1% increasing confidence in low frequency variant calls. **Conclusions:** Accurate variant calling at these levels is critical to track biologically relevant levels of ctDNA for early detection and monitoring of cancer, GcfDNA to screen for organ rejection after transplantation, and fetal DNA at early stages of pregnancy. This study highlights the power of MID technology in amplicon NGS library preparation to enable low frequency variant detection for cfDNA analysis.

#### G057. A WITHDRAWN

#### G058. Evaluation of a Single-tube, Long-read, Two-mode PCR Technology that Reports the Categorical Range of DMPK CTG Expansions and Resolves up to 2000 Repeats in Myotonic Dystrophy Type 1

B. Hall<sup>1</sup>, J. Cooley<sup>2</sup>, E. Bend<sup>2</sup>, J. Wisotsky<sup>1</sup>, J. Lee<sup>2</sup>

<sup>1</sup>Asuragen, Austin, TX; <sup>2</sup>Greenwood Genetics Center, Greenwood, SC.

**Introduction:** Myotonic Dystrophy is the most common adult-onset muscular dystrophy, and the most severe form is type 1 (DM1) which is caused by a pathogenic CTG expansion in the 3' UTR of the myotonic dystrophy protein kinase gene (*DMPK*). Patient phenotypes begin to appear in those with >50 repeats, and classic disease is associated with >100 repeats. DM1 molecular testing requires both PCR and Southern blot (SB) analysis because most laboratory-developed PCR tests (LDTs) cannot consistently amplify >100 repeats. Here, we describe results using a novel PCR amplification technology that surmounts this hurdle and reliably resolves normal and expanded alleles using two output modes: Capillary Electrophoresis (CE) for £200 repeats, and Agarose Gel Electrophoresis (AGE) for 200-1000 repeats. **Methods:** Forty residual clinical specimens were selected with genotypes independently determined using PCR and/or SB. These samples included those with ≤34 (normal), 35-49 (premutation), and expansions of 50-150 (mild), 100-

1000 (classic), and >1000 CTG repeats (congenital). Genomic DNA was amplified using pilot AmpliDeX PCR/CE *DMPK* Kit\* reagents (Asuragen) that were identical to final product formulations. FAM-labeled amplicons were resolved by CE (Thermo Fisher). Expansions larger than 200 repeats were resolved by AGE. **Results:** The AmpliDeX PCR/CE *DMPK* assay identified categorical genotypes with 100% concordance to reference results for all 40 samples. Sizing of repeat expansions using PCR/AGE were consistent with SB for clinical samples with between 200 and 1000 repeats. Samples with known mosaicism appeared as a smear on the PCR/AGE assay, similar to SB. Multiple sample comparisons highlighted agreement in sizing specific expanded alleles across *DMPK* PCR/CE, PCR/AGE, and SB. **Conclusions:** Current PCR methods fail to amplify moderate-to-large repeat expansions in DM1, and allele drop-outs may be indistinguishable from frequently-encountered homozygous samples. We report a single-tube, long-read PCR technology that can resolve zygosity and size primary and mosaic expansions by two modes: CE for sizing up to 200 repeats with flagging of larger expansions, and AGE for many larger expansions. This *DMPK* PCR can genotype normal and expanded alleles using a fast and simple assay and analysis workflow compared to conventional methods.

\*Research use only. Not for use in diagnostic procedures.

#### G059. ACMG Incidental Findings at the CLIA-certified Colorado Center for Personalized Medicine Biobank: Data from the First 10,000 Subjects

S.J. Wicks, K.C. Barnes, K.R. Crooks

University of Colorado - Anschutz Medical Campus, Aurora, CO.

**Introduction:** The Colorado Center for Personalized Medicine (CCPM) has been established to uncover advancements in genomics that can improve diagnosis and treatment of disease. To hasten discoveries in personalized medicine, the CCPM has established a CLIA-certified human specimen Biobank. Accompanying biospecimen collection, we harness patient health information to support a broad range of research, operational, and clinical quality improvement agendas. To date, we have enrolled over 62,000 adult subjects from across the University of Colorado Health (UCHHealth) system into the Biobank and collected blood samples from over 27,000 subjects. **Methods:** DNA extracted from patient samples undergoes microarray genotyping using a custom Infinium Multi-Ethnic Global Array SNP chip (MEGA, Illumina, Inc.). Among the ~2.1 million genetic variants on the microarray are several thousand with potential clinical relevance. The CCPM Biobank follows the current American College of Medical Genetics and Genomics (ACMG) recommendations for reporting of incidental genetic findings (Kaila *et al.*, Genet Med 2017). Here we present information on the incidence and characteristics of reportable incidental findings from the first 10,000 genotyped patient samples. **Results:** MEGA content includes variants classified in ClinVar as "pathogenic" or "likely pathogenic" in each of the 59 ACMG genes. The content varies by gene, with 56 genes having between 1-80 potentially reportable variants (average 17). The remaining 3 genes, *BRCA1*, *BRCA2*, and *COL3A1*, have greater representation on the microarray, with 225, 250, and 258 pathogenic/likely pathogenic variants, respectively. Samples with identified variants have then undergone orthogonal confirmation by Sanger sequencing. After consenting to the return of these results, participants will be informed of the results by genetic counselor or clinical geneticist. The average incidental finding rate is approximately 1% to date. As expected given the microarray content and estimated allele frequencies, *BRCA1* and *BRCA2* are relatively over-represented and together account for >50% of the incidental findings. The remainder is distributed among several genes, including *GLA*, *DSG2*, *LMNA*, *MYBPC3*, and others. **Conclusions:** The MEGA microarray combined with Sanger confirmation provides a powerful tool for identifying pathogenic or likely pathogenic reportable genetic variants in a general population Biobank setting.

#### G060. Phenotypic and Genotypic Study of Patients with Hermansky-Pudlak Syndrome

J.A. Majerus, L.M. Coon, R.K. Pruthi, R. He, D.M. Warad, D. Chen  
Mayo Clinic, Rochester, MN.

**Introduction:** Hermansky-Pudlak syndrome (HPS) represents a group of autosomal recessive disorders due to mutations in genes involved in

intracellular vesicular trafficking. Clinical presentations include oculocutaneous albinism and bleeding diathesis. There are 10 genetic subtypes of HPS: type 1 (due to mutations in *HPS1*), type 2 (*AP3B1*), type 3 (*HPS3*), type 4 (*HPS4*), type 5 (*HPS5*), type 6 (*HPS6*), type 7 (*DTNBP1*), type 8 (*BLOC1S3*), type 9 (*BLOC1S6*), and type 10 (*AP3D1*). Eventually all patients with HPS1, 2 and 4 develop pulmonary fibrosis and may require lung transplant. Since severe platelet dense granule deficiency is considered a characteristic feature of HPS, platelet whole mount transmission electron microscopy (PTEM) has been considered a good initial screening test. Potential HPS positive cases detected by PTM still need to be confirmed and further classified by genetic testing. The goal of this study is to assess the HPS gene mutation status of the PTM identified HPS cases in our institution. **Methods:** Nine patients with PTM documented severe dense granule deficiencies and one patient with ocular albinism but normal dense granules by PTM were included in this study. Next Generation Sequencing (NGS) was performed using a targeted panel (Agilent Technologies) encompassing 9 of the HPS genes (*HPS1*, *AP3B1*, *HPS3*, *HPS4*, *HPS5*, *HPS6*, *DTNBP1*, *BLOC1S3*, and *BLOC1S6*). DNA library preparation was performed using the SureSelect<sup>XT</sup> Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library (Agilent Technologies). The enriched indexed DNA sample was then sequenced on an Illumina MiSeq or HiSeq 2500 platform. The CLC Bio Genomics Server was used for sequence alignment and variant calling, which was then uploaded into NGS Workbench (Mayo Clinic) for review. All sequence variants were classified following the current American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines. **Results:** All 10 patients had oculocutaneous albinism and bleeding diathesis (age range 8-80 years, 3 male). The average mean dense granules/plt of the 9 PTM positive patients was less than 0.1 (normal range >1.2). Pathogenic mutations were found in all 9 PTM positive patients, *HPS1* (*n*=5), *HPS3* (*n*=2), *HPS5* (*n*=1) and *HPS6* (*n*=1). These included both reported (*n*=5) and novel mutations (*n*=4). No pathogenic mutations were found in the patient with ocular albinism but negative PTM findings. **Conclusions:** An algorithmic approach of employing both PTM and genetic testing can accurately identify and classify Hermansky-Pudlak syndrome to assist clinical management in such patients. The late diagnosis of many HPS patients in this cohort underscores the importance of availability of both PTM and molecular genetic testing in clinical laboratories.

#### G061. Performance Characteristics of High-resolution Human Leukocyte Antigen (HLA) Typing Using TruSight Next-generation Sequencing (NGS) Technology

A. Budhai, X. Zhang, S. Hodgson, J. Zhuge, J. Fallon, A.N. Arnold, F. Ye  
Westchester Medical Center, Valhalla, NY.

**Introduction:** It is widely accepted that high-resolution, allele-level, HLA matching reduces patient morbidity and mortality after hematopoietic stem cell transplantation (HSCT). Recently, next generation sequencing (NGS)-based HLA typing strategies have been proposed as an advancement by PCR-based molecular methods such as SSP, SSO, SBT. Compared to SBT, NGS types more HLA loci and sequences more exons at each locus. Although NGS is routinely used in molecular genetics, few clinical HLA laboratories use the technology. Therefore, it is a great challenge for HLA laboratories to develop new NGS-based HLA typing strategies. **Methods:** In this study, we evaluated the performance characteristics of high-resolution HLA typing assay using the TruSight HLA v2 Panel on Illumina MiSeq platform. Briefly, 11 HLA locus specific amplicons were generated using the TruSight HLA v2 assay, and then prepared for sequencing following the manufacturer's instruction and loaded onto a MiSeq for paired-end sequencing (2x150 bp). Our average run size was 15 samples with HLA A\*, B\*, C\*, DRB1\*, DRB3\*, DRB4\*, DRB5\*, DQA1\*, DQB1\*, DPA1\*, DPB1\* loci evaluated per run sequenced using a micro flowcell. HLA genotyping analysis was performed using Assign 2.1 with IMGT/HLA database 3.27. A total of 206 samples, previously characterized for HLA typing, were included in this study. The NGS quality metrics were evaluated along with assay sensitivity, reproducibility and accuracy. **Results:** Sequencing of a total of 206 samples established the criteria of QC metrics for each NGS run and each HLA locus, including cluster density, % of read >Q30, coverage, percent Base Call with >Q30, etc. Our sensitivity study showed that the assay had

a consistent limit of detection at 5 ng/LR-PCR (corresponding to the DNA concentration of 1 ng/μL). The reproducibility study showed the assay had 100% intra-run, inter-run and inter-technologists reproducibility in detecting HLA alleles. 90 of the 206 samples (78 blood samples and 12 buccal samples) were analyzed for overall accuracy. A total of 1,507 alleles were identified (1,291 alleles from blood and 216 alleles from buccal swab). The accuracy was 99.8% (1,289/1,291) for blood samples and 99.1% (214/216) for buccal swab samples when compared with the two-field reference results. The overall ambiguity rate was 3.4% (45 out of 1323). Of 45 ambiguities, 37 (82.2%) were from DPB1\*, 6 (13.3%) were from DRB1\*, and 2 (4.4%) were from DQB1\*. In addition, 11 alleles (0.83% of total identified alleles) required manual review and editing of the sequence to generate HLA typing during analysis. **Conclusions:** In summary, the TruSight HLA v2 Panel is a sensitive and accurate method, with minimal ambiguities, that can be used for high-resolution HLA typing of peripheral blood and buccal swab samples.

#### G062. Magnis, a Fully Automated NGS Sequencing-ready Library Preparation System

S. Bigdeli<sup>1</sup>, B. Buehler<sup>1</sup>, K. Yeap<sup>2</sup>, S. Im<sup>1</sup>, H. Hogrefe<sup>1</sup>, P. Sheffield<sup>1</sup>, R. Bagga<sup>1</sup>, Y. Lee<sup>1</sup>, J. Halladay<sup>3</sup>, S. Jandle<sup>3</sup>, K. Poon<sup>4</sup>, P. Maunary<sup>1</sup>, P. Webb<sup>4</sup>, R. Allen<sup>1</sup>

<sup>1</sup>Agilent Technologies, La Jolla, CA; <sup>2</sup>Agilent Technologies, Penang, Malaysia; <sup>3</sup>Agilent Technologies, Cedar Creek, TX; <sup>4</sup>Agilent Technologies, Santa Clara, CA.

**Introduction:** Agilent's Next Generation Sequencing (NGS) target enrichment kits for preparation of sequencing-ready libraries are in widespread use in laboratories around the world. Automation of these complex protocols eliminates the need for training specialists, improves turnaround time, library quality, and reproducibility for NGS libraries. In a large collaborative effort between several groups within Agilent, we are developing a walk away liquid handler system Magnis capable of preparing up to eight NGS sequencing-ready libraries per run without the need of human intervention during the run. We have programmed Magnis and optimized Agilent's HaloPlex HS and XT HS chemistries to prepare automated Illumina-ready libraries. The system also comes with pre-aliquoted plated reagents for each chemistry made by Agilent's production facility. Prepared libraries can be kept sealed and refrigerated on the deck for up to 72 hours post-run. In a clinical setting, a decreased turnaround time can expedite patient diagnosis and aid doctors to closely monitor a patient's disease status and update the treatment plan frequently.

**Methods:** We have made modifications to the manual target enrichment protocols to optimally generate libraries on Magnis using different baits while utilizing innovative techniques for pipetting to prevent well to well contamination. An example we use for testing the Magnis system is one of Agilent's latest baits, AIO Lung, which is a target enrichment All-in One panel addressing lung cancer specific genetic aberrations. **Results:** Both chemistries have been fine-tuned and optimized, and libraries have been tested for yield and reproducibility. Sequencing has confirmed reliable coverage, specificity, uniformity, and duplicate reads. We have demonstrated that the integrated germicidal UV bulb installed on the deck removes >99.5% of the amplifiable DNA at all tested deck positions in less than 30 minutes. We have also developed qPCR-based spike-in assays to confirm sample to sample contamination below the limit of detection (less than 0.001%). We have also used sequencing to address potential run to run, and index contamination between samples. Protocol run time is currently under 8 hours for HaloPlex HS and under 9 hours for XT HS. All yield and sequencing specs are targeted to meet the criteria for manually run protocols. **Conclusions:** The fully automated Magnis NGS sequencing-ready library prep system, the pre-loaded target enrichment chemistry protocols, the variety of panels, and the pre-aliquoted reagent plates can expedite turnaround times for results. Due to its reduced need for human intervention and minimal training, libraries can be reliably prepared from both regular and FFPE quality patient samples with minimal hands on time.

**G063. Multiplex Synthetic Reference Material for Monitoring the Analytical Performance of Highly Complex Variant Detection of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) using Next Generation Sequencing**

R. Mihan, C. Rundell, J. Gordon, J. Salem, M. Steffen, S. Nesbitt, T. Spennhauer

Maine Molecular Quality Controls, Saco, ME.

**Introduction:** The ability of next generation sequencing (NGS) to deliver quantitative and qualitative analysis of nucleic acids in a single test with increased diagnostic potential has made it the system of choice for many clinicians but the errors introduced during sample preparation, library construction and bioinformatics analysis make the implementation of NGS challenging. This is further compounded by the inability of NGS technology to accurately detect challenging variants particularly in regions of low sequence complexity and high GC/AT ratios. In 2017, AMP and CAP published a joint guideline regarding validation of NGS panels and pipelines to mitigate these problems. To this end, MMQCI has developed a comprehensive synthetic reference panel to be used as positive control for monitoring the analytical performance of complex NGS panels examining the CFTR gene. **Methods:** MMQCI designed a synthetic control containing 27 CFTR exons plus intronic borders representing 188 variants including 146 single nucleotide variants, 42 insertion/deletions (indels) and homopolymers up to 9Ts. The synthetic sequences were ligated into MMQCI vectors. Multiple plasmids with known variants were mixed to create heterozygous or homozygous alleles, further diluted to a copy number approximately equivalent to that of an extracted human sample. All plasmid variants were confirmed by bidirectional Sanger Sequencing. MMQCI's NGS CF Control Panel G211 v1.1 was tested using Illumina's MiSeqDx CF 139-Variant Assay, alongside samples comprised of single synthetic plasmids spiked into known genomic background and genomic DNA alone. All samples were prepared according to MiSeqDx CF 139-Variant Assay protocol and tested on MiSeqDx platform. All results were comparable and expected variants were detected. **Results:** MMQCI has successfully manufactured synthetic reference material for use in multistep NGS analysis. Since the reference panel is processed exactly like an extracted patient sample, it undergoes the same biases introduced through the various steps of NGS assay. All except one sample used in this study resulted in 100% call rate thus accounting for all the mutations detected by the MiSeqDx CF 139-Variant Assay with an overall coverage of >1000x. ~7.2 million reads were obtained from the run with 90% of the reads aligned to the reference genome. The cluster density for the run was 739 K/mm<sup>2</sup> (ClustersPF-95.3%). In addition, the reference panel can account for all the mutations detected by the assay. **Conclusion:** MMQCI can manufacture complex NGS synthetic reference material to aid in design, development, optimization, and validation of NGS assay. The reference materials harbor horizontally and vertically complex variants, in addition to homopolymers in contiguous regions of CFTR gene routinely missed by various NGS assays.

**G064. Low Input Microfluidic Library Preparation Platform for Targeted CFTR Using Blood, Buccal Swabs and Saliva Samples**

N. Ramalingam, D. Wang, C. Sanada, B. Hunt, J. Brockman, T. Lu, P. Chen, T. Goralski

Fluidigm, South San Francisco, CA.

**Introduction:** Cystic fibrosis (CF) is an autosomal recessive disorder affecting more than 70,000 people worldwide. There is an unmet need to support the use of buccal and saliva samples, as these typically yield genomic DNA (gDNA) of lower quantity and purity. Additionally, the workflow must be cost-effective and efficient. Integrated fluidic circuit (IFC) technology addresses these needs by lowering the gDNA input and miniaturizing reactions to nanoliter volumes, thereby reducing the cost per sample. IFC technology also enables automated preparation of a barcoded library from 48 to 1,536 samples per week that is ready for next-generation sequencing (NGS). **Methods:** We describe development and validation of the Advanta CFTR NGS Library Prep Assay with Preamp using as little as 5 ng gDNA. Sample and assay mixes are dispensed into the IFC, which is loaded on the Juno system. Juno automates reaction assembly, target amplification by multiplex PCR, and barcode addition (all on-IFC). Amplicons are harvested and pooled in a single tube for

cleanup, adapter addition, and QC prior to NGS on the Illumina MiSeq system with 300 cycle sequencing chemistry. The assay contains a set of eight primer pair pools designed to cover 256 CF-causing variants that include large deletion and insertions. Two IFC formats (LP 48.48 and LP 192.24) were tested that can process from 48 to 192 samples per run.

**Results:** High-quality data was generated using 112 genomic DNA samples that contain CFTR variants (Coriell). In addition, gDNA extracted from blood, buccal swabs and saliva samples was tested. Testing according to protocol resulted in amplicon uniformity (5x within mean) of 99.7%, 99.7%, 98.9% and 99.1% for Coriell gDNA, whole blood, buccal swabs, and saliva samples respectively. Sensitivity of 98.4% single-nucleotide variants (SNVs; Coriell) and 97.9% insertion/deletions (indels; Coriell) was observed for variant calling performance. Using National Institute of Standards and Technology (NIST) reference materials NA12878, NA24143, and NA24149 (Coriell), we obtained 100% accuracy and specificity of variant calling for both SNVs and indels. Reproducibility of the assays by repeat testing was conducted in a manner that accounted for sources of variability that include operator, day, instrument, and reagent lot. We noted 99.9% reproducibility for Coriell gDNA and 100% reproducibility for whole blood, buccal swabs and saliva samples.

**Conclusions:** The Advanta CFTR NGS Library Prep Assay with Preamp, which utilizes integrated fluidic circuits (IFCs), demonstrates excellent performance and can achieve 98.4% sensitivity (SNVs) and 100% accuracy, specificity, and reproducibility. Advanta CFTR NGS Assay Pools include primers that target large deletions and insertions relevant for CFTR variant analysis.

**G065. Validation of a Next-generation Sequencing Gene Panel for Inherited Platelet Disorders**

W. Zhang, J. Denton, C. Wei, J. Han, E. Ulm, J. Wagner, S. Nortman, J. Palumbo, L. Luchtman-Jones, B. Dawson

Cincinnati Children's Hospital Medical Center, Cincinnati, OH.

**Introduction:** Inherited platelet disorders (IPDs) are a heterogeneous group of genetic qualitative and quantitative disorders impacting the platelet membrane and receptor assembly, signal transduction, granule content and release, as well as adhesion and aggregation. Diagnosis for the majority of IPDs, particularly those with a milder phenotype, thrombocytopenia, or suspected in young children, is challenging due to imperfect precision and accuracy of functional platelet testing as well as the requirement for a traumatically obtained, large volumes of platelet rich plasma and the exclusions of remote testing and thrombocytopenic patients. Incorporating molecular genetic testing into the platelet disorder diagnostic algorithm should improve speed and accuracy of diagnosis, and may optimize therapy. We developed and validated a Platelet Disorders Gene Sequencing Panel which utilizes Whole Exome Sequencing (WES) technology to identify inherited forms of platelet dysfunction. **Methods:** Platelet Disorders Gene Sequencing Panel uses the Agilent SureSelect WES kit to capture the exonic regions of genes from the genomic DNA. Targeted regions are sequenced using the Illumina HiSeq sequencing system. Sequence reads are mapped and compared to human genome build UCSC hg19. Variants within exons and flanking sequences of 53 clinically curated platelet disorder genes are identified and evaluated by a validated in-house developed bioinformatics analysis. Data quality is assessed to confirm it has a minimum coverage of 20x for >95% of targets of interest. **Results:** We validated this test using 14 validation samples. The average of whole exome targets of interest with a minimum coverage of 20x, was 96.70% with a range of 95.36% to 98.32%. We detected all 6 pathogenic, likely pathogenic and variants of unknown significance, including substitutions and insertion/deletions (indels) in the positive validation samples. We detected four additional variants of unknown significance in two samples, which may help further clarify the genetic etiology underlying the clinical phenotype, particularly in children of consanguineous background. All variants were confirmed by Sanger sequencing. We detected all 76 variants in our platelet targeted region in the GIAB/NIST NA12878 reference sample. In sum, the analytical sensitivity and specificity for substitutions and small indels of this next generation sequencing (NGS) test are 100%. This WES based panel sequencing also offered flexibility to add more clinical relevant genes in the future when new clinical evidence is available, by eliminating the need for redesign for customized



panels. **Conclusions:** We have demonstrated that our Platelet Disorders Gene Sequencing Panel is highly sensitive in detecting sequence variants in the platelet disorders genes.

#### G066. Identification of Mutation Signatures in Tumors Using Panel-based Targeted Sequencing

S. Rana, S. Middha, S. Chavan, A. Penson, K. Huberman, O. Hampton, A. Viale, D. Solit, M. Berger, A. Zehir  
Memorial Sloan Kettering Cancer Center, New York, NY.

**Introduction:** Endogenous and/or exogenous factors can give rise to specific mutations in cells leading to cancer initiation and progression. Examining the patterns of these mutations can help identify the underlying mutagenic processes, helping guide diagnosis, and further informing treatment modalities. Multiple algorithms and classifications have been developed to uncover mutational signatures (MS) based on whole genome/exome next-generation sequencing (NGS) data. However, the reliability of signature detection from panel-based targeted NGS data has not been addressed yet. Here, by utilizing samples with whole exome sequencing (WES) and MSK-IMPACT data, we develop criteria to confidently identify MS from targeted sequencing data. We also show different signatures require different numbers of mutations underlying the challenges associated with MS detection in panel-based NGS data.

**Methods:** A total of 543 tumor samples which underwent clinical sequencing using MSK-IMPACT, a 468 gene targeted panel, were also recaptured and sequenced using a whole exome capture kit (IDT). After processing the sequencing reads through a similar bioinformatics pipeline, the mutation calls were used to identify MS based on the previously published set of 30 signatures using an in-house developed pipeline. MS from WES data with contributions of at least 50% (major signature) were evaluated for concordance in corresponding data generated from MSK-IMPACT data. 22 of these MSK-IMPACT samples were selected based on the highest and lowest percent contribution of each available signature. Mutations from these selected samples were down-sampled to determine the limit of detection for each signature type. **Results:** In the cohort of 543 samples, we detected 196 samples with a major signature which we consider as the truth set. While the signatures were varied, the main ones were aging (n=99), BRCA-deficiency (n=24), mismatch repair (MMR) deficiency (n=22), smoking (n=19), and UV exposure (n=18). 86 out of 196 (44%) of the samples harbored the same major signature in the MSK-IMPACT data. By performing down-sampling analysis, we determined the minimum number mutations required to identify each signature confidently. We further show the contribution of clonal and subclonal mutations to the signatures determined. **Conclusion:** Concordance of major signatures detected from WES and MSK-IMPACT varied based on the signature. Each signature analyzed required different number of mutations to identify them. Overall, panel-based sequencing data can be used to identify MS, however, reliability of these signatures depend on several factors that needs careful interpretation.

#### G067. WITHDRAWN

#### G068. CleanPlex Amplicon-based Next Generation Sequencing Heredity Panels for Determining Genetic Predispositions

L. Lin, L. Lee, J. Liu, G. Liu, L.J. Zhang, L. Liu  
Paragon Genomics, Hayward, CA.

**Introduction:** As genetic testing becomes more widely accepted, medical professionals are increasingly interested in genetic testing as a means for guided testing and diagnostics of certain conditions. CleanPlex multiplex-PCR-based technology has made it possible to produce high-quality coverage of genomic targets using a fast and easy library-construction workflow. Here we present a panel targeting hereditary mutations that covers the full exons of 37 genes, in addition to two clinically relevant intronic mutations, using 1443 pairs of PCR primers. The panel paired with Paragon Genomics work flow can easily and quickly diagnose and monitor inherited cancers using small amount of genomic DNA. **Methods:** Paragon Genomics's CleanPlex 3-step work flow includes a multiplex PCR step with targeted primers, a cleanup step to remove byproducts, and a final PCR to add Illumina adapters and sample barcodes. Libraries were made using 10ng of genomic DNA. The hereditary panel contains primers divided into two pools for the multiplex PCR. Samples were

sequenced at ~ 1000 read depth via illumina's NextSeq. Sequenced reads were demultiplexed, mapping rate and on-target rates were calculated. Variants were called using Paragon Genomics's variant calling algorithm. **Results:** The samples exhibit 93% uniformity at 0.2x mean, and all exons were sufficiently covered to provide accurate variant calling information. **Conclusion:** Paragon Genomics's hereditary panel can be used for genetic monitoring of inherited cancers by using small quantities of genomics DNA. With the easy work flow and quick turnaround, this panel can make genetic monitoring easy, efficient, and economical.

#### Hematopathology

#### H001. The Detection of a BRAF Mutated Clone in Acute Myeloid Leukemia with Mutated Npm1 and Extensive Extramedullary Involvement

K. Gvozdzan, G. Chen, V. Merelo Alcocer, Z. Yang  
Penn State Milton S. Hershey Medical Center, Hershey, PA.

**Introduction:** Acute myeloid leukemia (AML) with mutated *NPM1* typically carries a favorable prognosis in the absence of concurrent *FLT3*-ITD and *DNMT3A* gene mutations. Although *BRAF* mutation has been associated with a worse prognosis in a small group of AML patients, the significance of *BRAF* mutation in AML with mutated *NPM1* is unknown. **Methods:** We report the cytogenetic and molecular findings in a 45 year-old man with extensive extramedullary involvement by AML with mutated *NPM1*, and emphasize significant pathologic and clinical implications. Next-generation sequencing (NGS)-based mutational analysis of 54 genes associated with myeloid neoplasms was performed on peripheral blood sample at initial diagnosis and during relapse. *NPM1* mutation testing for minimal residual disease on a post-induction bone marrow aspirate was performed by fragment analysis method. The extent of leukemic involvement was evaluated during postmortem examination. **Results:** The karyotype at initial diagnosis was 46,XY [20]. NGS mutational analysis showed the following mutations and allele frequencies at diagnosis: *NPM1* c.859\_860insTCTG (44.8%), *DNMT3A* c.2645G>A (39.1%), *NRAS* c.35G>A (27.2%), and *BRAF* c.1799T>A (2.5%). *NPM1* mutation was undetectable on post-induction bone marrow sample. Follow-up NGS mutational analysis during relapse (4 months after the initial diagnosis) showed the following mutations and allele frequencies: *NPM1* c.860\_863dupTCTG (4.3%), *DNMT3A* c.2645G>A (44.0%), *TP53* c.818G>A (85.2%), and *BRAF* c.1799T>A (38.3%). *FLT3*-ITD and TKD mutations were not detected at diagnosis or at relapse. Karyotype abnormality was seen at relapse, including 46,XY,add(6)(q21),10,+mar[13]/46,sl,t(1;9)(q23;q32)[5]/46,XY[2]. The patient expired 4.5 months after the initial diagnosis despite receiving multiagent chemotherapy, including a hypomethylating agent. Postmortem examination showed extensive leukemic infiltrate involving virtually all organs examined. **Conclusions:** The molecular results suggest two different leukemic clones at the time of diagnosis, including a clone harboring *NPM1* c.859\_860insTCTG mutation and the second, small clone harboring *BRAF* c.1799T>A (V600E). The *NPM1*, *DNMT3A*, and *NRAS* mutated clone was suppressed by the induction chemotherapy, while the *BRAF* and *DNMT3A* mutated clone persisted. The biallelic *TP53* mutation newly detected at relapse may suggest preferential selection by cytotoxic chemotherapy for the clone that was too small to be detected at initial diagnosis. The *TP53* and *BRAF* mutations are plausible culprits in widespread extramedullary tissue invasion. The presence of even a small *BRAF* clone at diagnosis may suggest an aggressive disease. Testing for *NPM1* alone might not be sufficient for minimal residual disease detection.

#### H002. Short Tandem Repeat Aberrancies in Hematopoietic Stem Cell Transplant Recipients

K. Gvozdzan, H. Casey, C. Mowery, L. Kumer, C. Fisher, S. Naik, C. Ehmann, M. Shin, M. Khawaja, H. Shike  
Penn State Milton S. Hershey Medical Center, Hershey, PA.

**Introduction:** Short tandem repeat (STR) analysis is used to monitor

donor engraftment after hematopoietic stem cell transplantation. By testing multiple STR loci on different chromosomes, we may detect unexpected aberrancies, in addition to engraftment status. We, therefore, studied the frequency and clinical significance of STR aberrancies in bone marrow transplant recipients. **Methods:** Two hundred sixty-one post-transplant hematology patients tested for chimerism by STR over a 2-year period were included in the study. A 24-locus multiplex STR assay (Promega PowerPlex Fusion, Madison, WI) was used to determine baseline genotypes of donors and recipients, and post-transplant chimerism. **Results:** Unexpected STR results were found in 2/261 patients. In patient 1, loss of heterozygosity (LOH) at one of the STR loci upon acute myeloid leukemia (AML) relapse was detected. In patient 2, neither of the son's (donor) alleles at one of the STR loci was detected. Patient 1 was a 59 year-old man with history of AML with myelodysplasia-related changes, cytogenetically 42-45,XY,add(3)(p13),-5[7],add(5)(q11.2)[12],dic(6;16)(p21.3;p13.3),-15,add(17)(p11.2),-18,-22,+0-3mar[cp19]/46,XY[1]. Two alleles [AGAA]<sub>12</sub> and [AGAA]<sub>16</sub> were present at D18S51 locus (18q) in his pre-transplant peripheral blood DNA (induction day 30, no circulating blasts). 3 months post double cord transplant, complete donor chimerism was detected, with donor alleles [AGAA]<sub>14</sub> and [AGAA]<sub>19</sub> at D18S51 locus. However, post-transplant month 4, patient developed mixed chimerism (54% recipient) without the recipient's [AGAA]<sub>16</sub> at D18S51 locus, indicating relapse and LOH. Peripheral blood smear was consistent with relapsed AML. Patient 2 was a 72 year-old man with polycythemia vera in accelerated phase. Cytogenetics showed 45-46,XY,add(X)(p11.2),add(X)(p11.2),add(11)(p11.2),add(14)(p11.2),-17,add(17)(p11.2), 18,add(21)(q22),+mar[cp20]. Next-generation sequencing revealed JAK2, ASXL1, TET2, and TP53 gene mutations. The patient received a haploidentical stem cell transplant from his son. At least one allele from each of the 23/24 STR loci were shared between son and the father, as expected. However, at Penta E locus (15q), only one allele, [AAAGA]<sub>13</sub> was detected in the patient's pre-transplant DNA and buccal swab, while the patient was missing both [AAAGA]<sub>7</sub> and [AAAGA]<sub>14</sub> alleles detected in his son's DNA. This finding indicated that the father was homozygous and the son had uniparental heterodisomy of chromosome 15. **Conclusions:** STR discrepancy is infrequent and can be caused by multiple mechanisms, such as malignancy and meiotic missegregation. Correlation with buccal swab, pre-transplant (remission) or family member's DNA is critical in discerning the clinical significance of a "homozygous" result.

### H003. A Strategy for Implementing Sensitivity Controls for qPCR Chimerism Monitoring

J. Tyler, M. Fasnacht, C. Fisher, H. Casey, H. Shike, L. Kumer, M. Maybach

Penn State Milton S. Hershey Medical Center, Hershey, PA.

**Introduction:** Chimerism test is used to monitor post-stem cell transplant chimerism status and risk of relapse. Quantitative real-time PCR (qPCR) utilizes TaqMan probe that detect insertion/deletion variation and provides higher sensitivity than short tandem repeat (STR). CAP requires sensitivity control as QC parameter for each chimerism assay (standard: HSC.38150). Since two selected ins/del markers are used to follow patients by qPCR, the qPCR sensitivity controls must represent the ins/del variations used in each donor-recipient cases. Such controls are not commercially available. We manufactured sensitivity controls and developed a system to verify sensitivity for each assay. **Methods:** KMRTYPE kit (GenDx) was used for genotyping 30 insertion/deletion markers (4-color multiplex) on QuantStudio 12K Flex Real-Time PCR instrument (Thermo Fisher). Nine volunteers were typed as a potential source of artificial DNA mixtures. We evaluated combinations between two volunteers that simulate donor-recipient pairs and chose the combinations that minimize the number of pairs required to provide at least one sensitivity control for all 30 qPCR markers. Volunteer DNA concentration was quantitated by Qubit. Sensitivity controls (100ng/reaction, 0.1% Recipient) was created and tested by KMRTYPE (GenDx) on the Roche LightCycler480 Real-Time PCR instrument (one-color). **Results:** We found 5 combinations of "Recipient-Donor" pairs that are sufficient to serve as sensitivity controls for all 30 qPCR markers, each covering 5-7 qPCR markers. All of the manufactured "0.1% Recipient"

were detected by corresponding qPCR markers, while none of the "0% Recipient ("Donor only") were detected. Therefore, 0.1% sensitivity controls were validated and used for the subsequent testing. Since implementation, the marker specific sensitivity controls were detected in 31/31 assays (100% success rate). **Conclusions:** Artificial DNA combinations (0.1% Recipient and 99.9% Donor) can serve as sensitivity controls for qPCR chimerism. By maximizing the numbers of markers that each sensitivity control covers, we found five combinations of "Recipient-Donor" sufficient to be used for all 30 markers. This eliminates the need of preparing actual patient-donor DNA mixtures as sensitivity controls in each assay.

### H004. Genetic Profiling of Adult Acute Myeloid and Lymphoid Leukemia Cases in a Major Referral Center in Lebanon

S. Halabi, N. Assaf, R. Mahfouz

American University of Beirut Medical Center, Beirut, Lebanon.

**Introduction:** Recurrent genetic abnormalities confer distinct morphologic features and play a role in determining the clinical behavior, prognosis and adequate treatment of acute leukemia. The frequency of the most common translocations and mutations is published by the WHO. However, numbers diverge in different areas of the world. In the MENA region, only one study targets the frequency of genetic modifications in AML, reporting a higher occurrence of acute promyelocytic leukemia in Lebanon. In our study, we describe the genetic profile of all adult acute leukemia cases presenting to our tertiary care center over a 10 year period. We determined the frequency of translocations and gene mutations in acute myeloid and lymphoid leukemia cases in an adult patients' population in Lebanon and compared the resultant genetic profile with the published international molecular behavior of adult acute leukemia. **Methods:** Laboratory results of adult patients diagnosed with AML or ALL presenting to AUBMC for genetic profiling between years 2006 until June 2016 were reviewed. Genetic profiling of AML cases in our molecular diagnostics laboratory consists of a lab developed RT-PCR for the detection of t(8;21)(q22;q22), inv(16)(p13.1q22) or t(16;16)(p13.1;q22), t(15;17)(q22;q12), t(9;11)(p22;q23) and mutations in the FLT3 receptor, NPM1, c-kit and CEPBA genes. The ALL panel tests for the presence of t(9;22)(q34;q11.2); BCR-ABL1, t(v;11q23); MLL rearranged, t(12;21)(p13;q22); TEL-AML1 (ETV6RUNX1), t(5;14)(q31;q32)IL3;IGH, t(1;19)(q23;p13.3); E2A-PBX1 (TCF3-PBX1). **Results:** We obtained 580 AML and 175 ALL cases. In the AML cohort, the M:F ratio was 1.3:1 with a mean age of 50 years. t(15;17) was present in 7.3%, t(8;21) in 4%, inv(16) in 3.7%, t(9;22) in 2.5% and t(9;11) in 0.7% of cases. FLT3 mutation (ITD or TKD) was present in 25.2% of all cases and 30.1% of normal karyotype (NK) patients. Mutations of the NPM1 gene was present in 31.4% of AML cases and in 43.8% of NK patients. Double positive (NPM1+/FLT3+) cases accounted for 20% of NK patients. CEPBA and c-kit mutations were detected in 7.3% and 2.4% respectively. In the ALL cohort, the mean age was 37 years. B- and T-lymphoblastic leukemia constituted 81% and 19% of ALL cases and the M:F ratio was 1.2:1 and 3:1 respectively. B-ALL patients were positive for t(9;22) in 14.7%, t(4;11) and t(1;19) in 2.8% each, and t(12;21) in 1.4%. T-ALL patients were positive only for t(4;11) (12.9%). **Conclusion:** Except for a mild increase in t(9;22) positive AML prevalence and a lower age at presentation, the genetic profile of our acute leukemia cohort is concordant with the international literature. Full molecular profiling by next generation sequencing is required for further classification into prognostic categories.

### H005. Clinical Implementation of T-cell Clonality Testing by Next-generation Sequencing: Improved Detection Sensitivity and Reliability in Initial Diagnosis and Minimal Residual Disease Detection of T-cell Malignancies

J. Yao, M. Syed, W. Yu, L. Maciag, C. Ho, K. Petrova-Drus, C. Moun, A. Zehir, M. Roshal, A. Rosado, D. Ahmet, K. Nafa, M. Arcila

Memorial Sloan Kettering Cancer Center, New York, NY.

**Introduction:** T-cell receptor (TCR) clonality assessment is often essential in the initial diagnosis of T-cell lymphomas (T-NHL). Current widely used capillary electrophoresis (CE) cannot separate multiple rearrangements with same size and has no or limited utility in the detection of minimal residual disease (MRD). Next generation sequencing (NGS) offers unique advantage in differentiating clonal populations

sharing the same fragment size. Furthermore, the disease associated unique sequences can be tracked in subsequent samples for monitoring and detection of MRD. In this work, we describe our clinical implementation of TCR gamma gene (TRG) based NGS clonality testing, with direct comparison to CE assay at diagnosis and flow cytometry (FC) for disease monitoring. **Methods:** Clinical samples submitted for routine TCR clonality testing over a period of one year were selected for the study. Testing was performed with LymphoTrack TRG kits (Invivoscribe) using primers flanking the conserved V $\gamma$  and J $\gamma$  regions, followed by sequencing on Illumina MiSeq instrument. Data was analyzed by LymphoTrack MiSeq Software and in-house developed MSK-LymphoClone (LC) bioinformatics pipelines. Performance characteristics were evaluated by directly comparing to CE assay (BIOMED-2 primers) and FC. Data of corresponding clonality testing by CE and FC were collected. **Results:** A total of 172 clinical samples (98 initial characterization and 74 monitoring) from 93 patients with confirmed diagnosis of T-cell malignancies were tested, including: 13 angioimmunoblastic T-cell lymphoma (AITL), 13 peripheral T-cell lymphoma (PTCL), 13 mycosis fungoides/Sézary syndrome (MF/SS), 6 cutaneous T-cell lymphoma (CTCL), 15 T-cell large granular lymphocytic leukemia (T-LGL), 7 T-cell prolymphocytic leukemia (T-PLL), and 10 acute T-cell leukemia/lymphoma (T-ALL). Among 86 initial characterization samples with testing by CE, the concordance rate was 92% (79/86): 1 sample was clonal by CE but oligoclonal by NGS; while 6 cases with prominent peaks by CE were confirmed to be clonal by NGS. Among 74 monitoring case, corresponding FC was available on 69. The concordance of NGS vs FC was 80% (55/69), with all 14 discrepant cases detected by NGS but not FC. **Conclusions:** TRG NGS testing by LymphoTrack demonstrated higher sensitivity in comparison to the conventional CE assay and FC, at initial diagnosis and in monitoring of disease, respectively. The unique ability to search for specific clonal sequences, greatly facilitates early and accurate detection, both for monitoring and staging with distinct advantages to patient management.

#### H006. Performance Evaluation of a Custom DNA/RNA Next-generation Sequencing (NGS) Assay for Hematologic Malignancies

J.X. Kars, X. Yu, F. Sabato, V. Williamson, W. Tang, P. Anderson, P. Morris, C. Margaritini, C. Jackson-Cook, G. Lai, A. Chesney, A. Ferreira-Gonzalez

Virginia Commonwealth University Health System, Richmond, VA.

**Introduction:** Characterization of hematologic malignancies continues to rapidly develop. Single nucleotide variants (SNV), fusions and copy number variations (CNV) are increasingly recognized as driver gene alterations in a wide range of leukemias. Given the heterogeneity and increase in number of clinically relevant mutations discovered, detection methods such as fluorescence *in situ* hybridization (FISH) and qRT-PCR are becoming unsuitable for comprehensive assessment as they are laborious and low throughput. Here we describe the validation of Oncogenomics Dx Heme Two (ODH2), a custom targeted next generation sequencing (NGS) assay for SNV, fusion and CNV detection in hematologic malignancies, allowing simultaneous assessment of 163 genes. **Methods:** We evaluated the performance of ODH2 using 19 characterized clinical samples (DNA and RNA) to identify all actionable SNVs, fusions, and CNVs. In addition, we evaluated 3 commercially available reference materials that contained SNVs, fusions and CNVs (SeraSeq Myeloid DNA, RNA, SeraSeq Breast CNV Mix). We utilized negative reference panels from Coriell and ATCC cell lines (NA12878 for DNA negative control, CCRF for RNA negative control). Samples were separately extracted (Qiagen EZ1 Advanced XL extraction for DNA, Trizol extraction for RNA) and a consolidated work-flow with multiplex PCR (VCU Custom and HemeV2, ArcherDx Boulder, CO) was utilized for library preparation. All samples were sequenced on an Illumina NextSeq500 instrument with the mid output kit using paired end sequencing (Illumina, San Diego, CA). Data analysis was performed with Archer Analysis software 5.1.8. ODH2's performance was correlated with results from Oncogenomic Dx One Plus Heme assay (Ampliseq Hot Spot v 2.0 by ThermoFisher, Walltham, MA) and FISH results as well as expected fusion and CNV variants in the controls provided by SeraCare. **Results:** All 19 samples were successfully analyzed from ODH2. We detected 43/44 SNVs for a sensitivity of 97.8% and specificity of 100%

compared to our previous panel and expected SeraCare results. Additionally, we detected 12/12 fusions, 6/6 internal tandem duplications (ITDs) and 1/1 CNVs for 100% sensitivity and specificity for these mutations. Known variants were detected at allele frequencies down to 4.72%. Further studies will be included with serial dilutions of positive controls to establish the limit of detection and reproducibility for this assay. **Conclusions:** This assay demonstrated excellent ability to detect SNVs, and ITDs, fusions and CNVs. This assay provides several advantages, including high multiplexing capability and scalability, detection of SNV, CNV, rare fusions, and characterization of fusion genes involving heterogeneous partners, which may provide insight into hematologic disease.

#### H007. Evaluation of Performance of Two Commercially Available BCR-ABL Real-time PCR Assays for Deep Molecular Response in International Scale.

B. Das, F. Ahmad  
SRL Ltd., Mumbai, India.

**Introduction:** Presence of BCR-ABL fusion is the genetic hallmark of Chronic Myeloid Leukemia (CML) patients and three clinically relevant variants are observed: p210 (major), p190 (minor), and p230 (micro) isoforms. Achievement of deep molecular responses is an emerging treatment goal becoming attainable for more patients due to the availability of second-generation tyrosine kinase inhibitors (TKIs), and these patients and may be enrolled into treatment-free protocols provided they show sustained DMR. Here, we compared the BCR-ABL1 levels determined using international scale (IS)-based two commercially available assays. **Methods:** BCR-ABL1 quantitation was done using Asuragen and TRUE PCR (3B Black bio) for performance evaluation. Three secondary controls were run with consecutive logs to check the linearity. Precision analysis was determined as inter and intra assay variability using five positive specimens. Correlations between the two assays were done using 20 specimens. Result acceptance criteria included percent Coefficient of Variants (CV) difference within  $\pm 0.5\text{Log}_{10}$  among two assays. On the basis of performance, a total of 2232 samples were further tested using TRUE PCR assay. **Results:** Both the assays showed good linearity across three consecutive logs of the secondary controls. Precision analysis on the basis of inter and intra assays showed % CV difference  $< \pm 0.5\text{Log}_{10}$  among different runs. Of the 20 samples taken for comparison of results, there was complete concordance in 90% of the cases. Interestingly, TRUE PCR assay picked up very low level BCR-ABL transcripts in two cases which were missed by Asuragen assay, indicating improved sensitivity of TRUE PCR kit for low level transcripts. In addition, TRUE PCR assays could also give additional information about the transcript type in the assay which was not possible with Asuragen assays, and needed fragment analysis. Asuragen and TRUE PCR results showed an overall good linear correlation with a tendency for the TRUE PCR to better sensitivity than Asuragen assays. A total of 2232 samples were further reported using TRUPCR assay. BCR-ABL1 fusion transcript was found in 63.75 % (1423/2232) samples. Out of 1423 positive cases, 47.7% (679/1423) were  $\leq 1\%$  transcript level with the lowest transcript level being as low as 0.0007%. Majority of the samples were positive for BCR-ABL1 major transcript, followed by minor and micro transcripts. **Conclusions:** In conclusion, TRUE PCR seems to have better performance for detecting DMR (upto 4.5 log) and supported by transcript differentiation in the same run. Secondly, TRUE PCR assay seems to be cost effective as well in comparison to Asuragen.

#### H008. Fusion Detection by Next-generation Sequencing from Methanol/Acetic Acid Fixed Cell Pellets in the Setting of Acute Lymphoblastic Leukemia Workup

X. Qu<sup>1,2</sup>, O. Sala-Torra<sup>2</sup>, C. Yeung<sup>2</sup>, M. Fang<sup>2</sup>

<sup>1</sup>Seattle Cancer Care Alliance, Seattle, WA; <sup>2</sup>Fred Hutchinson Cancer Research Center, Seattle, WA.

**Introduction:** Methanol/acetic acid fixation is routinely used in cytogenetic laboratories to preserve and archive clinical specimens. Fixed pellets have been successfully used for DNA based microarray testing and simple RNA-transcript detection. Here we present results of RNA-sequencing based fusion detection from archived fixed cell pellets in acute lymphoblastic leukemia (ALL). **Methods:** RNA was extracted using



ReliaPrep RNA Cell Miniprep System (Promega) from 33 specimens representing 29 ALL patients: One fixed cell pellet each for 25 patients and paired fresh and fixed specimens for four. Fixed specimens were leftover pellets from cytogenetic workup that had been stored at -20°C for 1-5 years. Blast percentages ranged from 2.6% to >80%. In one specimen, the RNA yield was too low for Qubit quantification and was not processed further. For the remaining 32 RNA samples from 28 patients, up to 100 ng of RNA were used. Libraries were prepared using the FusionPlex ALL kit targeting 44 fusion genes (ArcherDX), sequenced on MiSeq, and analyzed using Archer Analysis 5.1. Results were compared with karyotype, fluorescence *in situ* hybridization (FISH), RT-PCR, and Chromosome Genomic Array Testing (CGAT). **Results:** All 32 samples passed PreSeq QC. Each of the 4 pairs of frozen and fixed specimens showed the same results, including *BCR/ABL1* in two patients and *IKZF1* Δ4-7 in all four. Among the 28 fixed specimens, fusion and oncogenic isoform were detected in 17. Using karyotype and FISH as the reference, *BCR/ABL1* (n=7), *KMT2A/AFF1* [t(4;11), n=2], *EBF1/PDGFRB* (n=1) were detected with 100% sensitivity and specificity. FusionPlex uniquely identified *P2RY8/CRLF2* and *STIL/TAL1* (n=1 each). For *IKZF1* deletion: 8 samples were shown to have *IKZF1* Δ4-7 by FusionPlex and the corresponding exons 4 through 7 deletion by CGAT; one sample, with approximately 20% tumor, was shown *IKZF1* Δ4-7 positive by FusionPlex, but not CGAT; and FusionPlex did not detect haplo-insufficiency deletion of *IKZF1*, e.g. deletion of exon 1 or 8, which were detected by CGAT (n=4). **Conclusions:** Our study demonstrated that FusionPlex is highly sensitive and specific in detecting recurrent fusions in ALL. FusionPlex and CGAT are complementary in detecting *IKZF1* deletion, such that FusionPlex is more sensitive in detecting deletion that leads to oncogeneic isoform namely *IKZF1* Δ4-7, while CGAT can also detect deletion that leads to haplo-insufficiency. Methanol/acetic acid preserved specimens can be successfully processed for RNA-based fusion detection using next generation sequencing, enabling the use of commonly archived cytogenetic cell pellets in cases where fresh/frozen tissue is not available for RNA sequencing fusion detection.

#### H009. Fluorescence *in situ* Hybridization as a Tool for Minimal Residual Disease Testing in Multiple Myeloma

S. Golem, D. Persons

University of Kansas Medical Center, Lenexa, KS.

**Introduction:** Multiple myeloma (MM) is a malignant neoplasm of plasma cells and accounts for 1.8% of all cancers. MM is a heterogeneous disease at the genetic level (as indicated by the associated cytogenetic abnormalities) and in terms of clinical outcome. Fluorescence *in situ* hybridization (FISH) is currently one of the leading standards for detection of genomic (chromosomal) aberrations as well as the identification of some cryptic changes associated with prognosis in MM. Available data show minimal residual disease (MRD) negativity demonstrates a powerful predictor of therapy response, progression-free survival and overall survival in MM. For MRD detection, multi-parameter flow-cytometry (FCM) in MM is the current standard. However, the role of FISH in MRD detection in MM is not clear. In the current study, we aim to analyze the sensitivity of FISH on enriched plasma cells compared to FCM, for the detection of MRD in cases with genetic abnormalities that are detectable by commercially available FISH probes. **Methods:** Plasma cell enrichment was performed on bone marrow samples of MM patients at diagnosis or during surveillance using Stemcell Technologies (Seattle, Washington) CD138+ selection kit, followed by FISH analysis for high-risk markers including deletion of 1p, gain of 1q, deletion of *TP53*, *FGFR3/IGH*, and *IGH/MAF* rearrangement; additional FISH probes involved *IGH/CCND1* and 13q deletion probes (Abbott or Cytocell). FCM results from all these cases were also evaluated. **Results:** A retrospective study was performed on 183 bone marrow cases comparing FISH and FCM results in MM patients in which at least one FISH probe was abnormal at diagnosis. The cases involved 74 females and 109 males. Of the 183 cases, 33 were initial diagnosis and the remaining were follow-up studies, including post-transplant studies. Of the 183 cases, 66 cases were positive for both FISH and FCM, 10 cases were FISH positive and FCM negative, 14 cases were negative by FISH and positive by FCM, and 33 cases were negative by both FISH and FCM. **Conclusions:** This

study provides important clinical evidence that use of FISH in addition to FCM increases the sensitivity for detection of MRD in MM patients. FISH can be a more sensitive assay for detection of MRD compared to FCM in select patients with genetic abnormalities that are detectable by commercially available FISH probes. This study demonstrates that FISH can be a useful adjunct to FCM for detection of MRD in select patients with MM.

#### H010. Reproducibility of Clinical Samples by the Illumina TruSight Myeloid Next-generation Sequencing Panel

L. Commander, J.K. Booker, L.B. Thorne, M.L. Guley, N.D. Montgomery

University of North Carolina Hospitals, Chapel Hill, NC.

**Introduction:** Mutation information generated from massively parallel sequencing (MPS) panels is a critical element of standard of care for myeloid malignancies with potential to impact diagnosis, prognosis, and therapy options. The Illumina TruSight Myeloid (TSM) 54-gene panel is the library preparation most commonly used for characterizing hematologic malignancies in the College of American Pathologists proficiency surveys. However, reproducibility of variant calls in the TSM assay using the manufacturer's bioinformatics pipeline has not been thoroughly evaluated in the literature. To ensure reproducibility of variants reported on this platform, we instituted a policy to run clinical samples in duplicate as part of testing at the University of North Carolina Hospitals. Here, we report detailed reproducibility data from our clinical experience. **Methods:** We reviewed data from 104 consecutive samples run in duplicate over a three-month period at our institution. Patient samples from two flow cells during the study interval were excluded due to technical problems, leaving 101 duplicate clinical samples on 31 flow cells for evaluation. TSM libraries were sequenced using v3 chemistry on a MiSeq instrument. Binary alignment and variant call files were generated using MiSeq Reporter and manually reviewed using Variant Studio (v3.0). After excluding known recurrent artifacts and filtering for read depth  $\geq 200$ , population allele frequency  $< 0.1\%$ , and variant allele fraction (VAF)  $\geq 5.0\%$ , variants in 34 genes were reviewed in replicate runs to assess reproducibility. **Results:** A total of 201 variants were identified in the 'A' replicate of 101 patient samples. While 89% of variants repeated in the 'B' replicate, 11% failed to replicate. Unreplicated variants were characterized by lower VAF (6.6% vs 39.5%,  $P < 0.0001$ ), with half of all variants identified between 5 and 10% VAF in the 'A' sample failing to repeat to a limit of detection of 1% VAF in the 'B' sample. Similarly, unreplicated variants were associated with lower read depth (550 vs 10549,  $P < 0.0001$ ) and lower alternate allele read depth (35 vs 3980,  $P < 0.0001$ ). Moreover, unreplicated variants were all single nucleotide variants or 1-base pair insertion/deletions (indels), whereas all large indels replicated. Finally, unreplicated variants were predominantly ASCO/AMP/CAP Tier III variants (95%), as compared to replicated variants, which were most commonly Tier I or II variants (60%) ( $P < 0.0001$ ). **Conclusions:** A subset of somatic variant calls on the Illumina TSM panel are sequencing artifacts that do not reproduce in replicate analyses. Our observations suggest that confirming low level variants ( $< 15\%$  VAF) by orthogonal methods or in replicate analyses is necessary to ensure accurate variant calling on the TSM platform.

#### H011. Extended Myeloid Mutation Profiling Using NGS in Triple-negative Myeloproliferative Neoplasms: Single Institution Experience at a High Volume National Reference Laboratory

A. Jhurane, J. Bissonnette, K. Barber, L. Liang, E. Antoniou, B. Tandon, J. Zhao

BioReference Laboratories Inc., Elmwood Park, NJ.

**Introduction:** Approximately 90% of classical myeloproliferative neoplasms (MPNs) harbor oncogenic driver mutations involving *JAK2*, *CALR* and/or *MPL*, and mutational profiling is therefore a primary component of routine clinical diagnostic evaluation. MPNs lacking alterations in these three genes are referred to as "triple-negative" MPNs. For example in primary myelofibrosis (PMF), triple negative mutation status has been established as an indicator of adverse clinical prognosis associated with inferior overall survival and progression to acute leukemia. Studies delineating the somatic mutational landscape among triple negative MPNs are relatively limited, however. Herein, we report molecular profiling results among >2000 MPNs sequenced at our

institution, including a large cohort of triple negative MPNs. **Methods:** We performed a retrospective analysis of all GenPath OncoSight next-generation sequencing (NGS) 17-gene MPN panel testing on myeloid neoplasm samples in the past three years. Molecular profiles among triple-negative MPNs was studied. **Results:** 2,157 cases were analyzed, of which, 36% were abnormal (n=776). 70% of the abnormal cases were positive for either a *JAK2*, *MPL* or *CALR* alteration. Consistent with published literature, *JAK2* alterations were most prevalent (73%), followed by *CALR* (20%) and *MPL* (7%). Interestingly, although generally considered mutually exclusive, co-occurrence of mutations in *JAK2*, *CALR*, and/or *MPL* were noted among 1% of total abnormal cases. In aggregate, among triple-negative MPNs studied (30% of remaining abnormal cases, n=232), pathogenic mutations were most frequently identified in *DNMT3A* (27%), *ASXL1* (23%), *SRSF2* (10%), *TP53* (7%), *SF3B1* (6%), *SETBP1* (6%) and *EZH2* (5%). Alterations in *DNMT3A* and *ASXL1* were most prevalent among triple negative PV (49% and 44% of the 41 triple-negative PV cases, respectively). A similar pattern was seen among confirmed triple negative ET, with *ASXL1* and *DNMT3A* alterations each representing the most frequent somatic alterations (31% of ET cases, n=26). On the other hand, in triple-negative PMF cases, *SRSF2* (50%) alterations were most common. **Conclusion:** This data illustrates the clinical utility of extended myeloid mutational profiling using NGS in triple-negative MPNs. Alterations in genes such as *ASXL1*, *DNMT3A* and *SRSF2* are generally considered to be adverse prognostic markers in various myeloid neoplasms. Our findings demonstrate that alterations in these genes are recurrent in triple-negative MPNs and therefore, may contribute to the worse prognosis of this class of MPNs. Overall, NGS panel testing for MPNs beyond *JAK2*, *CALR* and *MPL* may serve as a useful diagnostic adjunct to further inform clinical prognostication and treatment decisions in patients with triple-negative MPNs.

#### H012. Validation of a Custom, Focused Next-generation Sequencing Panel for Lymphoma

M. Kluk, S. Cheng, W. Tam

Weill Cornell Medicine, New York Presbyterian Hospital, New York, NY.

**Introduction:** Next Generation Sequencing (NGS) is becoming increasingly important in diagnostic hematopathology. The use of NGS in the clinical and pathologic work up of myeloid disorders (e.g., AML, MDS) has become widespread, while the use of NGS for lymphoproliferative disorders continues to expand as more is learned about the genetic abnormalities that are relevant for diagnostic, prognostic and therapeutic purposes in lymphoma. Herein, we describe the design and ongoing validation of a custom, focused, NGS panel for use in the clinical and pathologic workup of lymphoproliferative disorders. **Methods:** An amplification-based, custom, NGS panel targeting 66 genes, which are known to be relevant in lymphoproliferative disorders, was designed for the Illumina TruSeq Custom Amplicon platform, to detect single nucleotide variants (SNV) and small insertion/deletions (In/Dels). The assay includes 1,523 amplicons targeting either hot spot regions or all coding exons for a gene. Validation samples included a variety of patient samples and reference sample of known genotypes. DNA was isolated from frozen and formalin-fixed paraffin embedded (FFPE) tissues and library preparation was performed according to manufacturer instructions. Sequencing was performed on the MiSeq and analysis was performed using the MiSeq Reporter and Illumina VariantStudio. **Results:** The cluster density ( $1,145 \pm 440$  K/mm<sup>2</sup>), percent clusters pass filter (92 +/- 5%), number of reads pass filter ( $25 \pm 8$  M), percent reads (read 1)  $\geq$  Q30 (90 +/- 3%) and percent reads (read 4)  $\geq$  Q30 (87 +/- 4%) have shown good performance over 8 runs. The inter-assay comparison for VAF (ie, method comparison vs. external reference lab assay) has shown good concordance ( $y=0.93(x) + 1.24$ ,  $R^2 = 0.86$ ) across a range of VAF (5-50%), including different variant types (>20 variants) in multiple genes in a variety of patient samples, including formalin-fixed, paraffin-embedded (FFPE). Similarly, the intra-assay reproducibility for VAF (ie, samples repeat tested on different in-house MiSeq runs) has shown excellent agreement ( $y=1.09(x) - 1.30$ ,  $R^2 = 0.98$ ). Testing of a commercially available formalin fixed reference sample over 5 different MiSeq runs has also show very good concordance

( $y=1.22 - 1.60$ ,  $R^2 = 0.96$ ) with expected VAF across a range of values (5-35% VAF). Dilution studies with cell lines has shown detection of In/Dels and SNVs to approximately a 5% VAF limit of detection. **Conclusions:** These findings indicate the successful design and on-going validation of a focused, custom, amplification-based NGS panel for use in the clinical work up of lymphoproliferative disorders in diagnostic hematopathology. Such testing will become increasingly important as the understanding of the genetic alterations in lymphoproliferative disorders continues to expand.

#### H013. Identification of FLT3 ITD Using Next-generation Sequencing (NGS): A Single Institution's Experience

A. Campbell<sup>1</sup>, W. Crowe<sup>1</sup>, J. Spence<sup>2</sup>, J. Spence<sup>1</sup>, P. Rothberg<sup>1</sup>, Y. Ding<sup>1</sup>

<sup>1</sup>University of Rochester, Rochester NY; <sup>2</sup>NGS Tech, Albany, NY.

**Introduction:** Internal tandem duplication (ITD) involving the fms-related tyrosine kinase 3 gene (*FLT3*) is a recurrent somatic mutation frequently found in acute myeloid leukemia (AML). *FLT3* ITD mutation has been shown to be one of the most pertinent prognosticators for overall survival in AML patients. Although recent advances in high-throughput sequencing technologies have enabled genome-wide detection of various alterations in cancer cells, detection of insertions in short read NGS data is still a challenge because insert-containing reads often fail to align to the reference genome using common NGS read mappers. The performance of most NGS analysis tools for identifying medium-size insertion such as *FLT3* ITD mutations is also largely unknown. **Methods:** 37 AML samples positive for various mutations as detected by prior sequencing were selected for study using the TruSight Myeloid Sequencing Panel and the ArcherDx VariantPlex Myeloid Panel. Of these samples, 31 were known positives for *FLT3* ITD with length ranging from 15-201bp as determined by electrophoresis. To improve the ability of detecting large insertions with the TruSight Myeloid Sequencing Panel, we used MiSeq Reagent Kit v3 chemistry, which permitted 2x301bp read lengths. The Illumina MiSeq Reporter software can only detect insertion/deletions (indels) up to 25bp in length, thus a large fraction of the *FLT3*-ITDs would have been missed. To address this deficit, we developed a customized pipeline, Insertion-Deletion Detected Using Paired End Reads (InDelDuper). For the ArcherDx VariantPlex Myeloid Panel, we used the MiSeq Reagent Kit v3 chemistry but used 2x151bp read lengths and the Archer Analysis software to detect *FLT3* ITDs. We compared the performance of InDelDuper, Archer Analysis pipeline and two other variant callers (VarDict and Pindel) that have utility for detecting indels. **Results:** The TruSight Myeloid Sequencing Panel, in conjunction with the customized pipeline InDelDuper, and the ArcherDx VariantPlex Myeloid Panel and Archer Analysis software detected 100% (31 of 31) of the tested *FLT3* ITD with no false positives. Both outperformed the other two programs evaluated. When comparing the ITD size obtained by NGS, the results from the ArcherDx VariantPlex Myeloid Panel and analysis software were more consistent with those obtained by fragment analysis. **Conclusions:** Compared to the TruSight Myeloid Panel, the ArcherDx VariantPlex Myeloid Panel is a very sensitive tool which can detect partial, large, and complex *FLT3* tandem duplications. It can also provide a precise location of the duplication breakpoints without increasing read length or implementation of a customized sequence analysis pipeline.

#### H014. Mate Pair Sequencing: Ushering Cytogenetics Into the Era of Personalized Medicine

N. Hoppman, B. Pitel, S. Smoley, S. Johnson, J. Smadbeck, M. Webley, S. Koon, C. Zepeda-Mendoza, J. Peterson, G. Vasmatazis, E. Thorland, W. Sukov, H. Kearney, R. Rowsey, P. Greipp, R. Ketterling, R. Jenkins, L. Baughn  
Mayo Clinic, Rochester, MN.

**Introduction:** Cytogenetic testing is a gold standard of care for patients with hematologic malignancies, as it yields important diagnostic, prognostic, and therapeutic information. For decades, karyotype and fluorescence *in situ* hybridization (FISH) studies have been heavily utilized for characterization of hematologic malignancies. Chromosomal microarray has also become increasingly utilized over the last several years. However, even with the combination of all three methodologies, clinicians' patients are often left with results of uncertain significance and/or incomplete cytogenetic characterization of their

neoplasm. The Mayo Clinic Genomics Laboratory recently launched a novel clinical test, mate pair sequencing (MPseq), to help clarify uncertain results obtained from chromosome, FISH, and/or microarray studies. This assay couples whole genome MPseq with patient-specific, customized analysis to provide clearer, more accurate results to the patient.

**Methods:** To date, we have performed MPseq on 27 patients with hematologic malignancies to clarify FISH, chromosome, and/or microarray results. Of these, 16 had a diagnosis of B-ALL, 3 T-ALL, 4 AML, and 4 patients were described as having other myeloid neoplasms (one PV, one APL, one MDS/MPN unclassifiable, and one with a questionable diagnosis of a "low-level myeloid process"). MPseq was performed using the Nextera library preparation method, followed by paired end sequencing on the Illumina HiSeq 2500. Data was analyzed using BIMA, and the region(s) of interest were visualized using either SVAtools or Ingenium, both in-house developed softwares. **Results:** MPseq was able to identify and further characterize the abnormality of interest in 24 of 27 cases. The three cases in which the abnormality was missed had either a low-level abnormality (approximately 10%) or breakpoints in regions with significant repetitive DNA; both of which are known technical limitations of MPseq. Of the remaining 24 cases in which the abnormality was identified, MPseq yielded additional significant prognostic information in 7 cases (29%) and information regarding additional therapeutic options or effectiveness of a particular therapy in eight cases (33%). Finally, in 11 cases (46%), other potentially useful information was revealed, including novel rearrangements not previously described in hematologic malignancies as well clarification of atypical FISH results.

**Conclusions:** This summary of our clinical experience with MPseq thus far highlights the unique ability of this assay to clarify uncertain or unusual results obtained by conventional cytogenetic methods in a large percentage of patients and highlights its utility in delivering important prognostic and therapeutic information that would not have been appreciated using traditional technologies.

#### H015. Evaluation of *NPM1* Mutation Detection by Droplet Digital PCR for Minimal Residual Disease Detection

R.Y. Walder, A.A. Stence, K. Sompallae, R. Sompallae, N.V. Guseva, D. Ma, A.N. Snow, A.D. Bossler

University of Iowa Hospitals and Clinics, Iowa City, IA.

**Introduction:** Insertion mutations in the nucleophosmin 1 gene (*NPM1*) are frequently found in cytogenetically normal patients with acute myeloid leukemia (AML). The TCTG (type A) insertion mutation in the last coding exon is the most abundant of these mutations. Quantification of *NPM1* mutation has been useful in tracking minimal residual disease (MRD) in AML. We evaluated a laboratory developed droplet digital PCR (ddPCR) assay for detection and quantification of the *NPM1* type A mutation.

**Methods:** Extracted DNA using the Gentra Puregene blood kit (Qiagen) was amplified and measured using ddPCR (QX200, Bio-Rad) with an assay for simultaneous measurement of the *NPM1* mutated and wildtype sequences. *NPM1* primers and a FAM-labeled linked nucleic acid probe for the type A mutation were duplexed with a HEX-labeled linked nucleic acid probe for wildtype *NPM1*. Data was analyzed using QuantaSoft Analysis software and results were represented as the ratio of mutant to wildtype copies/μl. **Results:** Testing of positive and negative controls demonstrated appropriate differentiation between the mutant and normal *NPM1* alleles. Negative control samples demonstrated a low mutant background signal of 0.0006% mutant/wildtype copies/μl. This assay showed 4-log detection of *NPM1* mutant in peripheral blood using serial dilution of mutant DNA specimen with wildtype DNA with reproducible detection down to 0.01% mutant/wildtype copies/μl (n=24). Serial dilution of input DNA was reliably detected down to a minimum of 1ng input DNA.

**Conclusions:** The ddPCR format allows the absolute quantitation of the *NPM1* type A mutation and obviates the need for normalization to a separate gene. Quantitation is linear, has a lower LOD of 0.01% mutant/wildtype copies/μl, and is highly reproducible across the range of the assay. This method is reliable for monitoring MRD and treatment responses in clinical samples.

#### H016. Detection of *CRLF2* Rearrangements in B-cell Acute Lymphoblastic Leukemia in Children with Down Syndrome

A. Garcia<sup>1,2</sup>, T. Qdaisat<sup>1</sup>, H. Sayeed<sup>2</sup>, I. Ghandi<sup>1</sup>, J. Reuther<sup>1,2</sup>, S.C. Reshmi<sup>3</sup>, E. Stonerock<sup>3</sup>, I. Chen<sup>4</sup>, Y. Dai<sup>5</sup>, R.C. Harvey<sup>4</sup>, C.L. Willman<sup>4</sup>, J.M. Gastier-Foster<sup>6</sup>, M. Devidas<sup>5</sup>, A.J. Carroll<sup>6</sup>, N.A. Heerema<sup>7</sup>, N.J. Winick<sup>8</sup>, W.L. Carroll<sup>9</sup>, K.E. Fisher<sup>1,2</sup>

<sup>1</sup>Baylor College of Medicine, Houston, TX; <sup>2</sup>Texas Children's Hospital, Houston, TX; <sup>3</sup>The Ohio State University and Nationwide Children's Hospital, Columbus, OH; <sup>4</sup>University of New Mexico School of Medicine, Albuquerque, NM; <sup>5</sup>University of Florida, Gainesville, FL; <sup>6</sup>University of Alabama at Birmingham, Birmingham, AL; <sup>7</sup>The Ohio State University, Columbus, OH; <sup>8</sup>University of Texas Southwestern Medical Center, Dallas, TX; <sup>9</sup>New York University Medical Center, New York City, NY.

**Introduction:** Philadelphia-like B-cell acute lymphoblastic leukemia (Ph-like ALL) is a B-ALL subtype with a *BCR-ABL1*-like gene expression profile and genetic alterations that dysregulate cytokine receptor and kinase signaling pathways. It is associated with poor outcome and is common in young adult, Hispanic, and Down syndrome (DS) patients. In DS-ALL, *CRLF2* rearrangements (*CRLF2-R*) comprise a majority of Ph-like molecular alterations, yet optimal methods for detection are unclear. We therefore assessed the correlation of low density array (LDA) *CRLF2* overexpression (*CRLF2-Hi*) with the presence of *CRLF2-R* in a cohort of pediatric DS-ALL patients. **Methods:** We identified 33 high-risk DS-ALL cases with *CRLF2-Hi* (delta Ct <6.0) by 15-gene LDA. For cases that lacked *P2RY8-CRLF2* fusion by RT-PCR (n=11), fluorescence *in situ* hybridization (FISH) was performed for *IGH@-CRLF2* rearrangements using locus-specific break apart probes (200 interphase nuclei scored). For cases that were negative for both *P2RY8-CRLF2* and *IGH@-CRLF2* (n=2), next generation sequencing (NGS) libraries were prepared from extracted total RNA using the Archer FusionPlex Heme v2 anchored multiplex PCR-based NGS library with molecular barcoding protocol. Illumina paired-end indexed libraries were multiplexed and sequenced on a MiSeq. Data were analyzed using vendor-provided analysis pipelines and custom-developed scripts. ANNOVAR was used for breakpoint annotation. **Results:** Of the 33 cases that were LDA *CRLF2-Hi*, 32 harbored *CRLF2-R*: 20 *P2RY8-CRLF2* fusions by RT-PCR, 10 *IGH@-CRLF2* confirmed by FISH, 1 *P2RY8-CRLF2* fusion detected by NGS alone, and 1 *CRLF2-R* by FISH without *IGH@* gene rearrangement or defined partner (*CLRF2-U*). In one case, no *CRLF2-R* was detected by RT-PCR, FISH, or NGS, but gain of the X chromosome (+X) was observed on karyotype. Six (18.2%) LDA *CRLF2-Hi* cases were not classified as Ph-like by the LDA; 4 *P2RY8-CRLF2* fusions (3 RT-PCR, 1 NGS), the *CRLF2-U* case, and the +X case. **Conclusions:** LDA *CRLF2-Hi* combined with RT-PCR, FISH analysis, and targeted NGS identified all *CRLF2-R* in this DS-ALL cohort. These results highlight the need for a combination of several established diagnostic modalities for comprehensive characterization of *CRLF2-R* in DS-ALL patients, and support the use of *CRLF2-Hi* to identify a subset of *CRLF2-R* cases that are not Ph-like by LDA. Molecular confirmation of LDA *CRLF2-Hi* results is needed to confirm *CRLF2-R*, and cytogenetics may identify fusion-negative LDA *CRLF2-Hi* attributable to +X. In rare cases of LDA *CRLF2-Hi* with no identifiable *CRLF2-R* by conventional methods, targeted NGS may assist in confirming a *CRLF2* fusion partner. These diagnostic tools may become increasingly important if use of targeted therapies such as JAK inhibitors becomes more widespread in this ALL subtype.

#### H017. Standardization of *FLT3*-ITD Mutation Allelic Ratio Reporting in the Clinical Laboratory Setting

S. Bhattacharyya<sup>1</sup>, E. Castro-Echeverry<sup>2</sup>, L.M. Kelly<sup>1</sup>, C.L. Burnes<sup>1</sup>, C. Gentile<sup>3</sup>, S. Roy<sup>1</sup>, M. Nikiforova<sup>1</sup>, C.D. Watt<sup>3</sup>, N. Aggarwal<sup>1</sup>

<sup>1</sup>University of Pittsburgh Medical Center, Pittsburgh, PA; <sup>2</sup>Baylor Scott & White Medical Center, Marble Falls, TX; <sup>3</sup>Hospital of the University of Pennsylvania, Philadelphia, PA.

**Introduction:** In acute myeloid leukemia (AML), a high allelic burden of *FLT3*-ITD (internal tandem duplication) confers a significantly worse overall prognosis. The cut-off for high versus low allelic ratio (AR) varies across studies (ranging from 1:1 [AF = 0.5] to 1:1.3 [AF = 0.8], mutant/wild-type *FLT3*) [Thiede *et al.*, Blood 2002; Ho *et al.*, Biol Blood Marrow Transplant 2016; Gale *et al.*, Blood 2008]. The exact method for calculating and reporting the allelic burden in a clinical setting is also not



well described in the literature. Here we describe our experience in validating FLT3-ITD allelic ratio reporting. **Methods:** The juxtamembrane regions of *FLT3* were amplified with fluorescently labeled primers and evaluated by capillary electrophoresis (ABI-3730 genetic analyzer, range: 150–10,000 RFU; Saturation Limit: 30,000 RFU). The AR was calculated as the rounded ratio of mutant allele to wild type (ratio of peak heights and area under the curve [AUC]). Serial dilutions were performed to determine limits of detection (LOD). Subsequently, an inter-institutional blinded 20-specimen exchange was conducted to compare results for assay validation. These comparisons were based on ITD repeat size, AR calculation, and the allelic fraction of ITD signal. It is worth noting that there were only minor differences in instrumentation (ABI-3500) and methodology (AR calculated from AUC only) between the two laboratories. **Results:** A series of dilution studies confirmed reproducibility and linearity of AR, and established a LOD of AR = 1:100-1:200 (AF = 0.01-0.02). There was no significant difference between AR calculated using peak height or AUC ( $r^2 = 0.9998$ ). Data from the Sample exchange demonstrated 100% (20/20) concordance of qualitative (Positive [17] vs. Negative [3]) results as well as number of ITD mutations present. There was mild variability in the reported ITD repeat size, with 5 cases showing concordance, 11 cases with a difference of  $\pm 1$  bp, and 1 case with a difference of  $\pm 2$  bp. However, these results are within the acceptable variability range for this assay. Overall, there was good inter-institutional concordance of calculated AR (16/17 = 94%;  $r^2 = 0.9722$ ). The single discordant sample was reported as an AR of 1:1 vs 1:2 (AF = 0.5 vs 0.33). This variability attributed to differences rounding of values during calculation. **Conclusions:** A FLT3 PCR assay performed on an instrument with a large signal intensity range can reliably and accurately provide detection of both ITD mutation status, repeat size, as well as estimated allelic burden, which may be calculated using either a ratio of peak heights or AUCs. These results may provide improved prognostic and therapeutic significance to this assay.

#### H018. A Limited FISH Panel is a Useful Surrogate for Metaphase Analysis to Rapidly Identify Patients with AML-MRC

N.D. Nelson, A. Bagg, S.M. Luger, A. Ganetsky, J.J. Morrisette  
Hospital of the University of Pennsylvania, Philadelphia, PA.

**Introduction:** FDA approval of CPX-351, a liposomal encapsulation of daunorubicin and cytarabine, for treatment of secondary acute myeloid leukemia, including AML with myelodysplasia-related changes (AML-MRC) and therapy-related AML (t-AML), highlights the need for rapid diagnosis. Eligible AML-MRC patients have either prior myelodysplastic syndrome (MDS) or an MDS-related cytogenetic abnormality. Metaphase analysis is too time intensive to impact induction therapy, so we incorporated rapid fluorescence *in situ* hybridization (FISH) testing for -5/5q, -7/7q, and -17/TP53 deletion for all patients age 60-75 in our AML pathway to identify CPX-351 candidates. We present an analysis of the MDS-FISH findings and correlation with chromosome analysis in CPX-351 patients at our center. **Methods:** Over a 9-month period, we performed rapid MDS-FISH testing, which includes *EGR1*(5q31)/5p15.2, *D7S486*(7q31)/CEP7, and *TP53*(17p13.1)/*NF1*(17q11.2) probes (MetaSystems) on 20 patients. Results were correlated with metaphase chromosome analysis. Patients who did not receive rapid MDS-FISH testing were identified from a database of patients treated with CPX-351 over the same time period. **Results:** Of the 20 patients who had MDS-FISH performed, 5 were positive; del(5q) was seen in all 5 patients. In all 5 patients conventional karyotype revealed complex cytogenetics and at least one MDS defining cytogenetic feature, with a monosomal karyotype in 4 patients. Cytogenetic abnormalities included 1 patient with concurrent del(5) del(7) del(17), 1 with concurrent del(5q) del(6;17), and 1 with concurrent del(5q) del(7) del(17). Of the 15 that were negative by MDS-FISH, one had another MDS-defining cytogenetic abnormality, isolated deletion 11q. An additional cohort of 7 patients with newly diagnosed AML, no FISH testing, and no history of MDS were found to have MDS-defining cytogenetic abnormalities by conventional karyotype. Of these 7 patients, 4 had cytogenetic abnormalities that would not have been identified by FISH testing, including del(12p), del(13q), and two with complex karyotypes. **Conclusions:** With the release of novel directed therapies, development of new laboratory protocols is necessary to allow rapid identification of eligible patients. We describe a FISH panel that

allows identification of MDS related cytogenetic changes within 4 hours of sample receipt. This panel identified 5/6 tested patients with MDS related cytogenetic changes, with a sensitivity of 83%. However, in patients with MDS-defining cytogenetic abnormalities who did not have FISH testing, this panel would have missed 4/7 patients, yielding a combined sensitivity of 62%. This panel is now performed at our institution for all patients between ages 60-75 with a new AML diagnosis, and continues to be refined.

#### H019. IntelliGEN Myeloid 50 Gene Panel Validation and Testing Experiences

L. Cai<sup>1</sup>, S. Parker<sup>1</sup>, U. Geigenmüller<sup>2</sup>, A. Leo Kenyon<sup>2</sup>, M. Mooney<sup>1</sup>, K. Wagner<sup>1</sup>, A. Walker<sup>2</sup>, Z. Zhang<sup>2</sup>, W. Chen<sup>1</sup>, S. Gardner<sup>1</sup>, N. Nagan<sup>1</sup>, S. Letovsky<sup>2</sup>, M. Eisenberg<sup>1</sup>, T. Richman<sup>1</sup>, H. Dong<sup>3</sup>, D. Boles<sup>1</sup>, A. Chenn<sup>1</sup>

<sup>1</sup>Laboratory Corporation of America Holdings, RTP, NC; <sup>2</sup>Laboratory Corporation of America Holdings, Westborough, MA; <sup>3</sup>Laboratory Corporation of America Holdings, New York, NY.

**Introduction:** The development of advanced molecular platforms, bioinformatics tools and the rapidly growing number of biomarkers that are the potential targets for new therapies have contributed to a rapid increase in cancer testing. Acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and myeloproliferative neoplasms (MPN) are clonal disorders of hematopoietic stem cells. The simultaneous detection of somatic mutations in multiple candidate target genes as markers of the neoplastic clone by the next generation sequencing (NGS) IntelliGEN Myeloid panel can provide information in diagnosis, prognostic risk stratification and eligibility for targeted therapy. In this study, the clinical and analytical performance features of this assay are evaluated. **Methods:** The IntelliGEN Myeloid panel utilizes amplicon-based next generation sequencing to identify alterations: single nucleotide variants (SNVs) and insertion/deletions (InDels) in 50 genes and whole-gene copy number alterations (CNAs) in 13 genes. DNA from blood, bone marrow and cell pellet were used to evaluate the assay's accuracy, repeatability, reproducibility and analytical sensitivity. Identified reportable mutations were confirmed by a secondary method. **Results:** Of the 60 specimens tested during validation, 97 variants identified were confirmed by a secondary method and showed 92.8% concordance. Discordances were most likely due to low detection sensitivity of the confirmation method. Repeatability (intra-assay precision) and reproducibility (inter-assay precision) were 100% for SNVs and InDels, and 89% for CNAs. The sensitivity of this assay is 5-10% variant allele fraction for SNVs and InDels. This assay can detect whole-gene CNAs of 25% or greater when the input DNA is 200ng or more. The IntelliGEN Myeloid panel has been offered as a clinical test based on the successful performance features. In a set of 176 clinical specimens, the number of mutations identified in each specimen ranged from 0-11 with 13% having no mutation identified, 72% 1-5 mutations, and 15% 6-11 mutations. 90% of specimens with mutations had clinically important information: 55% diagnostic, 88% prognostic and 58% therapeutic. Mutation profiles identified in related disease categories were comparable to similar studies. Patients who underwent targeted therapeutic treatment indicated by the test results showed successful response to the treatment from the follow up testing. **Conclusions:** The IntelliGEN Myeloid panel is a robust and reproducible assay using blood, bone marrow and cell pellet. The molecular alterations provided by this assay can assist in diagnosis, prognosis and making cancer treatment decisions involving targeted therapies.

#### H020. Mutational Analysis of Myeloid Neoplasms in Paired Peripheral Blood and Bone Marrow by Next-generation Sequencing

P. Michaels, A. Kim

Brigham and Women's Hospital, Boston, MA.

**Introduction:** During the diagnostic evaluation of myeloid neoplasms (MNs), peripheral blood (PB) and bone marrow (BM) are often submitted for next-generation sequencing (NGS). It is the aim of this study to reveal the concordance of mutations in the PB and BM and the types of mutations that might be present specifically in one tissue type over the other. We present the initial results of an analysis for mutational differences in paired PB and BM that were evaluated by a clinical NGS panel. **Methods:** This is a retrospective study of electronic pathology records for testing utilizing a NGS myeloid-directed panel assay (95

genes, 1330 amplicons, 5% variant allele fraction (VAF) typical threshold for new variants) from July 2014 to December 2017 at the Center for Advanced Molecular Diagnostics (CAMD) at Brigham and Women's Hospital (BWH). The following inclusion criteria were applied: 1) samples were tested for evaluation of MNs and 2) separate PB and BM samples were tested within two weeks of each other (defined as paired). Analysis of mutations in paired PB and BM was performed and were categorized based upon concordance and, if discordant, further categorized based upon the differences. Statistical analysis was performed using the GraphPad QuickCalcs website. **Results:** A total of 90 paired PB and BM samples were found that met inclusion criteria. Discordant mutations were seen in 24 pairs (27%). Of the 24 discordant samples, the PB showed additional mutations in 9 cases (37.5%, 14 total new mutations) while the BM displayed additional mutations in the remaining 15 cases (62.5%, 23 total new mutations). Of discordant cases, there was no significant difference in the average mutational burden (PB = 2.38 mutations, BM = 2.88 mutations,  $t$ -test  $p=0.12$ ). The PB harbored mutations that may have been undercalled in the BM (low VAFs or areas of poor coverage). Genes mutated in PB or BM encompassed similar pathways, but five mutations in *TP53* (22%) were identified solely in the BM ( $t$ -test  $p = 0.022$ ).

**Conclusions:** Our analysis of MNs submitted for clinical NGS in paired PB and BM has revealed that discordances can occur in 27% of cases. While most mutations that were PB-specific may be technical in nature, the BM seemed to have variants that may imply more of a stem cell phenotype with *TP53* mutations never identified in the PB alone. These data highlight the acquisition of mutations within the BM in MNs as well as stress the importance of BM submission for NGS for thorough mutation assessment.

#### H021. Guideline-adherent, Evidence-based NGS Workflow for Myeloid Leukemia

R. Kohle

Augusta University, Augusta, GA.

**Introduction:** Myeloid leukemias are a group of bone marrow disorders driven by various somatic mutations in a set of oncogenic and tumor suppressor genes. While Next Generation Sequencing (NGS) has proven to be a useful tool to understand leukemia molecular profile and its potential correlation to disease characteristics, it remains a subject of debate to what extent each mutation is clinically actionable and thus practically important for pathology labs to test. Increasing panel size also needs to be balanced with higher costs and data analysis burden. Furthermore, as professional organizations including ASCO, AMP and CAP continue to update their guidelines, it is increasingly challenging for laboratories to remain abreast of all current data and information. To address these issues, we established and compared several NGS workflows in parallel to identify evidence-based dataset for sequencing. We also cross-referenced multiple interpretation databases to ensure real-time access to updated guideline information. **Methods:** For the first workflow, we used QIAseq myloid panel with MiSeq sequencer. For the second workflow, we implemented the GeneReader NGS system and QIAGEN Clinical Insight (QCI) software suite for variant analysis and interpretation. The same set of samples were run in parallel on each workflow. Results were compared using both sequencing data and interpreted reports, then cross-referenced to ASCO, AMP and CAP guidelines. **Results:** Our refined myeloid panel contains 25 genes. All variants identified using this panel were supported by guideline and other evidence. A high level of concordance in sequencing results was achieved between the two workflows. Each interpretation software offered unique advantages in assigning variant clinical significance and incorporating guideline information. **Conclusion:** Our NGS workflows provide a reliable solution for routine implementation of myeloid sample testing. The content as defined in our experience offers a 'necessary and sufficient' set of data supported by clinical evidence. By incorporating a robust interpretation software we are able to access and adhere to latest guidelines. Our lab's experience provides an example for others that may wish to implement myeloid NGS testing.

#### H022. Evaluation of Targeted Next-generation Sequencing Panels for Myeloid Malignancies-Focusing on *CEBPA* and *FLT3* Genes

R. Akabari, M. Hussaini, D. Qin

Moffitt Cancer Center, Tampa, FL.

**Introduction:** Next generation sequencing (NGS) has been effectively used in detecting mutations in myeloid leukemia. However, the detection *CEBPA* and *FLT3* mutations poses a challenge for NGS assay due to high GC content in *CEBPA* and different sizes of Internal Tandem Duplication (ITD) in *FLT3*. Traditionally, orthogonal single gene tests would need to be performed alongside NGS panels to ensure detection of the variants in these two challenging genes. However, advances in bioinformatics and NGS chemistry have been made recently, which offers the possibility to surmount these challenges. In this study, we compared three NGS panels in detecting *CEBPA* and *FLT3* mutations. **Methods:** Three NGS panels were compared in covering and detecting mutations of *CEBPA* and *FLT3*. These panels are: Panel A: A 30 gene hybrid-capture assay, with 150 bp paired-end sequencing; Panel B: A 28 gene hybrid-capture assay, with 300 bp paired-end sequencing; Panel C: A 78 gene nested PCR capture assay, with 150 bp paired-end sequencing. **Results:** Seven *CEBPA* positive and ten *FLT3* positive cases were identified using two criteria: 1. tested positive in single gene test, and 2. tested negative in an amplicon NGS assay. In 7 *CEBPA* positive cases, two of them have point mutations and 5 of them have insertion/deletions (indels). In 10 *FLT3* positive cases, the length of ITD is 21 to 75 bp. The cases were tested using these three NGS panels. The average reads, the coverage of *CEBPA* and *FLT3*, and the detection of *CEBPA* or *FLT3* mutations were compared among these three panels. **Results:** All three panels detected all 7 *CEBPA* mutations and 10 *FLT3* ITD with 100% sensitivity and specificity in this setting. The average reads and the coverage of *CEBPA* and *FLT3* will be presented at the meeting. **Conclusions:** The results show that the NGS capture assay are able to detect *CEBPA* mutations and *FLT3* ITD that are not detected by regular NGS amplicon assay. NGS capture assay may be used for myeloid leukemia without additional orthogonal single gene tests for *CEBPA* and *FLT3*.

#### H023. Novel *PML-SYK* Fusion in Acute Myeloid Leukemia Constitutively Activates Targetable Pathways

J. Mosquera<sup>1,3</sup>, M. Sugita<sup>1,3</sup>, D. Wilkes<sup>1</sup>, R. Bareja<sup>1</sup>, K.W. Eng<sup>1</sup>, J. Croyle<sup>1</sup>, T.Y. MacDonald<sup>1</sup>, R. Jimenez Flores<sup>1</sup>, Z. Noorad<sup>1</sup>, D. Pancirer<sup>1</sup>, S. Cheng<sup>1</sup>, J.Z. Xiang<sup>1</sup>, L. Olson<sup>1</sup>, K. Van Besien<sup>1</sup>, D. Rickman<sup>1</sup>, W. Tam<sup>1</sup>, G. Chiosis<sup>2</sup>, M.A. Rubin<sup>1</sup>

<sup>1</sup>Weill Cornell Medicine, New York, NY; <sup>2</sup>Memorial Sloan Kettering Cancer Center, New York, NY; <sup>3</sup>Englander Institute for Precision Medicine, New York, NY.

**Introduction:** Syk is a widely expressed nonreceptor tyrosine kinase that initiates signaling through the Ras, PI3K and PLC- $\gamma$  pathways. The role of Syk alterations in B-cell lymphomas is common and better understood but its oncogenic driver function in myeloid neoplasia is rare. **Methods:** We report a 61 year-old female with incidental elevated white blood cell count (WBC) 4 yrs before, which work up revealed a hypercellular bone marrow (>95%) with myeloid proliferation and full maturation, and 6% blasts in aspirate. Cytogenetics revealed a t(9;15) involving the *PML* gene. Molecular studies were negative for *BCR-ABL*, *PDGFRA/B* and *FGFR1* rearrangements, and *JAK2* mutation. The diagnosis of unclassified myeloproliferative disorder (MPD) was rendered. Patient started hydroxyurea and received allogeneic stem cell transplantation (matched unrelated donor), and obtained remission but relapsed within one year. Post-relapse marrow showed granulocytic hyperplasia, megakaryocytic atypia with increased CD34-positive cells and persistent t(9;15). Patient was referred to the Englander Institute for Precision Medicine. Whole-exome sequencing (86x coverage) with control buccal swab (92x coverage) was performed. No somatic alterations in clinically relevant genes were found. However, a *PML-SYK* fusion was detected by RNA-seq (FusionSeq). Due to increasing leukocytosis (WBC 100,000 and peripheral blasts 18%), there was concern for accelerated phase. The patient did not respond to conventional treatment and progressed to acute myeloid leukemia (AML). **Results:** The *PML-SYK* fusion candidate was validated (fluorescent *in situ* hybridization and Sanger sequencing), and Syk overexpression was demonstrated (RNA-seq). The *PML-SYK* fusion transcript (PCR) involves exon 4 and exon 11

(kinase domain), respectively. Flow cytometry analysis of patient's cells revealed a constitutive activation of p-Syk, p-STAT5 and p-ERK when compared to normal cells. Thus, in addition to Syk inhibitors, the proposed therapeutic intervention included Hsp90 inhibitors given the apparent hyperactivated signalosome that would render tumor cells sensitive to epichaperome therapy. *In vitro* treatment of patient's cells with PU-H71 or Entospletinib resulted in cell death and decreased colony formation. Based on the results, the patient was enrolled onto PU-H71 clinical trial and started receiving this epichaperome inhibitor.

**Conclusions:** We describe and characterize a novel *PML-SYK* fusion in a patient with unclassified MPD who progressed to AML. Proven Syk overexpression and constitutive activation of several signaling pathways provided therapeutic alternatives to a patient refractory to standard of care treatment. Our findings have significant therapeutic implications for patients with *SYK*-induced MPD or AML.

#### H024. Validation of a Low Input Targeted NGS Assay for Lymphoma Across Multiple Specimen Types

A. Oran<sup>1</sup>, C. Paolillo<sup>1</sup>, S. Deihimi<sup>2</sup>, D.B. Lieberman<sup>2</sup>, A. Bigdeli<sup>2</sup>, A. Chittur<sup>2</sup>, J.J. Roth<sup>2</sup>, J. Rosenbaum<sup>2</sup>, J.J. Morrisette<sup>2</sup>, R.T. Sussman<sup>2</sup>  
<sup>1</sup>University of Pennsylvania, Philadelphia, PA; <sup>2</sup>University of Pennsylvania Hospital System, Philadelphia, PA.

**Introduction:** A variety of malignancies originate in cells of lymphoid lineages and are associated with genetic variants specific to lymphomas. There is currently a deficit in the availability of next generation sequencing (NGS) panels which specifically test for somatic DNA variants seen in lymphoid malignancies. The molecular characterization of a patient's tumor allows for an individualized, patient-specific treatment plan. We have validated a targeted sequencing panel which offers prognostic, therapeutic, and diagnostic information for the care of lymphoma patients.

**Methods:** The Illumina TruSight Lymphoma assay was used for the validation of a 40-gene panel designed for the detection of somatic DNA single nucleotide variants (SNVs) and small (<25bp) insertions/deletions (indels) relevant to lymphoid malignancies. The validation included 62 samples from different subtypes of lymphomas and a variety of sample types including formalin-fixed paraffin-embedded (FFPE) tissue, blood, bone marrow, and fresh tissue. Validation samples had previously been characterized on laboratory-developed tests specific to hematological malignancies and/or solid tumors, which had common targets in 18 genes. The lymphoma assay is designed for low DNA input and dual-stranded sequencing which allows for distinction between true variants and artifacts introduced from formalin fixation. This panel was evaluated for analytical validity, accuracy, precision, specificity, sensitivity, limit of quantification and linear dynamic range. **Results:** Of the 133 SNVs expected, 127 (95%) were detected and 38 out of 39 (97%) expected indels were detected. Missed calls can be attributed to allele frequencies falling below the lower limit of detection or to low depth of coverage. Three samples were repeated multiple times by the same technologist and 7 samples were repeated by three independent technologists, resulting in minimal variability. The assay was validated over a range of inputs with a minimum input of 10ng of DNA. A probit analysis of both blood and FFPE samples was used to determine the limit of detection of the assay with 95% confidence. **Conclusions:** The development of a lymphoma-based targeted sequencing panel will allow for more specific and appropriate care of patients with lymphoid malignancies. We have validated the use of this assay using minimal amounts of DNA and a variety of specimen types. This assay is highly reproducible, accurate, sensitive and specific. The use of genomic information from NGS assays can be useful in upfront chemotherapy selection for lymphoma patients as well as monitoring of clonal evolution and therapy resistance.

#### H025. Ultradeep Error Corrected Next-generation Sequencing (NGS) of ABL1 Kinase Domain Mutations in BCR-ABL1 Positive Malignancies

N. Patkar, A. Mehta, S. Joshi, R. Kodgule, S. Chaudhary, G. Raval, B. Mishra, T. Battapadi, P. Tembhare, P. Subramanian, S. Gujra  
 Tata Memorial Center, Navi Mumbai, India.

**Introduction:** *BCR-ABL1* kinase domain mutations (KDM) are the most common cause of resistance to targeted therapy in chronic myeloid leukemia (CML). Most published studies till date have employed Sanger sequencing (SS) to detect *ABL1* KDM. Next generation sequencing (NGS)-based *ABL1* KDM assays have many advantages over SS such as an ability to detect mutations at lower frequency and distinguish between polyclonal and compound mutations. A distinct disadvantage of NGS assays is an inability to distinguish sequencing errors from true mutations especially at lower variant allele frequencies (<5% VAF). The application of error correction by using unique molecular identifiers (UMID) to libraries allows us to overcome this hurdle. However, till date such data remains unpublished. **Methods:** The *BCR-ABL1* molecule was amplified and used as a template for KD amplification. The *ABL1* KD was amplified in a multiplexed reaction containing 5 tiled amplicons. Here we incorporated sequencing motifs, 8bp UMID and locus specific primers in a primary amplification step. Thus, every *ABL1* molecule was "tagged" with a unique molecular barcode. During subsequent amplification, we incorporated Illumina adapters as well as sample specific dual indices. This library was sequenced at equimolar concentrations using the Illumina V2 chemistry. Variant calling was done on a customized pipeline by creating consensus read families. Read families specific to a targeted *ABL1* locus were combined for variant calling and annotation. Assay linearity was demonstrated on a *BCL-ABL1* plasmid that had a T315I mutation. KDM results were validated on patients of CML with known KDM. **Results:** The assay could detect KDM as low as 0.1%. Validation was done on 17 patients of therapy resistant CML that had 22 proven (SS) *ABL1* KDM. The median coverage for 17 samples was 1706x (range 1130 – 7058). We could detect 20 out of 22 SS-proven *ABL1* KDM. Furthermore, using NGS, we could identify ten additional mutations that were described in literature at a VAF below the detection limit of SS. (Median VAF 1.23%, range 0.41 – 8.41). In samples with high coverage (> 5000x) we detected 4 novel mutations that could potentially contribute to therapy resistance. **Conclusion:** We describe a novel ultradeep error corrected sequencing based assay for detection of *ABL1* KDM in *BCR-ABL1* rearranged malignancies that detected 50% additional mutations as compared to SS. The validation of low VAF KD mutations is challenging. The clinical relevance of detecting low VAF KD mutations will need to be determined.

#### H026. Routine Clinical Monitoring of Disease Status Through NGS Measurement of Clonal Architecture in AML and MDS

P.D. Velu<sup>1</sup>, R.T. Sussman<sup>1</sup>, C.R. Soderquist<sup>2</sup>, A. Bagg<sup>1</sup>, S. Luger<sup>1</sup>, M. Carroll<sup>1</sup>, J. Morrisette<sup>1</sup>

<sup>1</sup>University of Pennsylvania, Philadelphia PA; <sup>2</sup>Columbia University Medical Center, New York NY.

**Introduction:** Routine use of clinical cancer next-generation sequencing (NGS) in diagnosis and disease monitoring can track changes in mutation profiles of individual patients, either in response to therapy or at disease progression. Myeloid malignancies such as acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) may be characterized and treated based on specific mutation profiles identified at diagnosis. Here we determined how NGS profiling at diagnosis and interval follow-ups may anticipate early failure in therapy and potential for relapse or progression. **Methods:** Using sequencing data from the Penn myeloid malignancy NGS panel, we selected patients whose blood or marrow specimens who had NGS studies done between February 2013 and February 2017 in which at least two studies were found to have mutations. At initial testing, patients had either newly diagnosed *de novo* AML (n=105) or a diagnosis of MDS (n=45). At subsequent testing, *de novo* AML patients had either hematologic remission (CRi, n=51), refractory AML after initial therapy (refAML, n=15), or first relapse of AML (relAML), and MDS patients had either persistent MDS or progression to secondary AML (sAML). A subset of patients who achieved CRi had subsequent testing while still in remission (CRi-sus, n=11). Diagnoses and timelines were determined



using bone marrow hematopathology reports and clinical notes from the electronic medical records. Mutational profiles were analyzed and visualized with R scripts. The institutional review board approved this study. **Results:** At initial diagnosis, mutational profiles of AML patients with sustained remission and no documented relapse within the study period were enriched in mutations in spliceosome complex genes compared to AML patients who relapsed (7/26 patients versus 2/21 patients). Mutational profiles at relAML were essentially stable compared to those at initial diagnosis, with infrequent fluctuations in signaling pathway (SP) and tumor suppressor genes. Of 10 MDS patients with mutations in SP or *NPM1* progressed to sAML, 1 was lost to follow-up, and 1 underwent an allogeneic bone marrow transplant and is continuing to be followed with no evidence of MDS. All *NPM1* mutations and 4/34 SP mutations were undetectable at CRi, and patients with sAML showed a significant gain in SP mutations (5 to 20 mutations,  $p=0.003$ ) after transformation from MDS. **Conclusions:** Mutational shift occurs in AML and MDS in tandem with changes in disease state and in response to therapy. Routine genetic profiling of patients in the clinical setting using multi-gene targeted NGS panels can be useful in monitoring AML and MDS patients as the type of mutation detected and the changes in mutation status correlate with response, relapse, and progression.

#### H027. Peripheral T-cell Lymphoma: Understanding and Characterizing the Phenotypic Behavior Using Molecular Tools

O. Shetty, K. Rai, S. Epari, S. Desai, N. Padte, H. Jain, M. Sengar, T. Shet

Tata Memorial Hospital, Mumbai, India.

**Introduction:** Advances in the field of T-cell lymphomas in the last decade have revealed many new genetic abnormalities and targets that seek to change the abysmal course of this disease. **Methods:** The study was performed on Formalin Fixed Paraffin Embedded (FFPE) lymphoma cases ( $n=42$ ) which were categorized histologically into PTCL NOS ( $n=23$ ) and Angioimmunoblastic T-cell lymphoma (AITL;  $n=19$ ) with a view of understand prevalence of these in our patient population. Gene sequencing for *RHOA* and *IDH2* was performed. Gene expression profiling (GEP) for NFkB and PIK3CA pathway was performed using quant studio 12K flex. Data was analyzed using Chromas lite software and Expression suite software. The data was correlated with histological, molecular, and clinical findings. **Results:** The age range was between 35-76 years. Among the 42 cases sequencing data could be obtained for 37 cases only. *RHOA* G17V mutation was observed in 3/13 AITL cases (23%) and 1/16 PTCL cases (6.2%). *IDH2* mutation was observed in 2/13 AITL cases (15. 3%). In two cases double mutation for *IDH2* and *RHOA* was observed. All the PTCL NOS with *RHOA* mutation showed nodules of clear cells and follicular helper T cell phenotype. The disease was progressive in 54% of the cases with bone marrow involvement. AITL cases with dual mutation was found to be associated with poor prognosis, high proliferation index followed by bone marrow involvement. GEP studies have been performed on 10 cases and the study is ongoing in order to obtain the designated panel. Differential expression of *FCGR2a*, *LCK*, *ITK*, *KRAS*, *PIK3* family of genes, *TLR4*, *GSK3B*, *MAPK1*, *VAV1*, *IRAK4*, *RPS6KB1* was observed for the PI3K pathway GEP. Similarly, the following genes *FASLG*, *TBK1*, *PTGS*, *RELB*, *TLR2*, *CXCL1*, *PTGS2*, *TNFSF11*, *CHUK*, *CSF3*, *BCL10*, *ZFP36*, and *NOS2A* were differentially expressed for the NFkB GEP. This data will be used for designing a signature panel for characterizing the PTCL cases. **Conclusion:** *RHOA* and *IDH2* mutation is seen less frequently in our patient population and cases with dominance of clear cells and this morphologic feature can help us in differential classification of the disease. The molecular profile derived from this will help in predicting the prognosis as well as offering targeted therapies and thus help in better patient care management.

#### H028. Myeloseq One: A Cost Effective Integrated Next-generation Sequencing Assay for Myeloid Malignancies

R. Kodgule, G. Raval, C. Kakirde, P. Bhanshe, S. Kadechkar, F. Naeem, V. Shetty, S. Chaudhary, S. Joshi, P. Tembhare, P. Subramanian, S. Gujral, N. Patkar

Tata Memorial Centre, Navi Mumbai, India.

**Introduction:** Introduction of next generation sequencing (NGS) platforms towards clinical implementation has occurred at a rapid pace globally. However, in India the mass applicability of NGS based cancer diagnostics continues to be bottlenecked mainly by the high cost of library preparation. Here, we have standardized and validated MyeloSeq One, a cost-effective targeted NGS assay for diagnosis and prognostication of myeloid malignancies. This is a laboratory developed assay that detects somatic mutations on genomic DNA as well as unknown fusions using RNA sequencing. **Methods:** We designed 1140 single molecule molecular inversion probes (smMIPS) to detect mutations in 49 genes implicated in pathogenesis of myeloid malignancies. After evaluation of initial capture efficiencies, non-performing smMIPS were discarded. The final panel (151kb) consisted of 1014 smMIPS. These smMIPS were balanced to ensure similar capture efficiencies of targeted regions. The panel was validated by comparing its performance in 25 cases of adult *de novo* AML against Illumina TruSight Myeloid Panel. For detection of mutations in *CEBPA* (high GC content) we developed an error corrected assay that employed 8bp unique molecular identifiers. For detection of fusions we developed assay that ligated a hemi functional Y-adaptor to enzymatically sheared cDNA, followed by amplification with a universal primer and a gene specific primer allowing us to detect unknown partner of a gene implicated in a gene fusion. A fusion panel was created that detected fusions across 44 genes. **Results:** Validation of MyeloSeq One was done on three different sets of samples with confirmed somatic mutations and fusions using orthogonal techniques. smMIPS were tested on 25 cases of adult *de novo* AML, already analysed with TruSight Myeloid Panel. 52 variants were detected using both assays with 35 single nucleotide variants & 17 small insertion/deletions (indels). Three variants were missed by smMIPS assay whereas 2 variants were missed by TruSight myeloid assay. Linear regression analysis for Variant allelic frequency of concordant variants showed high degree of correlation ( $R^2 = 0.83$ ). Targeted RNA sequencing was done on cases known to harbour common translocations occurring in myeloid malignancies. Fusion transcripts were detected in all the cases by RNA sequencing. Out of 25 cases known to harbour *CEBPA* mutations we could detect mutations in 24 cases by the NGS assay. **Conclusions:** The library preparation and sequencing costs for our assay are less than US \$100. Using targeted NGS approach we could cost-effectively interrogate a large focused subset of molecular pathways in the clinical laboratory set up; thereby demonstrating feasibility of comprehensive molecular profiling of disease in the era of personalized medicine.

#### H029. Minimal Residual Disease in AML can be Monitored Utilizing Cell-free DNA

L.M. Chamberlain, Z. Xie, A. Carson, V. McClain, O. Kiya, B. Patay, M. Blankfard, T. Stenzel, J. Miller

Inivoscribe, San Diego, CA.

**Introduction:** Cell-Free DNA (cfDNA) isolated from plasma is a source of circulating tumor DNA (ctDNA) for diagnostic use. ctDNA with a molecular profile similar to that of bone-marrow tumor cells has been observed in cfDNA from multiple myeloma patients. Additionally, in diffuse large B-cell lymphoma, remission is detected more frequently via cfDNA than circulating cells. We have developed a panel-based next generation sequencing (NGS) assay (MyMRD) that identifies pathogenic variants in acute myeloid leukemia (AML), confirming remission status. MyMRD targets mutation hotspots in 23 genes associated with AML. It identifies driver mutations that cause relapse in >90% of all AML patients, as well as common drivers in other myeloid neoplasms and myelodysplastic syndromes. Here we developed a MyMRD cfDNA assay and demonstrate sensitivity in both contrived cell line and clinical samples. Additionally, increased sensitivity through deeper sequencing and error correction methods is explored. **Methods:** cfDNA collection, processing, and extraction were optimized prior to processing samples from patients, healthy donors, and synthetic plasma. DNA fragments with sizes similar to

cfDNA were generated for initial feasibility studies. Genomic DNA was fragmented by sonication and selected for a mean size of ~160bp. Whole genome libraries, generated from cfDNA and fragmented DNA, were hybridized with MyMRD probes. Enriched libraries were sequenced using Illumina platforms. Sequencing data was analyzed using proprietary Invivoscribe MyInformatics software. **Results:** We observed stable cfDNA concentrations following incubation of whole blood in preservation tubes for up to 3 days. cfDNA concentrations from AML patients are significantly higher than those from healthy donors (2.3 ng/uL vs 0.9 ng/uL,  $p=0.0056$ ). The assay shows linearity ( $R^2=0.888-0.998$ ) for multiple variants in the range of VAFs (0.1–20%) tested. With sample DNA input of 25ng, the LOD was established to be 0.5% for targeted single-nucleotide variant (SNV) sites, and 1% for targeted insertion/deletion (indel) variant sites. Application of these methods to patient samples resulted in sufficient coverage across 99.7% of the panel for an LOD of 1% and 87.3% of the panel for an LOD of 0.5%. When sequenced with greater DNA input to a greater depth with error correction, variants were identified with VAFs as low as 0.125%. **Conclusions:** MyMRD can provide monitoring of residual disease using cfDNA. The assay detects clinically important driver variants, has excellent linearity, and low LOD for targeted regions. Higher sensitivity can be obtained through deeper sequencing, but is limited by background noise at certain bases. This assay can potentially replace invasive BM sampling and provide an alternative test for monitoring of patients receiving targeted therapy.

### H030. Rosai-Dorfman Disease Co-existing with Lymphoma in the Same Lymph Node: A Localized Histiocytic Proliferation with MAPK/ERK Pathway-induced Cyclin D1 Upregulation

S. Garces, C. Yin, K.P. Patel, J.D. Khoury, J.T. Manning, S. Li, J. Xu, L. Medeiros

The University of Texas MD Anderson Cancer Center, Houston, TX.

**Introduction:** Rosai-Dorfman disease is a rare histiocytic disorder recently shown to carry kinase-activating mutations in genes encoding for the MAPK/ERK pathway in up to a third of cases. In a small subset of patients, Rosai-Dorfman disease is detected concomitantly with lymphoma in the same biopsy specimen. The underlying molecular mechanisms including mutation status of focal Rosai-Dorfman disease occurring in the setting of lymphoma has not been investigated.

**Methods:** We analyzed the clinicopathologic features of 6 cases of Rosai-Dorfman disease coexisting with lymphoma in the same lymph node. In 4 cases, the highly specific antibody phospho-p44/42 MAPK (Thr202/Tyr204) (D13.14.4E) p-ERK was used to assess for the presence of nuclear and cytoplasmic phosphorylated p44 and p42 MAPK (Erk1 and Erk2). In addition, in 3 cases with available material, cyclin D1 antibody was used to assess for the expression of this marker. We performed mutation analysis using a panel of 146 genes (including *ARAF*, *BRAF*, *CCD1*, *ERBB1*, *ERBB2*, *ERBB3*, *KRAS*, *MAP2K1*, *NRAS*, *PIK3CA*, and *SRC*) by next-generation sequencing in microdissected Rosai-Dorfman disease foci of 5 cases. **Results:** There were 4 women and 2 men (median age, 56 years; range, 23–72 years). The lymphomas included classical Hodgkin lymphoma ( $n=3$ ), nodular lymphocyte predominant Hodgkin lymphoma ( $n=1$ ), chronic lymphocytic leukemia/small lymphocytic lymphoma ( $n=1$ ) and T-cell large granular lymphocytic leukemia ( $n=1$ ). No patients had evidence of Rosai-Dorfman disease at other sites. All cases were negative for mutations. Nevertheless, two of four cases showed p-ERK expression, and the three cases analyzed for cyclin D1 showed strong nuclear expression. Five patients with available information received therapy for their lymphoma and no patients developed Rosai-Dorfman disease in other anatomic locations (median follow-up, 31 months). **Conclusions:** These findings suggest that Rosai-Dorfman disease coexisting with lymphoma is a clinically benign histiocytic proliferation. These lesions are associated with MAPK/ERK pathway activation, but are not associated with gene mutations identified previously in Rosai-Dorfman disease.

### H031. Clinical Utility of Targeted Next-generation Sequencing in Evaluation of Cytopenias of Undetermined Significance

R. Beck, N. Sadri, K. Oduro, H. Meyerson, E. Moore  
University Hospitals of Cleveland/Case Western Reserve University, Cleveland, OH.

**Introduction:** Utility of molecular analysis in evaluation of bone marrow (BM) biopsies from patients with unexplained cytopenias is not established. The role of mutations in diagnosis of myelodysplastic syndrome (MDS) is currently only defined for MDS with ring sideroblasts (RS) and *SF3B1* mutation. We developed a 30-gene targeted Myeloid Next Generation Sequencing (NGS) Panel, which was performed routinely on BM biopsies drawn for unexplained cytopenia, to examine whether NGS results would aid in pathologist evaluation. **Methods:** From a 16-month period, we identified 133 BM biopsies drawn for evaluation of  $\geq 1$  cytopenia and which did not have a definite diagnosis of myeloid neoplasm; all biopsies had Myeloid NGS and genetic testing (MDS fluorescence *in situ* hybridization (FISH) panel and/or karyotype). Based on pathology reports, cases were classified as having either 1) no notable dysplastic changes (ND), 2) "mild" dysplasia (MiD) when  $<10\%$  dysplasia was present in a cell lineage, or 3) "moderate" dysplasia (MoD) when dysplasia approached or exceeded 10% but was limited to a single lineage or clinical history indicated a plausible explanation for the dysplasia. NGS and genetics results as well as report addenda incorporating these results were reviewed. Known pathogenic variants (PVs) were called at variant allele frequency (VAF)  $\geq 2\%$ . Cases with  $\geq 1$  PV were designated clonal cytopenia of undetermined significance (CCUS) or MDS RS if *SF3B1* mutation and RS were present. **Results:** Fifty-six percent (75/133) of biopsies had mild (59) or moderate (16) dysplasia. Of 59 MiD cases, 15 (25%) had  $\geq 1$  PV by NGS, 12 (80%) of which had  $\geq 1$  VAF  $\geq 10\%$ . Of 16 MoD cases, 8 (50%) had  $\geq 1$  PV, of which 7 (88%) had  $\geq 1$  VAF  $\geq 10\%$ . Of the 23 dysplastic cases with  $\geq 1$  PV, 4 were classified as MDS RS and 19 as "CCUS with dysplasia" (CCUS-D). Of 52 dysplastic cases without PV by NGS, only 1 had a pathogenic genetic abnormality. Of 58 ND cases, all lacked disease-defining genetics but 15 (26%) had CCUS by NGS (CCUS-ND), of which only 6 (40%) had  $\geq 1$  mutation with VAF  $\geq 10\%$ . CCUS with  $\geq 2$  PVs was found in 3/16 ND, 6/15 MiD and 5/8 MoD cases. **Conclusions:** NGS raised pathologist concern for presence or future development of MDS in 23/75 biopsies with mild or moderate dysplasia, indicating potential usefulness for routine NGS testing when at least mild dysplastic changes are present. PVs occurred with highest frequency in MoD cases but with similar frequency between MiD and ND cases. However, incidence of  $\geq 2$  PVs or PVs having VAF  $\geq 10\%$  rose significantly with increasing level of dysplasia, suggesting correlation between degree of dysplasia with VAF and multiple mutations. Future studies with long term follow-up are warranted to assess ultimate risk for MDS in CCUS-D versus CCUS-ND patients.

### H032. Myeloid Neoplasms with Ring Sideroblasts without SF3B1 Mutation

S. Bhavsar, N.G. Bailey, Y. Liu, W.E. Coyne, N. Aggarwal  
University of Pittsburgh Medical Center, Pittsburgh, PA.

**Introduction:** *SF3B1* mutations are seen in 60%–80% of myelodysplastic syndromes with ring sideroblasts (MDS-RS) and myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T) and are associated with favorable outcome. Conversely, MDS with RS and wild-type *SF3B1* are characterized by multilineage dysplasia and unfavorable prognosis. This study explores the molecular genetic alterations in myeloid neoplasms with ring sideroblasts lacking the *SF3B1* mutation further. **Methods:** Clinical features, peripheral blood and bone marrow findings, classical cytogenetics (CCG), fluorescence *in situ* hybridization (FISH) for -7,+8, del5q13, del7q31, del20q12, *RUNX1T1/RUNX1*, *KMT2A*, *CBF* rearrangements and next-generation sequencing of 37 genes [Illumina MiSeq] were evaluated in 242 patients with clinically suspected myeloid neoplasm excluding acute myeloid leukemia (10/2016 to 04/2018). **Results:** Ring sideroblasts were present in 60/242 (24.7%) cases (20–92yrs, M: F 1.8:1). Of these 60, 30 had *SF3B1* mutations. Four of these 30 cases had more than one spliceosome mutation (1 case with *SF3B1* and *U2AF1* and 3 cases with *SF3B1* and *SRSF2* mutations). Four cases with RS were negative for all genetic alterations tested.

Among the *SF3B1* wild-type cases, the percentage of RS ranged from 1-47% of the erythroid precursors, with 60% (18/30) cases showing >5% RS. The most common diagnosis in this group was MDS with excess blasts (MDS-EB) (9/30) and only 3/30 *SF3B1* wild-type cases were MDS-RS with single lineage dysplasia (MDS-RS-SLD). Fourteen of 30 (47%) *SF3B1* wild-type cases had other spliceosome mutations, *U2AF1* (10/14) and *SRSF2* (4/14). Other mutations included *TP53* (9), *ASXL1* (8), *TET2* (4) and *JAK2* (3). *U2AF1* and *SRSF2* mutations were found to be mutually exclusive. Seven of nine *TP53* mutated cases showed >5% RS (9 >10%RS). Non-*SF3B1* spliceosome mutations showed a higher prevalence amongst cases diagnosed as MDS with multilineage dysplasia (MDS-MLD) (4/6) and MDS-EB; with 6/9 (66.7%) cases of MDS-EB showing *U2AF1* mutation (4 with concurrent *TP53* mutation). Among the cases without RS (n=182), 24.7% showed non-*SF3B1* spliceosome mutations (*U2AF1*=16, *SRSF2*=24 and *ZRSR2*=5) and 9% showed *TP53* mutations. **Conclusions:** Spliceosome mutations are over-represented in myeloid neoplasms with RS including among the non-*SF3B1* mutated cases. In comparison to the *SF3B1* mutated cases with RS, the *SF3B1* wild-type cases with RS were associated with higher blast count, multilineage dysplasia and higher prevalence of *TP53*. With the development of newer therapeutic strategies using splicing-modulating agents, further studies to identify disease groups with splicing factor mutations are required.

### H033. Clinical Use of Rapid Transcriptome (R-RNASeq) Analysis for Gene Fusion and Rearrangement Detection in Pediatric Leukemia

E.M. Azzato, S. Newman, Y. Li, J. Nakitandwe, J. Gu, S. Shurtleff, A. Silkov, S. Foy, M. Rusch, C. Mullighan, H. Inaba, T. Gruber, D. Ellison, J. Zhang  
St. Jude Children's Research Hospital, Memphis, TN.

**Introduction:** Fusion detection is critical for leukemia diagnosis, prognosis and therapy prediction. Well-characterized, recurrent fusions are often evaluated with single or multi-analyte approaches; however, there is an expanding list of fusions with prognostic and therapeutic impact, including *ABL*-class fusions and other rearrangements associated with Ph-like ALL, rearrangements with complex regions (e.g. *DUX4*) and rare fusion events, that are not targeted by standard molecular diagnostic assays. We clinically validated a Rapid RNASeq (R-RNASeq) assay to detect recurrent or novel fusion events, as well as internal tandem duplications (ITDs) within a 15-day turn-around-time, replacing our standard RT-PCR and fluorescence *in situ* hybridization (FISH) approaches. Here we present our 14-month clinical experience utilizing this platform in a pediatric oncology setting. **Methods:** The Illumina TruSeq Stranded Total RNA LT Kit was used to generate libraries using RNA extracted from patient samples (tumor purity ≥40%). All libraries were sequenced using a paired end 2 x 125 bp cycle protocol and SBS technology on Illumina HiSeq and/or NextSeq Instruments. RNA sequencing data was aligned against human reference sequence (hg19) utilizing a cloud-based bioinformatics pipeline ([https://platform.stjude.cloud/tools/rapid\\_rna\\_seq](https://platform.stjude.cloud/tools/rapid_rna_seq)). Samples were sequenced to obtain at least 20% of bases at 30X depth of RNA sequencing coverage. Rearrangements predicted to deregulate an oncogene, with at least 2 reads supporting the junction, were reported. **Results:** During the first 14 months of testing, 136 pediatric leukemia patients had R-RNASeq performed (81 B-ALL, 15 T-ALL, 40 AML). 70 (52%) were positive for a rearrangement; of those, only 40 (57%) had recurrent fusion transcripts, with the remainder either being infrequent or novel (12, including *NUP214-ABL1*, *PICALM-MLL110*, *MEF2D-BCL9*, *ETV6-FOXO3*) or involving enhancer proximity swapping (18, e.g. *IGH@-CRLF2*, *IGH@-DUX4*). 120 of the samples later underwent whole genome sequencing or other confirmatory testing, which revealed 10 false negative cases. The majority (n=8) involved enhancer proximity swapping, 2 of which were observed in R-RNASeq but did not meet quality thresholds for reporting. The other 2 false negatives had low tumor purity/transcript levels, and were clinically tested by alternative methods. 38 samples also underwent fragment analysis for *FLT3*-ITD (7 positive) and showed complete concordance with R-RNASeq. **Conclusions:** R-RNASeq is a feasible clinical assay for timely leukemia fusion and ITD detection, with flexibility to incorporate new

discoveries. As our experience demonstrates, a substantial proportion of pediatric leukemia cases (n=38, 28%) have infrequent fusions or enhancer proximity effects, which may be missed by traditional assays.

### H034. Mutational Signatures Differ between Cytogenetic Risk Groups of *de novo* AML

R.T. Sussman, P.D. Velu, A. Bigdeli, C. Paolillo, A. Bagg, M. Carroll, J.J. Morrisette  
University of Pennsylvania, Philadelphia, PA.

**Introduction:** Cytogenetic studies are a well-established part of the workup for acute myeloid leukemia (AML), with the pattern of chromosome abnormalities highly associated with prognosis. At our institution, next-generation sequencing (NGS) has increasingly become part of the routine testing algorithm in myeloid malignancies, using a custom 68-gene panel. Recurrently mutated genes belong to several distinct pathways. We have assessed patients with *de novo* AML (dnAML) who have had both cytogenetic and NGS testing performed on the diagnostic specimen and assigned functional categorization to the genes on our panel. We present a correlation of the variants with specific chromosomal prognostic categories and show differences in the mutational profiles within cytogenetic subgroups. **Methods:** Electronic medical records (EMR) were used to determine which patients in the cytogenetics laboratory database had both a karyotype and NGS test performed at the time of diagnosis of *de novo* AML (dnAML). Karyotypes were categorized into "favorable", "intermediate", and "adverse" according to the Medical Research Council (MRC) guidelines. Disease-associated variants detected from NGS were grouped into functional categorizations, including normal for studies where no variants were detected. The prevalence of variants in functional categories was compared between MRC categories and MK. The institutional review board approved this study. **Results:** A total of 132 patients with dnAML had a karyotype and matched NGS study at the time of diagnosis. Of these, 21 had a favorable karyotype (mean DNA variants from NGS/case = 1.6), 82 had an intermediate karyotype (2.8 variants/case), and 29 had an adverse karyotype (1.8 variants/case). The favorable group was enriched for variants in signaling pathway genes (52% of variants), especially *FLT3*, *KIT* and *NRAS*, and lacked variants in DNA methylation genes, which made up 20% of variants detected in the intermediate group and 18% of variants detected in the adverse group. *NPM1* variants were only observed in the intermediate group, and variants in chromatin regulation were more prevalent in this group than the others (12% in intermediate v. 3% in favorable and 4% in adverse). Variants in tumor suppressors were enriched in the adverse group (29%), most of these (24% of all variants detected in the adverse group) were in *TP53*. Normal sequencing studies were enriched in the favorable group (16% v. 1% in intermediate and 6% in adverse), in contrast to those with normal karyotypes (intermediate group). **Conclusions:** The addition of mutational information to cytogenetic findings demonstrates that there are different mutational profiles based on the cytogenetic risk category of AML.

### H035. Therapy-related Acute Myeloid Leukemia, Characterized by t(8;16)(p11;p13);MYST3-CREBBP and Co-occurring TET2 and ASXL1 Mutations

A. Alsuwaidan, P. Koduru, F. Fuda, M. Vusirikala, N. Sadeghi, C. Zhang, W. Chen  
The University of Texas Southwestern Medical Center, Dallas, TX.

**Introduction:** Therapy-related acute myeloid leukemia (t-AML) with t(8;16) is rare with scanty molecular genetic data in the literature. Herein, we presented one case to explore co-operative genetic abnormalities in leukemia pathogenesis and targeted treatment. **Methods:** A 69-year-old woman was diagnosed with t-AML following chemotherapy for breast cancer and diffuse large B-cell lymphoma. Morphological, immunophenotypic, cytogenetic and molecular studies were performed. **Results:** Morphological and immunophenotypic analyses by flow cytometric analysis revealed an AML (acute monoblastic leukemia) expressing CD117, CD33, CD64, LILRB1 and LILRB4, but lacking CD34 and MPO. Cytogenetics studies reveal an abnormal female karyotype containing multiple structural abnormalities: 46,XX,t(8;16)(p11.2;p13.3),add(9)(q34). Massively parallel DNA sequencing by FoundationOne Heme revealed *TET2* mutation



(C677fs\*22, splice site 3501-1G>T), ASXL1 mutation (R693\*-subclonal), and MYST3-CREBBP fusion. She was treated with Azacitidine plus Pracinostat. Unfortunately, she developed neutropenia and septic shock, and died four months after the diagnosis. **Conclusions:** Our patient was diagnosed with t-AML with t(8;16)(p11;p13);MYST3-CREBBP, TET2 and ASXL1 mutations. AML with t(8;16) may occur as *de novo* AML or t-AML with frequent monocytic differentiation (confirmed by novel markers of LILRB1 and LILRB4 in our case) and worse prognosis. The findings in our case fall in the spectrum of the described features in the literature. To the best of our knowledge, this is the first case to demonstrate multiple genetic abnormalities affecting epigenetic remodeling pathway: t(8;16)(p11;p13);MYST3-CREBBP, TET2, and ASXL1 mutations. t(8;16)(p11;p13) results in fusion of MYST3 with CREBBP. Both genes encode proteins with histone acetyltransferase activity. TET2 encodes a methylcytosine dioxygenase, which is crucial in the DNA demethylation. ASXL1 regulates epigenetic marks and transcription. The truncating mutation as seen in this patient has been reported to lack of ASXL1. These combined genetic abnormalities may therefore disturb chromatin remodeling by dysregulation of DNA methylation (by TET2 mutation) and histone deacetylation (by MYST3-CREBBP fusion and ASXL1 mutation). Furthermore, testing for these genetic abnormalities will identify a subgroup of patients who may benefit from targeted treatments, such as DNA methyltransferase inhibitors (Azacitidine and Decitabine) and histone deacetylase inhibitor (Pracinostat). In summary, our case highlights the importance of integrated approach of morphology, immunophenotypic, cytogenetics, and molecular findings in the diagnosis, classification, prognostication, and targeted therapy in AML.

### H036. Variant Characterization for a Clinical Lymphoma Sequencing Panel

S. Deihimi, A.R. Oran, A. Bigdeli, D. Lieberman, J.J. Morrisette, R.T. Sussman

Hospital of the University of Pennsylvania, Center for Personalized Diagnostics, Philadelphia PA.

**Introduction:** At our institution, next generation sequencing (NGS) and genomic variant characterization is part of routine testing for solid tumors and hematological malignancies to inform diagnosis, prognosis and therapeutic choice. However, the panels used by our lab and others often lack lymphoma-specific genes. We validated a targeted sequencing panel for lymphomas that had limited overlap with existing assays, making the characterization of clinically relevant variants in lymphoma samples difficult. To prepare for clinical testing of lymphoma specimens, we curated a variant knowledge base with interpretations of novel lymphoma-specific variants detected during the validation of this NGS panel. **Methods:** The Illumina TruSight Lymphoma assay is designed for low input and is dual-stranded, generating a sequence alignment file (BAM) for each strand. A single variant call file (VCF) is generated from variants present in both BAMs at a variant allele frequency (VAF) > 3%. Variants were confirmed to be present in both strands in the analyzed data through visual inspection using Integrative Genomic Viewer (IGV). Variants found in the normal population at  $\geq 0.1\%$  allele frequency were internally classified as benign. To characterize somatic disease associated variants, we searched for functional evidence in the literature and the online databases COSMIC, cBioPortal and ClinVar. **Results:** Within the 40 genes on this lymphoma panel, 21 genes were not present on orthogonal assays and, hence, required interpretation of their clinical significance. Newly-detected variants in these 21 genes were characterized and added to our clinical variant knowledge base. From 72 lymphoma specimens, 55 unique variants were characterized and, based on the level of evidence, classified as benign (n=14), likely benign (1), variant of uncertain significance (VOUS, 26), probably disease associated (2), and disease associated (12). The twelve disease associated variants included 5 insertion/deletions (indels) and 7 single nucleotide variants (SNVs: 4 nonsense, 2 splice site and 1 missense). **Conclusions:** Genomic characterization of cancer-associated genes will offer prognostic and therapeutic benefit to lymphoma patients. To better serve the lymphoma community, we validated a targeted lymphoma NGS panel, and as part of the validation process, developed a

lymphoma variant knowledge base by characterizing the lymphoma-specific variants not covered by our other NGS panels.

### H037. Post-remission NGS-based MRD Surveillance is Critical for Early Detection of Impending Relapse in B-ALL

S. Cheng, W. Tam

Weill Cornell Medicine, Cornell University, New York, NY.

**Introduction:** Next generation sequencing (NGS) has emerged as a new and ultra-sensitive method for minimal residual disease (MRD) assessment in malignance of B lymphoid origin, including B precursor acute lymphoblastic leukemia/lymphoma (B-ALL). Recent studies demonstrate that NGS-based MRD status at end of induction is a powerful predictor of relapse. However, clinical value of NGS-based MRD surveillance and MRD surveillance frequency in post-remission setting has not been explored. **Methods:** A total of 51 diagnostic or post-treatment bone marrow samples were selected and collected from 10 B-ALL patients and assessed for their tumor loads by a VDJ deep sequencing-based MRD method (ILGV assay). These patients were classified into two groups, surveilled (n=5) and non-surveilled (n=5), according to whether any post-treatment bone marrow specimens for ILGV assay are available within 6-7 months prior to clinical relapse. Criteria for selection of patients are as follows: (1) positive identification of leukemia-specific clonotypes; (2) achievement of complete molecular remission defined as undetectable MRD by NGS before relapse; (3) availability of sufficient amount of DNA from post CR, pre-relapse specimens; and (4) symptomatic clinical relapse after achieving CR. NGS-based MRD status is denoted as CPMRD if conversion to positive NGS-MRD from undetectable MRD. **Results:** In the surveilled group, patient #1, at day 186 post-initial diagnosis, achieved molecular MRD response, followed by two fold increase in MRD levels (0.0113% vs 0.0238%) during the 4-month interval and a 3,336.5 fold increase in tumor content in a span of 80 days. Patients #2-#5 achieved NGS-based molecular remission on day 80, 36, 171, 160 post initial diagnosis, converted to NGS-MRD positivity on day 358, 212, 338, 333 post initial diagnosis, respectively. All patients with CPMRD eventually relapsed, and the median interval between CPMRD to clinical relapse in the surveilled cases is 4.7 months. On the other hand, among the non-surveilled cases, all the patients had relapses despite the lack of demonstrable CPMRD. In this group of patients, CPMRD has escaped detection because of the lack of sampling in the immediate months before clinical relapse. In line with this explanation, the median interval between the most recent post-treatment sample and clinical relapse in this group of patients was 20 months (13.2 to 23.7 months), which was significantly longer compared to the interval between the detection of CPMRD and overt relapse in the surveilled patients (20 months vs 4.7 months,  $p=0.0001$ ). **Conclusions:** Our findings suggest the utility of more frequent MRD surveillance (4-5 months interval) based on IGH deep sequencing in a post-remission clinical setting is critical for the detection of early relapse.

### H038. Clinical Evaluation of the Archer VariantPlex Myeloid Panel for Mutation Profiling in Myeloid Neoplasms

A. Campbell, W. Crowe, P. Rothberg, Y. Ding

University of Rochester, Rochester NY.

**Introduction:** Mutation profiling has become standard of care for patients with myeloid neoplasms including myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN), MPN/MDS, and acute myeloid leukemias (AML) and plays important role in the disease classification, prognostication and stratification of disease management. Traditionally, single analyte testing is used to sequentially evaluate BCR-ABL1 negative samples for JAK2, CALR, and MPL to assist in the diagnosis of myeloproliferative disease. Introduction of next-generation sequencing assays with comprehensive coverage and analysis of genes relevant to myeloid malignancies is widely accepted as the preferred method to detect genetic abnormalities. We validated the Archer VariantPlex Myeloid 75 gene Panel and compared its performance to our New York State approved Trusight Myeloid panel on the Illumina MiSeq platform. **Methods:** Ninety myeloid neoplasms samples positive for single-nucleotide variants (SNVs), small and large insertion/deletions (INDELs) in various genes, especially in JAK2, CALR, CEBPA, NPM1, TP53, and FLT3, were selected for study. The included sample types were fresh

blood, fresh bone marrow aspirate and formalin-fixed paraffin-embedded (FFPE) tissue. The libraries were sequenced on an Illumina MiSeq sequencer using the MiSeq Reagent Kit v3 chemistry and a maximum of 8 samples per flow cell. Analysis was performed using the Archer Analysis software for the Archer VariantPlex Myeloid Panel. **Results:** In contrast to reflex or parallel testing of single genes which can take up to 10 working days from DNA preparation to reporting, results from the ArcherDx VariantPlex Myeloid Panel could be reported within 5 working days. For samples with SNVs and small INDELS in *CEBPA* (n=26), *FLT3* (n=38), *JAK2* (n=14), *NPM1* (n=25), and *TP53* (n=15), the Archer Analysis software detected 100% of the variants. For *CALR* positive samples (n=10), 100% of the small INDELS and large deletions were correctly identified. The ArcherDx VariantPlex Myeloid Panel also detected 100% of the tested *FLT3* ITDs ( $\geq 36bp$ ) (n=21). For *FLT3*, *JAK2*, *NPM1*, and *TP53*, 100% concordant results were obtained between Archer VariantPlex and Illumina TruSight Myeloid panels. For *CEBPA* and *CALR*, all results were confirmed with our stand-alone assays. In addition, we found that the known mutations were accurately identified by this panel from the FFPE samples. **Conclusion:** The ArcherDx VariantPlex Myeloid Panel and Archer Analysis software is a very sensitive tool which can detect partial, large, and complex tandem duplications in *FLT3* and INDELS in *CALR*. Coupled with the accurate identification of mutations in *CEBPA*, *JAK2*, *NPM1*, and *TP53*, this assay can be used for mutation profiling in myeloid neoplasms.

#### H039. Donor-derived Clonal Hematopoiesis of Indeterminant Potential Mutations are Detected in Transplant Recipients after Allogeneic Hematopoietic Stem Cell Transplant

J. Liu, Y. Wang, X. Wang, P. Raess, R. Press, G. Fan, J. Dunlap  
Oregon Health and Science University, Portland, OR.

**Introduction:** Allogeneic hematopoietic stem cell transplants (allo HSCT) are increasingly used to treat older patients with hematopoietic malignancies. Consequently, the use of older related donors has also increased. Older individuals are more likely to harbor somatic mutations in leukemia-associated genes, also known as clonal hematopoiesis of indeterminate potential (CHIP). CHIP is detected in approximately 10-15% of individuals over the age of 70. CHIP is an asymptomatic condition but confers an increased risk of progression to hematologic malignancy (0.5-1% per year) and increases all-cause mortality. CHIP mutations may be undetected in otherwise healthy donors and transferred to recipients of stem cell transplants. We describe seven patients with donor derived CHIP mutations after allo HSCT. **Methods:** We performed a retrospective search of the Oregon Health & Science University laboratory information system from 2014-2017 for patients treated with allo HSCT. Next generation sequencing (NGS) was performed on bone marrow aspirate from the diagnostic specimen (recipient) prior to transplant and in follow up bone marrow after transplantation. Sequencing was performed using Ampliseq libraries and an Ion-torrent PGM sequencer. Mutations were considered donor-derived if they were present in the first post-transplant sequencing study and were not present in any pre-transplant specimens at an LOD of 0.5% variant allele frequency (VAF). To date, confirmation with direct sequencing of donor specimens has been performed in three cases. **Results:** We identified 7 patients with donor-derived CHIP mutations following allo HSCT: 6 with *DNMT3A* mutations and 1 with a *TET2* mutation. *DNMT3A* mutations consisted of nonsense, frameshift, splice site and missense mutations, including one *DNMT3A R882H*. The average VAF was 13.4%. In four patients, NGS was performed at multiple time points post-transplant. CHIP mutations showed an increase in VAF over time. NGS was also performed on the donor specimens in three cases, which identified the same CHIP mutation at approximately the same allele frequency as seen in the recipient, confirming donor origin. Two patients died after transplant from sepsis and/or graft versus host disease. The other five patients are alive without evidence of hematologic malignancy and no significant CBC abnormalities. The average follow up was 829 days post-transplant. **Conclusions:** We identified donor derived CHIP mutations in seven patients after HSCT; five patients remain alive with no evidence of a hematopoietic malignancy. As the donor population ages, it is likely that so will the frequency of donor engrafted CHIP. Further studies are necessary to determine the clinical impact of donor-engrafted CHIP.

#### H040. Comprehensive Assessment of Variants in *SOCS1*, *JAK2* and *B2M* Using Anchored Multiplex PCR and Next-generation Sequencing

H.E. Robinson<sup>1</sup>, L. Stuart<sup>2</sup>, L.P. Le<sup>2</sup>, A.J. Iafrate<sup>2</sup>, A.R. Sohani<sup>2</sup>, J.K. Lennerz<sup>2</sup>

<sup>1</sup>Massachusetts General Hospital, Charlestown, MA; <sup>2</sup>Massachusetts General Hospital, Boston MA.

**Introduction:** Mutations in *SOCS1* are of prognostic relevance in diffuse large B-cell lymphoma (DLBCL) with potentially significant implications for therapy selection. Standard genotyping methods like Sanger sequencing limit the ability to accurately characterize low-level mutations in *SOCS1* due to >83% GC-content. Here, we present a method based on Anchored Multiplex PCR (AMP) which achieves sufficient sequencing coverage to uncover both known and uncharacterized mutations in *SOCS1*. **Methods:** Primers were designed to specifically amplify *SOCS1*, *JAK2* and control gene *B2M*. Next generation sequencing (NGS) libraries were prepared utilizing an AMP approach. DNA fragments from bone marrow and formalin-fixed, paraffin-embedded (FFPE) samples were ligated at random start sites with molecular-barcoded adapters. These adapter-ligated products were amplified with two nested PCR reactions utilizing the gene-specific primers. We used Novoalign to align reads and mark duplicates, and QC metrics were generated with HTQC to visualize in IGV for coverage analysis. We compared mean collapsed coverage (MCC) across both samples types and the 3 genes being targeted using student's t-test. Significance was defined as  $p < 0.05$ . **Results:** In five samples tested (2 bone marrow and 3 FFPE) we show that it is feasible to achieve an MCC of at least 200 over the *SOCS1* gene for both sample types including FFPE. A minimum of 200x MCC was established as the minimum required to accurately call variants at a required level of sensitivity. The MCC across *SOCS1*, *JAK2* and *B2M* for bone marrow samples was 339.8, 567.2 and 598.3 respectively. For the FFPE-based samples which would be the sample type submitted for malignant lymphoma analysis, the MCC across *SOCS1*, *JAK2* and *B2M* was 236.2, 386.6, and 344.3, respectively. **Conclusions:** We demonstrate that AMP-based NGS genotyping allows for adequate sequencing coverage to detect previously-described *SOCS1* mutations in FFPE samples, in addition to uncharacterized mutations. Identification of mutations that historically have been technically infeasible to detect may help to develop a revised prognostic and predictive stratification of DLBCL patients. Treatment implications could include the ability to mitigate high toxicity levels in patients whose specific disease may not require a highly aggressive regimen to be successfully treated. The prognostic relevance of *SOCS1* mutations supports the addition of *SOCS1* mutational analysis in NGS panels in the evaluation of malignant lymphomas.

#### H041. Longitudinal Monitoring of AML Tumors with High-throughput Single-cell DNA Sequencing Reveals Rare Clones Prognostic for Disease Progression and Therapy Response

D.J. Eastburn<sup>1</sup>, C.M. McMahon<sup>2</sup>, A. Viny<sup>3</sup>, R. Bowman<sup>3</sup>, L. Miles<sup>3</sup>, R. Durruthy-Durruthy<sup>1</sup>, R.L. Levine<sup>3</sup>, M. Carroll<sup>2</sup>, C.C. Smith<sup>4</sup>, A.E. Perl<sup>2</sup>

<sup>1</sup>Mission Bio, Inc., South San Francisco, CA; <sup>2</sup>University of Pennsylvania, Philadelphia, PA; <sup>3</sup>Memorial Sloan Kettering Cancer Center, New York, NY; <sup>4</sup>University of California, San Francisco, San Francisco, CA.

**Introduction:** AML (acute myeloid leukemia) is increasingly being treated with precision medicine. To better inform treatment, the mutational content of patient samples must be accurately determined. However, current tumor sequencing paradigms are inadequate to fully characterize many instances of the disease. A major challenge has been the unambiguous identification of potentially rare and genetically heterogeneous neoplastic cell populations, capable of critically impacting tumor evolution and the acquisition of therapeutic resistance. Standard bulk population sequencing is unable to identify rare alleles and definitively determine whether mutations co-occur within the same cell. Single-cell sequencing has the potential to address these key issues and transform our ability to accurately characterize clonal heterogeneity in AML. **Methods:** Previous single-cell studies examining genetic variation in AML have relied upon laborious, expensive and low-throughput technologies that are not readily scalable for routine analysis of the disease. We applied a newly developed platform technology to perform

targeted single-cell DNA sequencing on over 140,000 cells and generated high-resolution maps of clonal architecture from AML tumor samples. **Results:** Single-cell sequencing of multiple patient samples demonstrated that relapse clones acquired oncogenic RAS mutations. We utilized the high-throughput and sensitivity of our single-cell approach to more definitively assess where in the course of treatment these RAS mutated clones were acquired. Oncogenic RAS harboring clones, comprising between 0.4%, and 0.1% of tumor populations, were identified in patient samples either prior to or shortly after onset of treatment. Significantly, these RAS variant alleles were not detectable with targeted bulk sequencing. Throughout the course of treatment with the FLT3 inhibitor gilteritinib, the RAS mutant clones selectively expanded and were responsible for resistance to therapy and relapse. **Conclusions:** These findings point to the presence of underlying genetic heterogeneity in AML that contributes to therapy resistance and relapse. We demonstrate the utility of sensitively assaying clonal architecture with single-cell sequencing to better inform patient stratification and therapy selection.

#### H042. Next-generation Sequencing-based Detection of Clinically Significant *IKZF1* Deletions and *KMT2A* Partial Tandem Duplications

K.C. Floyd, M. Routbort, W. Chen, C. Lan, D.Y. Suose, M. Konopleva, C. Ok, J. Medeiros, R. Luthra

The University of Texas MD Anderson Cancer Center, Houston, TX.

**Introduction:** Clinically significant exonic deletions and duplications have been reported in *IKZF1* and *KMT2A* in hematologic neoplasms. Deletions involving exons 2 through 8 have been reported in B-acute lymphoblastic leukemia (B-ALL). These deletions have different functional and prognostic impact based on the specific exons deleted and the involvement of the DNA binding, activation and dimerization domains. Similarly, partial tandem duplications involving exons 2 through 10 have been reported in a variety of hematologic neoplasms and are associated with poorer prognosis. Detection of these alterations using targeted next generation sequencing (NGS) panels requires special design and informatics considerations. We validate for routine clinical use the detection of exonic deletion-duplications using targeted NGS panels. **Method:** We incorporated the goal of detecting exon-level deletion and duplication in the design of a targeted 81-gene NGS panel. For both *IKZF1* and *KMT2A*, specific targets involving commonly deleted or duplicated regions and control regions (not involved by copy number changes) including flanking introns were included in the panel design. Coverage analysis for target regions was performed in 100-bp segments to allow adequate sampling and normalization. Normalized coverage ratios of target versus control regions were computed for *IKZF1* deletion and *KMT2A*-PTD using in-house designed computational algorithms. Additional visualization tools were used to allow efficient screening and confirmation. **Results:** We identified 15 cases with *KMT2A*-PTD and 9 without *KMT2A*-PTD using the in-house developed algorithm. These findings were confirmed by chromosomal microarray analysis. All cases with PTDs consistently involved exon 3 in the duplication with no change in exon 27, consistent with reported literature. An additional 15 B-ALL cases with and without *IKZF1* deletions were detected using the algorithm. Confirmation of *IKZF1* deletions using an orthogonal (MLPA) assay is in progress. **Conclusion:** Incorporation of detecting copy number gains and losses involving entire exons in targeted NGS panels allows routine screening for clinically important alterations in *IKZF1* and *KMT2A*. A combination of targeted design and a customized informatics approach is required to detect these alterations.

#### H043. Development of Synthetic Secondary Standards for *BCR-ABL1* Quantification on GeneXpert BCR-ABL V2 and Xpert BCR-ABL Ultra Assays

R. Mihani, C. Rundell, J. Gordon, S. Nesbitt, T. Spenlinhauer, T. Nguyen  
Maine Molecular Quality Controls, Saco, ME.

**Introduction:** Chronic myeloid leukemia (CML) is characterized by the presence of Philadelphia chromosome (Ph) arising from translocation of *ABL1* gene on chromosome 9 and *BCR* gene on chromosome 22, resulting in *BCR-ABL1* fusion, an oncogenic tyrosine kinase protein. Quantitative monitoring of *BCR-ABL1* transcripts by RQ-PCR in patient blood is an important tool for measuring molecular response to therapy.

Current recommendations for monitoring CML patients call for measuring the levels of *BCR-ABL1* standardized to the International Scale (IS) to ensure harmonized reporting across laboratories. To this end, the 1st WHO International Genetic Reference Panel can be used. However, due to the limited supply of the WHO primary panel, development of reliable secondary reference standards is essential for monitoring of *BCR-ABL1* quantitative assays. **Methods:** In this study, we describe the development and performance of MMQCI's Xpert BCR-ABL IS Panel C130 to be used as reliable secondary standards calibrated and traceable to WHO primary panel for *BCR-ABL1* quantification. The Xpert BCR-ABL IS Panel C130 is designed for use with the GeneXpert BCR-ABL V2 and Xpert BCR-ABL Ultra assays which report on the international scale. The C130 Panel is designed to represent a range of *BCR-ABL1/ABL1* ratios at six levels, each containing fusion and control transcripts, to yield approximately 0.0%IS, 0.0032%IS, 0.01%IS, 0.1%IS, 1%IS and 10%IS. For calibration of Xpert BCR-ABL IS Panel C130, three lots of the panel were tested against all four levels of the WHO primary standards as per White *et al.*

**Results:** MMQCI successfully manufactured synthetic reference material Xpert BCR-ABL IS Panel C130 according to cGMP procedures with > 12 months of real time stability and two additional %IS levels. Following the procedures entailed in White *et al.*, the correction factor (CF) for the GeneXpert BCR-ABL V2 assay was determined to be 1.02. The CF was applied to all six levels of the C130 panel to assign IS values traceable to WHO primary panel. Furthermore, regression analysis showed no significant trend in the bias across all six levels of MMQCI WHO-Traceable Secondary Standards against WHO nominal values and WHO reported values on GeneXpert BCR-ABL V2 assay. **Conclusion:** MMQCI can reproducibly manufacture BCR-ABL1 secondary standards traceable to WHO BCR-ABL1 primary panel. Xpert BCR-ABL IS Panel C130 performs reliably on the GeneXpert BCR-ABL V2 assay and is stable for at least 12 months stored at -20°C. Thus, the Xpert BCR-ABL IS Panel C130 can be used to monitor the performance of GeneXpert BCR-ABL V2 and Xpert BCR-ABL Ultra assays.

#### H044. Validation of a Custom Next-Generation Sequencing (NGS) Panel for Characterizing Mutations in Ph-like ALL Using Anchored Multiplex PCR Technology

A. Guimaraes-Young, N.V. Guseva, K. Sompallae, A.A. Stence, R. Sompallae, A.N. Snow, D. Ma, S. Bhagavathi, A.D. Bossler  
University of Iowa, Iowa City, IA.

**Introduction:** Philadelphia-like acute lymphoblastic leukemia (Ph-like ALL) harbors fusions leading to kinase activation and mutations affecting JAK-STAT and RAS pathways. Using traditional assays, different tests are required for fusion, deletion, and point mutation detection. Knowledge of fusion partners is also necessary. This assay was developed to multiplex the detection of gene fusions and mutations characteristic of Ph-like ALL and for novel fusion partner detection using a custom anchored multiplex PCR (ArcherDx, Inc) RNA panel. **Methods:** RNA obtained from residual blood and bone marrow clot specimens and leukemia-derived cell lines (HL-60, REH, JURKAT, and SUP-B15) was extracted using either QIAmp RNA Blood or RNeasy formalin-fixed, paraffin-embedded (FFPE) mini kits (Qiagen, Inc). Libraries prepared using 250ng RNA were sequenced using MiSeq or NextSeq instruments (Illumina, Inc). Data were analyzed using Archer Analysis software. Fusions and variants were confirmed with fluorescence *in situ* hybridization, RT-PCR, or DNA sequencing. **Results:** The expected gene fusions were identified in 13 of 13 cases and expected variants in *KRAS*, *NRAS*, *JAK1*, and *PTPN11* were detected in 6 of 6 cases. Exon skipping was also detected for the *IKZF1* gene. All positive findings were confirmed by alternative method with 100% concordance. The assay demonstrated reproducible results across library preps, sequencing runs, and the two sequencing instruments. Analytical sensitivity reliably demonstrated detection down to 5 unique start sites for fusions and 1% mutant to wildtype reads for single nucleotide variants. The assay works with fresh and formalin fixed specimens. **Conclusions:** This Heme RNA-based next generation sequencing (NGS) assay is reliable for simultaneous fusion, exon skipping, and variant detection in Ph-like ALL. The ability to characterize these mutations simultaneously simplifies the workflow for the laboratory and will facilitate our ability to inform the clinical care of our patients.



#### H045. Detection of Clonal Rearrangements in Multiple Myeloma Samples Using LymphoTrack Assays

Y. Huang<sup>1,2</sup>, A. Jacobsen<sup>1,2</sup>, J. Panganiban<sup>1</sup>, E. Vigil<sup>1</sup>, K. Hutt<sup>1</sup>, J. Heilman<sup>1</sup>, M. Blankford<sup>1</sup>, T. Stenzel<sup>1</sup>, E.H. Rustad<sup>2</sup>, M. Hultcrantz<sup>2</sup>, O. Landgren<sup>2</sup>, J.E. Miller<sup>1</sup>

<sup>1</sup>InVivoScribe, San Diego, CA; <sup>2</sup>Memorial Sloan Kettering Cancer Center, New York, NY.

**Introduction:** Multiple myeloma (MM) is a type of cancer that affects white blood cells known as plasma cells. Traditionally multiparameter flow cytometry (MFC) is the tool used to detect and monitor MM in patients. Recently we developed next generation sequencing (NGS) based LymphoTrack Assays and bioinformatics software to detect and track clonal rearrangements. Here we report the results of a pilot study of clonal rearrangement detection from a 193 anonymized blinded MM samples using LymphoTrack Assays that identify B-cell populations (IGHV Leader, IGH FR1, FR2, FR3, and IGK), in order to assess their potential applications in MM. **Methods:** Genomic DNA from 193 MM samples at baseline were anonymized and blinded prior to testing with the four LymphoTrack Assays (IGH FR1, FR2, FR3 and IGK) on MiSeq. Libraries generated were purified, harmonized, pooled, and sequenced in a single MiSeq run. Fourteen samples that tested negative were reflex tested with the LymphoTrack IGHV Leader Assay. The most prominent clonal rearrangement (clonotype) identified by one of the LymphoTrack Assays was then tracked using a single assay to test the 30 subsequent samples that were available. LymphoQuant Internal control was added to each PCR reaction at 100 cell equivalency when testing these follow up samples to allow the estimation of cell equivalents within each sample. LymphoTrack Software – MiSeq and LymphoTrack MRD software was used to analyze the sequencing results from baseline and follow up samples, respectively. **Results:** Out of 193 baseline MM samples, 88 (46%), 81 (42%), 90 (47%), and 106 (55%) samples were detected as clonal positive by IGH FR1, FR2, FR3 and IGK, respectively. When combining all 4 targets, 79% (152/193) clonal positivity was achieved. The clonal status in baseline samples was compared with different MM disease stages. Among the 30 follow up samples, the baseline sample specific sequences were detected in 2, 9 and 14 of the subsequent samples by IGH FR2, IGH FR3, and IGK, respectively. This results in an overall detection rate of 83% (25 out of 30) for follow up samples by the LymphoTrack Assays. **Conclusion:** LymphoTrack Assays were shown to detect clonotype sequences in about 80% of MM baseline samples and in 83% subsequent follow up samples. This suggests that LymphoTrack Assays are potentially useful tools to identify and monitor disease status in MM samples. Unlike MFC, the LymphoTrack Assays and accompanying bioinformatics software can be submitted for approval to regulatory authorities worldwide.

#### H046. Pediatric Myeloid Sarcoma: A Single Institution Clinicopathologic and Molecular Analysis

T. Zho<sup>1,2</sup>, M.S. Bloomquist<sup>1</sup>, H. Voicu<sup>1</sup>, J. Reuther<sup>1,2</sup>, I. Gandhi<sup>1</sup>, M.S. Redell<sup>1,2</sup>, A.N. Marcogliese<sup>1,2</sup>, M.T. Elghetany<sup>1,2</sup>, P.H. Rao<sup>2</sup>, A. Roy<sup>1,2</sup>, D.H. Lopez-Terrada<sup>1,2</sup>, J.N. Punia<sup>1,2</sup>, C.V. Curry<sup>1,2</sup>, K. Fisher<sup>1,2</sup>

<sup>1</sup>Baylor College of Medicine, Houston, TX; <sup>2</sup>Texas Children's Hospital, Houston, TX.

**Introduction:** Myeloid sarcoma (MS) is a rare neoplastic condition composed of immature myeloid cells at an extramedullary site. The entity is understudied in the pediatric population, therefore little is known regarding the clinicopathological features and underlying chromosomal or molecular alterations. **Methods:** We retrospectively obtained clinical, pathologic, and cytogenetic data from a series of 35 pediatric MS cases identified via a pathology database search from 1984-2016 at our institution. Patients were classified as type 1, *de novo* MS (n=5); type 2, MS with concomitant acute myeloid leukemia (AML) (n=11); type 3, MS following other myeloid disorders (n=3, following JMML, atypical CML, and MDS); or type 4, MS as a recurrence of AML (n=10); 6 cases lacked data for classification. Six cases with normal karyotype (NK, n=4) or without cytogenetic data (n=2) underwent SNP array analysis on the Affymetrix OncoScan platform and next-generation sequencing (NGS) using a custom-designed 152 gene Roche NimbleGen SeqCap Target Enrichment probe set and sequenced on the Illumina MiSeq System. Survival analysis was performed using the Kaplan-Meier method. **Results:** Of the 35 MS

identified, karyotype/SNP array data were available for 27 patients. Chromosomal aberrations were detected in 22 patients (81.5%) with 5 normal karyotypes. KMT2A-rearrangements including t(9;11) were detected in 9 samples, 7 harbored complex karyotypes, 3 showed t(8;21), 1 gain of 8, and 1 inv(16). One patient had discordant cytogenetic abnormalities between the bone marrow (-Y, +7) and extramedullary myeloid sarcoma [t(8;21)]. NGS data of 6 cases showed a preponderance of alterations in tyrosine kinase pathway genes including mutations in *NRAS* (n=2), *PTPN11*, *NF1*, and *FLT3-ITD*. The median survival (MS) time was 482.0 days and the 5-year overall survival (OS) estimation was 0.29±0.11 (n=25, patients with karyotype data only). MS correlated with cytogenetic risk stratification groups: favorable [t(8;21), t(15;17) and inv(16), n=8, MS= 4700.0 days]; intermediate (NK and non-complex, n=10, MS=726.9 days); and adverse (complex, n=7, MS=150.0 days). In addition, disease relapse was an adverse prognostic factor for OS (p=0.013) while clinical types I-IV, very early age at onset (<1 year) and bone marrow involvement at diagnosis did not influence OS. **Conclusions:** This is the largest single institutional cohort of pediatric MS patients to date. Chromosomal abnormalities and mutations affecting tyrosine kinase pathway genes are frequent molecular events. The presence of complex chromosomal abnormalities at initial diagnosis and disease relapse are adverse prognostic factors, whereas clinical types I-IV, infancy onset, and bone marrow involvement do not influence the outcome.

#### H047. PTPN11 Mutation is Uncommon in Acute Myeloid Leukemia, but Associated with a Complex Karyotype, Co-mutations in KRAS or NRAS and Poor Prognosis

R. Ruiz-Cordero<sup>1,2</sup>, K. Floyd<sup>1,2</sup>, S. Loghavi<sup>1</sup>, R. Kanagal-Shamanna<sup>1</sup>, C.C. Yin<sup>1</sup>, Z. Zuo<sup>1</sup>, M.J. Routbort<sup>2</sup>, R. Luthra<sup>2</sup>, H.M. Kantarjian<sup>2</sup>, J.L. Medeiros<sup>2</sup>, K.P. Patel<sup>2</sup>, C.Y. Ok<sup>2</sup>

<sup>1</sup>MD Anderson Cancer Center, Houston, TX; <sup>2</sup>University of California, San Francisco, CA.

**Introduction:** The *PTPN11* gene encodes a cytoplasmic protein tyrosine phosphatase (SHP2), which activates the RAS-MAPK signaling pathway. Mutations in *PTPN11* have been reported in approximately 5% of patients with acute myeloid leukemia (AML), commonly (40%) co-mutated with *NPM1*, but mutually exclusive to *FLT3* internal tandem duplication mutation. However, previous study used Sanger sequencing with only few (n=14) *PTPN11* mutated patients. Therefore, updated systematic study is needed. **Methods:** We collected 224 consecutive patients with newly diagnosed AML who underwent clinically validated 81-gene next-generation sequencing (NGS) panel. Diagnosis was made according to the updated 2017 WHO Classification. Clinical, laboratory and cytogenetic information was searched using electronic medical record. **Results:** A total of 28 patients (12.5%) with *PTPN11* mutation were identified. The median mutant allelic frequency (MAF) was 10.8% (range: 1.4% to 44.9%). *PTPN11* mutation was present as a major clone (MAF >10%) in about two thirds of the patients (n=18). The median age was 66 years (range: 17 to 85 years). Male-to-female ratio was 1.8:1. AML with myelodysplasia-related changes (AML-MRC) was the most common subtype (n=17), followed by AML, not otherwise specified (n=3). AML transformed from underlying myeloproliferative neoplasm (n=3), AML with inv 16 (n=2), therapy-related AML (n=2), and AML with mutated *NPM1* (n=1). Complex karyotype was common (n=10, 36%), but diploid karyotype was rare (n=4, 14%). Mutations were most commonly found in the amino-SH2 domain (n=21), involving codons 60 (n=3), 61 (n=5), 69 (n=4), 72 (n=4), 73 (n=1) and 76 (n=4). Mutations in the protein tyrosine phosphatase (PTP) domain were less common (n=8), involving codons 265 (n=1), 461 (n=1), 491 (n=1), 502 (n=2) and 503 (n=3). All patients had mutations in other genes (median number of mutated gene: 5, range: 2 to 9). *DNMT3A* and *RUNX1* were the most commonly associated mutated genes (n=10), followed by *ASXL1* (n=7), *NRAS* (n=7), *FLT3* (n=6), *KRAS* (n=6), and *SRSF2* (n=6). Co-mutation in *NPM1* is not common (n=3). To the contrary, co-mutations in *KRAS* or *NRAS* common (39%), harboring additional activating signal in the RAS/MAPK pathway. Comparing overall survival (OS) from a separate AML cohort (n=37), *PTPN11* mutated patients had worse prognosis (median OS: 20.6 and 7.9 months, respectively, p <0.01). **Conclusion:** Using an NGS-based method, *PTPN11* mutation is present in 12.5% of newly diagnosed AML

patients. It is commonly seen in AML-MRC and is associated with a complex karyotype (36%). *PTPN11* mutation is a major clone in majority of patients (64%), but it can be present as a sub-clone. Co-mutations are present in all of these patients, including frequent (40%) *KRAS/NRAS* mutations.

#### H048. Accurate Detection of *FLT3*-ITDs and *CEBPA* Variants in Acute Myeloid Leukemia by Anchored Multiplex PCR and Next-generation Sequencing

N.M. Nair, J.D. Haines, M. Bessette, L. Johnson, K.E. Moore, L.M. Griffin, A. Licon, R. Walters  
ArcherDX, Boulder, CO.

**Introduction:** Acute Myeloid Leukemia (AML) is clinically and biologically heterogeneous, requiring the detection of mutations across multiple genes for characterization. *FLT3*-ITDs and *CEBPA* mutations represent important markers in AML, however they are difficult to detect by next generation sequencing (NGS) due to the highly variable nature of ITDs, the high GC content of *CEBPA*, and the difficulty in mapping repeated sequences to a wild-type reference. **Methods:** We developed Archer VariantPlex myeloid assays based on Anchored Multiplex PCR (AMP) to detect important mutations in myeloid malignancies, including *FLT3*-ITDs and *CEBPA* variants. AMP is a target enrichment strategy for NGS that uses molecular-barcoded adapters and single gene-specific primers for amplification, permitting open-ended capture of DNA fragments from a single end. This approach enables flexible and strand-specific primer design to provide better coverage of ITD-containing regions of *FLT3* and GC-rich regions of *CEBPA*. **Results:** *In silico* *FLT3*-ITD datasets enabled optimization of the Archer Analysis ITD detection algorithm. This algorithm used in combination with the VariantPlex Core Myeloid assay enabled detection of *FLT3*-ITDs down to <0.1% AF. Furthermore, we show 100% concordance of a VariantPlex Core AML assay with other methods commonly used to detect *FLT3*-ITDs from 25 blood and bone marrow samples. We detected concomitant non-ITD variants in *FLT3* and *NPM1* in some of these samples. Finally, we show >1000x unique molecule coverage across the entire coding region of *CEBPA* with 2M reads and 50ng input. **Conclusions:** AMP provides NGS-based detection of complex mutation types that are relevant in AML, including *FLT3*-ITDs and *CEBPA* variants.

#### H049. A Single NGS-based Assay for Simultaneous Identification of *BCR/ABL1* Fusion and *ABL1* Sequencing Detects Resistance Mutation and Subclones

R. Ruiz-Cordero<sup>1,2</sup>, N.R. Farhana<sup>1,2</sup>, W. Chen<sup>1</sup>, K. Floyd<sup>1</sup>, B. Barkoh<sup>1</sup>, M.J. Routbort<sup>1</sup>, J.E. Cortes<sup>1</sup>, D.Z. Hatfield<sup>1</sup>, R. Luthra<sup>1</sup>, J.L. Medeiros<sup>1</sup>, K.P. Patel<sup>1</sup>

<sup>1</sup>MD Anderson Cancer Center, Houston, TX; <sup>2</sup>University of California, San Francisco, CA.

**Introduction:** Chimeric *BCR/ABL1*, the pathogenomic molecular alteration resulting from t(9;22) in chronic myeloid leukemia (CML), has been successfully targeted with tyrosine kinase inhibitors (TKI). Unfortunately, a substantial number of patients (20-30%) treated with TKI develop TKI-resistant mutations in the kinase domain (SH1) of the chimeric *ABL1*. In addition, presence of alterations in SH2 and SH3 domains in *ABL1* and co-occurrence of >1 kinase domain mutation are shown to be clinically significant in some studies. Conventional methods for *ABL1* kinase domain mutation detection such as Sanger- and pyrosequencing are limited in the scope. We developed a single-step, next generation sequencing (NGS)-based assay to identify *ABL1* resistance mutations involving SH1, SH2, SH3 domains in cases with low *BCR-ABL1* fusion. **Methods:** We utilized cell line RNA to optimize the NGS-based detection of *BCR-ABL1* fusion and *ABL1* resistance mutations. Subsequent to optimization, peripheral blood (PB) and bone marrow (BM) samples from patients with CML were utilized to validate the assay. Briefly, complete *BCR/ABL1* fusion transcript was amplified using laboratory developed primers. Amplified cDNA fusion products were fragmented and tagged using tagmentation-based Nextera Flex kit (Illumina). Paired-end sequencing was performed using a V2 300 paired-end on a nano flow cell (Illumina). Fusion detection and variant calling were performed by in-house developed informatics pipeline (FusionMap and LoFreq). Results were compared against qRT-PCR and Sanger/pyro

sequencing results. **Results:** We successfully analyzed a total of 24 cDNA samples with p210 (e13a2, e14a2) and p190 (e1a2) fusion (peripheral blood: 5, bone marrow: 19) from 21 patients (women: 11) using laboratory developed NGS assay. The sample cohort included 8 wild-type *ABL1* cases, 7 positive for 1 *ABL1* mutation and 9 positive for >1 *ABL1* mutation by Sanger sequencing. A BCR-ABL1/*ABL1* percentage ratio of >1% provided adequate PCR products for sequencing. Of the 28 mutations detected by SS, 27 (96%) were detected by NGS. Interestingly, in 2 cases negative by SS, NGS identified 1 and 3 mutations at <2% VAF. Moreover, in one case the T315I mutation was only detected by our assay at 4.2% VAF. **Conclusion:** Our NGS-based assay provides a comprehensive tool for the assessment of *BCR-ABL1* fusion transcripts and clinically relevant *ABL1* mutations, thereby potentially increasing the window of opportunity to adjust therapy and improve outcomes.

#### H050. Clinicopathologic Characterization of Myeloid Neoplasms with Concurrent Spliceosome Mutations and MPN-associated Mutations

Y. Liu, N. Bailey

University of Pittsburgh, Pittsburgh, PA.

**Introduction:** Spliceosome genes (*SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*) are commonly mutated in myelodysplastic syndromes (MDS) while *JAK2*, *MPL*, and *CALR* mutations (Mut) are often associated with myeloproliferative neoplasms (MPN). Though *SF3B1* and MPN-associated Muts are well-known to co-occur in the rare entity MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T), myeloid neoplasms (MN) with concurrent spliceosome and MPN-associated Muts (SS-MPN) encompass other disease entities and are not well characterized. **Methods:** Twenty-two SS-MPNs were identified among 642 samples submitted for mutation analysis using amplicon target enrichment of 37 genes recurrently mutated in MN during 2016-2018. Nine MNs with concurrent *SF3B1* and MPN-associated Muts (*SF3B1*-MPN) and 13 MNs with concurrent non-*SF3B1* spliceosome and MPN Muts (non*SF3B1*-MPN) were identified. Among the 9 *SF3B1*-MPNs, 1 demonstrated concurrent *SF3B1*, *SRSF2* and MPN-associated Muts. Review of the clinical records and pathology material was performed. **Results:** There is a male predominance in non*SF3B1*-MPNs (M:F 12:1; 54-80 y/o) when compared with *SF3B1*-MPNs (M:F 4:5; 56-90 y/o). SS-MPNs only contain small numbers of MDS/MPN-RS-T (0/13 of non*SF3B1*-MPNs; 2/9 of *SF3B1*-MPNs). 6/13 non*SF3B1*-MPNs and 1/9 *SF3B1*-MPNs are AML. 3/6 AML in non*SF3B1*-MPNs and 0/1 AML in *SF3B1*-MPNs represent blast phase of MPN in which cells with non-*SF3B1* Mut appear to be a subclone of cells with MPN Mut. *SF3B1*-MPNs show higher platelet counts and higher MCV when compared with non*SF3B1*-MPNs (p=0.03 & 0.075). 4/15 non-AML SS-MPNs show absolute monocytosis, raising a difficult differential diagnosis between chronic myelomonocytic leukemia (CMML) and MPN with monocytosis. MNs with non-*SF3B1* spliceosome Mut are more likely to show dysgranulopoiesis (6/13 vs 1/7) and MNs with *SF3B1* Mut are more likely to show ring sideroblasts (6/6 vs 5/10). There is no significant difference in the number of additional Muts between non*SF3B1*-MPNs and *SF3B1*-MPNs (2.2 vs 1.9) though higher incidences of *ASXL1* and *IDH* Muts in non*SF3B1*-MPNs are noted (8/13 vs 1/9; 4/13 vs 0/9). 4/12 non*SF3B1*-MPNs and 0/7 *SF3B1*-MPNs show abnormal karyotype; only 1 abnormal karyotype carries MDS-defining abnormalities. There is no statistical significance between the survival between non*SF3B1*-MPNs and *SF3B1*-MPNs. **Conclusions:** SS-MPNs comprise many disease entities with only a small fraction being MDS/MPN-RS-T. A significant portion of non*SF3B1*-MPNs represent blast phase of MPN with only a subclone of cells demonstrating a spliceosome Mut, suggesting a non-*SF3B1* spliceosome Mut may contribute to the disease progression in these cases. SS-MPNs also enrich for the cases with differential diagnoses of CMML and MPN with monocytosis. Further validation of our findings is important.

#### H051. SNP Genotyping-based Stem Cell Engraftment Detection in Targeted NGS Testing

W. Chen, M.J. Routbort, Z. Zuo, L. Medeiros, R. Luthra, K.P. Patel  
MD Anderson Cancer Center, Houston, TX.

**Introduction:** Conventional stem cell engraftment studies rely on capillary gel electrophoresis-based detection of microsatellite (MST) markers. Use of single nucleotide polymorphisms (SNP) for PCR-based

detection of mixed chimera containing an admixture of host and donor DNA have been proposed. Routine clinical application of next generation sequencing (NGS)-based mutation profiling offers the opportunity to incorporate SNP genotyping to detect unsuspected mixed chimera reflecting engraftment failure and relapses. We tested whether NGS-based SNP genotyping can provide chimerism information in post-stem cell transplant samples being tested for mutation analysis using a clinical targeted NGS panel. **Methods:** We incorporated 49 SNPs shown to have utility in chimerism detection into the design of an 81-gene custom NGS panel with integrated unique molecular identifiers (UMI) for hematologic neoplasms to identify sample admixtures as a patient safety measure. NGS analysis was performed using paired-end sequencing (V3) on an Illumina MiSeq. We selected 17 patient samples with mixed chimera showing donor and host DNA by conventional chimerism studies, who also had NGS-based mutation profiling data available. Variant allele counts for SNPs covered on the NGS panel were used to compute the percentage deviation from heterozygous or homozygous distribution for each locus. Since this data represents only post-stem cell transplant samples, donor versus host allele determination was not possible and the values simply represent the percentage of genomic admixture present in the sample. No donor or pre-transplant host DNA was tested.

**Results:** Comparison of conventional MST and NGS-based SNP variant allele frequencies (VAFs) showed 100% concordance for the qualitative detection of mixed chimera. Both methods successfully detected chimera as low as 2%. The percentage of mixed chimera ranged from 2% to 45% on conventional MST testing. Using unselected SNP VAFs to compute the percentage DNA admixture, the NGS-based percentage of chimeras for the same set of samples ranged from 2% to 14%. The absence of donor and pre-transplant host testing limits accurate determination of informative alleles and accurate quantitation, but, closely represents a real-life scenario where donor and pre-transplant host profiles may not always be available. This approach provides a useful screening tool for the detection of an unsuspected chimera. **Conclusion:** Inclusion of carefully selected informative SNPs allows detection of mixed chimera in post-stem cell transplant samples using routine clinical NGS testing. Additional assessment including optimization of computational algorithms and testing of matched donor samples is required for quantitative chimerism assessment using NGS.

#### H052. Development of a Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) Assay for Nucleophosmin (NPM1) Minimal Residual Disease (MRD) Monitoring in Acute Myeloid Leukemia

M. Mai, D.S. Viswanatha, R. He  
Mayo Clinic, Rochester, MN.

**Introduction:** Acute myeloid leukemia (AML) is a genetically heterogeneous group of neoplasms. In the intermediate-risk group defined by cytogenetic findings, *FLT3* mutations are considered indicators of poor prognosis. However, in the absence of a *FLT3* mutation, the presence of *NPM1* mutation is associated with a more favorable prognosis. *NPM1* mutation is a common finding in *de novo* AML (25-30%) and consists of small insertion (typically 4 base pair) or insertion/deletion events involving exon 12. Three variants are highly recurrent, termed types A, B and D and account for ~90% of *NPM1* alterations in *de novo* AML. *NPM1* mutation serves as a sensitive MRD marker for evaluating minimal disease and therapeutic response following treatment. The aim of this study is to develop to sensitive quantitative MRD assay for *NPM1* monitoring in AML. **Methods:** As the somatic *NPM1* mutations in AML are insertion and/or deletions involving the last exon (exon 12), we developed a RT-qPCR using a common forward primer and probe, and mutation type-specific reverse primers covering the A, B and D mutation types. *ABL1* served as the reference gene. Relative *NPM1/ABL1* ratio was quantified using a  $\Delta\Delta C_t$  method. A synthetic oligonucleotide with 1:1 *NPM1* and *ABL1* ratio served as the assay calibrator which defined a 100% *NPM1/ABL1* ratio.

**Results:** Eighteen samples previously tested for *NPM1* by the qualitative fragment analysis (5% analytic sensitivity, 17 positive and 1 negative) and 20 samples previously tested negative from a myeloid-targeting next generation sequencing panel (5% analytic sensitivity) were used for accuracy study, and showed 100% positive/negative concordance. The

analytic sensitivity of this assay was first established by serial oligonucleotide dilution studies for all three types at 0.01% (*NPM1/ABL1* transcript copy number ratio), and then confirmed in a serial cell line dilution study and a serial high-positive patient sample dilution study. Analytic specificity was assessed in 52 normal donor samples and with a *NPM1* target  $C_t$  cut-off value of 39, 52/52 samples were negative. Inter and intra-assay reproducibility was acceptable in 8 positive and 1 negative samples tested with 100% of positive/negative concordance and <0.5 log quantitative differences in positive samples. Preanalytical variables were also evaluated. **Conclusions:** We have successfully developed a sensitive RT-qPCR assay for *NPM1* MRD monitoring in AML patients harboring this mutation, which will provide clinicians with an accurate and precise measurement of disease burden to facilitate relapse monitoring and guide treatment decisions.

#### H053. Next-generation Sequencing in Burkitt-like Lymphoma with 11q Aberration: A Clinicopathologic Correlation

A.N. Alsuwaidan, D. Xia, J. Malter, M. Chen

The University of Texas Southwestern Medical Center, Dallas, TX.

**Introduction:** Burkitt-like lymphoma with 11q aberration (BLL-11q) is a provisional entity in the 2016 World Health Organization classification with features that resemble Burkitt lymphoma (BL) without *MYC* gene rearrangement. They demonstrate chromosome 11q alterations, mostly proximal gains and telomeric losses. However, the genomic alterations and the clinical course of BLL-11q have not been well studied due to the limited number of cases. Here we contribute to further characterize this rare entity with molecular analysis and clinicopathological features.

**Methods:** A 24-year-old male presented with nasopharyngeal mass. Histologic and immunophenotypic findings were evaluated. Chromosomal analysis and fluorescence *in situ* hybridization (FISH) were performed. Our institutional 1385 genes Next-Generation Sequencing (NGS) panel was performed on formalin-fixed, paraffin-embedded tissue, sequenced with Illumina HiSeq 4000 instruments. Variants were identified using our institutional custom bioinformatics pipelines. Patient's clinical data were also reviewed. **Results:** Morphologic examination of the biopsy shows high grade B cell lymphoma resembling BL. The lymphoma cells lack *MYC* expression and show high proliferation rate. Chromosomal analysis reveal abnormal clone: 48, XY,+X,?der(11)t(1;11)(q25;q24),+12[8]. FISH studies are negative for *MYC*, *BCL2*, or *BCL6* rearrangements. NGS confirms the presence of chromosome 11q gain/loss and detects likely pathogenic hotspot variants in the following genes: *EZH2*, *KMT2D*, and *ERCC2*. No mutations in *MYC*, *FLI1*, *USP2*, *CBL*, and *ETS1* genes were detected. Our patient went into complete remission following 6 cycles of aggressive chemotherapy (etoposide, vincristine, and doxorubicin, with oral prednisone and cyclophosphamide plus rituximab (DA-R-EPOCH)). **Conclusions:** Our case confirms that BLL-11q usually show complex karyotypes, and lower levels of *MYC* expression compared to BL. The clinical course seems to be similar to BL, but the number of reported cases is limited and screening for 11q aberrations is not routinely performed. Our case reveals additional genomic alterations containing pathogenic variants including gain- of-function activating mutation in *EZH2*, loss- of-function frameshift detected in *KMT2D*, and splicing site variant found in *ERCC2*. These findings are significant not just in the diagnosis, but also to better understand the pathogenesis of this rare entity. Moreover, NGS can be helpful to match these cases with the available targeted therapy. For instance, these genomic alterations have been reported to be sensitive to *EZH2* inhibitors, and/or HDAC inhibitors. Finally, further studies are needed to fully understand the significant of these genomic alterations and define the clinical course of this rare entity.

#### H054. Integrative Analysis of Programmed Death-Ligand 1 DNA, mRNA, and Protein Status and their Clinicopathological Correlation in Diffuse Large B-cell Lymphoma

X. Zhou

Fudan University Shanghai Cancer Center, Shanghai, China.

**Introduction:** The Protein expression of Programmed Death-Ligand 1 (PD-L1) has been recognized a poor prognostic biomarker in diffuse large B-cell lymphoma (DLBCL). However, what DNA and mRNA status, whether they contribute to protein expression and their clinicopathological correlation in DLBCL are largely unknown. **Methods:** We detected PD-L1



status in three different levels by Fluorescence in situ hybridization, RNAscope and immunohistochemistry in 287 DLBCL samples with follow-ups, respectively. Their correlation and clinical pathological relevance was further analyzed. **Results:** 1.71% (3/175) patients had PD-L1 amplification, 19.86% (57/287) PD-L1 mRNA high expression and 11.84% (34/287) high protein expression. Both mRNA and protein high expression of PD-L1 was significantly elevated in non-GCB than that in GCB DLBCL ( $P<0.05$ ). In addition, the patients with PD-L1 mRNA or protein high expression but not DNA amplification have significantly poorer overall survival (OS) than that with PD-L1 low expression ( $P<0.05$ ). Furthermore, we found that PD-L1 mRNA and protein expression are highly correlated ( $P=0.012$ ), which was observed in all three samples with PD-L1 DNA amplification. **Conclusions:** This work suggested that PD-L1 DNA amplification is a rare event, PD-L1 mRNA mainly contribute to the protein high expression, and the latter two will serve as important biomarkers for predicting prognosis and selecting patients for immunotherapy in DLBCL.

#### **H055. Optimizing Diagnostic Algorithms for Pediatric Leukemia: Synergy Between Next-generation Sequencing, Chromosomal Microarray, and Conventional Cytogenetics**

M.C. Hiemenz, J. Ji, R.J. Schmidt, M.J. Oberley, D. Bhojwani, J.A. Biegel, G. Raca

Children's Hospital Los Angeles, Los Angeles, CA.

**Introduction:** Chromosomal microarrays (CMA) and next-generation sequencing (NGS) panels are increasingly used to complement standard cytogenetics (karyotyping) and fluorescence *in situ* hybridization (FISH) for diagnostic evaluation of bone marrow samples. To determine which combination of assays provides the highest diagnostic yield in pediatric leukemias, we analyzed 100 consecutive bone marrow samples by karyotyping, FISH, CMA and/or our OncoKids NGS panel. OncoKids is a DNA and RNA-based AmpliSeq pediatric cancer panel designed to detect sequence variants and RNA fusions. **Methods:** Sequencing was performed using the Ion S5 platform. The following pediatric leukemia types were tested: B-ALL (n=67), AML (n=23), T-ALL (n=6), and mixed phenotype acute leukemia (MPAL, n=4). **Results:** In B-ALL, as a single test the FISH panel had the highest overall diagnostic yield (61% of B-ALL cases), due to the high frequency of hyperdiploidy and *ETV6-RUNX1* fusions. However, the combined use of CMA and OncoKids<sup>SM</sup> had a higher overall detection of primary drivers (72% of B-ALL cases) than cytogenetics and FISH and also revealed secondary clinically significant variants. With the exception of two IGH rearrangements, these assays detected all of the alterations identified by cytogenetics and FISH, but also revealed primary abnormalities in eight cytogenetically negative cases. These alterations included "Ph-like" fusions (n=3), *ZNF384* fusions (n=3), a *MEF2D* fusion (n=1) and a *KMT2A* fusion (n=1). Important secondary alterations included *IKZF1* deletions (n=12) and *JAK1/JAK2* mutations (n=5). In AML, OncoKids had the highest yield for detection of both primary and secondary DNA mutations and RNA fusions (78% of AML cases). All of the abnormal fusions revealed by karyotype and FISH were detected. Additionally, in six cases, OncoKids identified the key driver that was not observed with cytogenetics/FISH. These six cases included four gene fusions (*NPM-MLF1*, *CBFA2T3-GLIS2* and two *KMT2A* fusions) as well as clinically significant sequence changes in *NF1* and *GATA1*. **Conclusions:** Our preliminary data highlights the complementarity between CMA/NGS and conventional cytogenetics/FISH in pediatric leukemia testing. Due to rapid TAT, FISH may be useful as an initial screening method in B-ALL, with FISH and karyotype negative or inconclusive cases proceeding for further testing. The disadvantage of this approach is that potentially valuable secondary abnormalities (like *IKZF1* deletions) could be missed in FISH-positive cases. Therefore, the optimal testing algorithm for B-ALL must integrate information regarding cost, TAT, and institutional resources. Our data also suggests that NGS testing with OncoKids may represent a superior alternative to karyotyping as a first line test in pediatric AML.

#### **H056. Variant Allele Frequency does not correlate with Marrow-based Leukemic Blast Proportions in Acute Myeloid Leukemia**

L.N. Toth, D.C. Green, J.D. Peterson, F.B. de Abreu, E.Y. Loo  
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

**Introduction:** There is ongoing effort in contemporary medical literature to associate mutant variant allele frequency (VAF) with prognostic outcomes for numerous hematologic diseases, including acute myeloid leukemia (AML). Many AML-focused studies assume that multiplexed PCR amplification of gene targets is relatively unbiased and will directly correlate with leukemic cell populations in the bone marrow. We retrospectively compared the VAF results from targeted gene panel sequencing studies against relative quantity of marrow-based blasts as assessed by morphology and immunophenotyping studies. **Methods:** We evaluated all Illumina TruSight Myeloid Sequencing Panel cases performed on bone marrow biopsies for acute myeloid leukemia, between 2014 -2018. Sequencing was performed on the MiSeq platform using at least 50 ng of genomic DNA. Base-calling and sequence alignment were performed using MiSeq Reporter Software and analyzed with VariantStudio v2.1. Mutant VAF was evaluated in relation to the relative proportion of blasts in all types of acute myeloid leukemia. **Results:** Sixty-nine of 75 cases were appropriate for evaluation, including primary AML (pAML, n=45) and secondary AML (sAML, n=24; includes AML with myelodysplasia related changes, therapy-related AML, and cases with prior low-grade myeloid neoplasm). Marrow aspirate myeloblast involvement ranged from 13 to 95%. No variants were found in eight pAML cases, but total of 196 variants were detected across all others. An  $R^2$  value was calculated for blast percentage versus VAF for each variant. No correlation was found in either pAML or sAML ( $R^2 = 0.05$  and  $0.02$  respectively). No significant differences were seen between pAML cases with normal karyotype versus those with recurrent cytogenetic aberrancies. No improvement in the  $R^2$  was appreciated after exclusion of cases with dysplasia, sorted case analysis based on genes with strong sAML association (*SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *BCOR*, *STAG2*, and *EZH2*), or in analysis arranged by gene-functional-group. **Conclusions:** Regardless of AML subtype or lack of apparent background myelodysplasia, mutant VAF by a common next-generation gene sequencing assay does not appear to correlate to relative blast proportions as assessed in the corresponding bone marrow biopsy. Thus, greater deliberation should be taken prior to broad incorporation of mutant VAF into prognostic systems. This may also confound the application of VAF to categorize dominant versus subclonal leukemic populations, suggesting that this practice should be performed with caution when using next-generation sequencing technologies.

#### **H057. BCOR Mutations Portend Poor Survival Independent of Concurrent Mutations in Other Epigenetic Modulators in Myelodysplastic Syndrome**

I. Badat<sup>1</sup>, S. Mirza<sup>1</sup>, J. Song<sup>2</sup>, M. Hussaini<sup>2</sup>

<sup>1</sup>University of South Florida, Tampa, FL; <sup>2</sup>Moffitt Cancer Center, Tampa, FL.

**Introduction:** Myelodysplastic syndromes (MDS) represent a group of myeloid neoplasms characterized by cytopenia(s), dyspoiesis, marrow hypercellularity, and unique (cyto)genetic abnormalities. These genetic abnormalities not only drive disease but also carry prognostic significance. While the role of single mutations has been extensively studied and incorporated into national guidelines, the impact of genetic aberrations in the context of other mutations is less well understood. BCOR (BCL6 Corepressor protein) is an epigenetic transcriptional co-repressor that regulates genes involved in repression of inflammation, differentiation cell cycle, and apoptosis. In MDS, BCOR mutations are rare and nonsense, frameshift, splice site, or missense (codon N1425) mutations are associated with poor prognosis (NCCN, MDS, v1.2018). While prior work has shown that increasing number of driver mutations portend worse prognosis, we hypothesized that additional concurrent mutations in epigenetic MDS-driver genes in addition to BCOR would not further contribute to prognosis. **Methods:** IRB approval was procured. Departmental next generation sequencing (NGS) databases (with >4000 cases) were queried for hematopoietic malignancies with associated NGS data (Genoptix 5-gene panel, Genoptix 21-gene panel, FoundationOne, Illumina TruSeq Myeloid) and the data analyzed to identify cases of MDS

with BCOR mutations only and MDS cases with BCOR mutations with concurrent mutation in one or more epigenetic modulators (namely, *TET2*, *ASXL1*, *DNMT3A*, *EZH2*, *IDH2*, *IDH1*, *BCORL1*, *ATRX*). Overall survival was determined by chart review. Fischer's exact test and unpaired t-test was performed for statistical analysis. **Results:** A total of 35 patients with BCOR mutations with concurrent mutations in epigenetic MDS genes were detected. Only four MDS patients with pure BCOR mutations were found. BCOR mutations occurred concurrently with 35 other myeloid genes the most common of which were: *DNMT3A* (40%), *RUNX1* (36%), *ASXL1* (32%), *TET2* (32%), *BCORL1* (23%), *U2AF1* (19%) and *SRSF2* (17%). One out of 4 patients (25%) with pure BCOR mutation transformed to AML while 11/35 patients with concurrent epigenetic mutations transformed to AML (31%) ( $p=1.00$ ). Overall survival was 16.1 months vs 17.7 months, respectively based on available OS data ( $p=0.74$ ; 95% CI: -19.26 to 13.93). **Conclusions:** Our data supports the notion that singular dysregulation *BCOR* gene in the epigenetic MDS driver class obviates the detrimental effect of additional changes in the same category on prognosis. Given the rarity of *BCOR* mutations, our data set provides an important contribution toward a more global understanding of the effect of multiple concurrent driver mutations in MDS.

#### H058. Isochromosome 17q in Acute Myeloid Leukemia and Myeloid Neoplasms

M. Kim, D. Ko

Ulsan University, Asan Medical Center, Seoul, South Korea.

**Introduction:** The isolated isochromosome (17q) is a rarely reported cytogenetic abnormality in myelodysplastic and/or myeloproliferative neoplasms (MDS/MPN) and *de novo* acute myeloid leukemia (AML). We described the clinicopathological features and survival rate of isochromosome 17q (i(17q)) in hematologic malignancies such as acute myeloid leukemia and myeloproliferative neoplasms in Asan Medical Center. **Methods:** Bone marrow samples were collected from 24 patients diagnosed and treated myeloid neoplasm or acute myeloid leukemia with i(17q)(10) in the Department of Hematology and Clinical Pathology, Asan Medical Center from 2001 to 2016. We analyzed bone marrow biopsy and bone marrow smear. And conventional cytogenetic studies were performed on more than 20 metaphase cells prepared to form bone marrow aspirate smears using standard techniques. **Results:** We evaluated total 24 patients; 13 patients were diagnosed acute myeloid leukemia and 11 patients were diagnosed myeloid neoplasm. For 188 month, total nine patients (37.5%) survived and 5 year survival rate was 23.4%. Median survival time was 15.0 month. And there is a significant difference in survival rate and median average survival time between complex karyotype and non-complex karyotype with i(17q). ( $P=0.006$ ) Two cases were normal karyotype at the time of the first diagnosis. But for follow-up period, i(17) was detected. Both patients had chromosomal changes when they were in a clinicopathologically bad condition. Despite receiving medical treatment, they did not show good clinical progression. **Conclusion:** In this study, we confirm a poor prognosis in i(17q), especially with the complex karyotype. In cases of detected i(17) during disease progression, The clinical condition of patients should be carefully observed.

#### H059. Comparison of Interpretive Guidelines for IGH and TCR Clonality by NGS in B and T-cell Cancers

L. Lay<sup>1</sup>, B. Stroup<sup>1</sup>, J. Payton<sup>2</sup>

<sup>1</sup>Barnes-Jewish Hospital, St. Louis, MO; <sup>2</sup>Washington University School of Medicine, St. Louis, MO.

**Introduction:** Cancers of B and T lymphocytes are the most common blood cancers in the US. More sensitive assays are needed for disease monitoring and improved diagnostic accuracy in cases of immunodeficiency or autoimmunity. Next generation sequencing (NGS) is a more sensitive method for assessing IGH and TCR clonality. However, the increased sensitivity raises questions regarding interpretive limits. Therefore, we sought to compare the effects of different interpretive cutoffs on concordance to the previously used PCR fragment assays.

**Methods:** We chose LymphoTrack (InvivoScribe) due to its similarity to the PCR fragment assays, its published use, and its compatibility with the Ion Torrent S5 system, which is not commonly used for NGS clonality.

Libraries were prepared from DNA extracted from 150 routine patient specimens (peripheral blood, bone marrow, or fresh/ formalin-fixed, paraffin-embedded (FFPE) tissue) using the Ion OneTouch 2 and Ion S5 (520 and 530 chips, read length 400bp), all following manufacturers' protocols. LymphoTrack-PGM Software was used to align and merge sequences with fewer than 2bp differences; merged sequences were ranked by percentage of total sequencing reads. Two sets of interpretive guidelines were compared for clonality reporting. **Results:** For IGH, Guidelines A and B gave similar interpretations that were highly concordant for clonal samples per the PCR fragment assay. Concordance for polyclonal samples was lower for both guidelines due to clonal results by NGS, which may be due to higher sensitivity of the LymphoTrack NGS assay. For TCRG, concordance was considerably lower for Guideline B, particularly for clonal samples (85% for A vs 57% for B). **Conclusions:** Overall, Guidelines A and B performed similarly in concordance to the PCR fragment assay for IGH clonality. However, TCRG concordance varied for clonal and polyclonal samples, which may be due to greater analytical sensitivity and higher resolution of the LymphoTrack NGS versus the PCR fragment assays. The apparent reduction in sensitivity for detection of clonal TCR samples by Guideline B raises concern for missed diagnoses. Given recent reports of the importance of early detection of TCR clonality in CTCL and the greater simplicity of Guideline A, we favor the use of a straightforward 2.5% cutoff. Initial feedback from ordering practitioners is positive regarding these interpretive guidelines.

#### H060. Impact of Single versus Multiple Spliceosome Mutations in MDS/CMML

M. Hussaini<sup>1</sup>, S. Mirza<sup>2</sup>, I. Badat<sup>2</sup>, J. Song<sup>1</sup>

<sup>1</sup>Moffitt Cancer Center, Tampa, FL; <sup>2</sup>University of South Florida, Tampa, FL.

**Introduction:** Myelodysplastic syndromes (MDS) represent a group of myeloid neoplasms driven by various classes of genetic abnormalities, most of which portend worse prognosis with the significant exception of SF3B1 (a spliceosome mutation). Spliceosome mutations are the most common category of genetic abnormalities in MDS, occurring in up to 60% of cases. Prior work has shown that an increasing number of driver mutations, regardless of whether they are subclonal, portend worse prognosis. However, mutations in the same class were not analyzed separately. We hypothesize that additional concurrent mutations in the spliceosome machinery are redundant and not further contribute to prognosis. **Methods:** IRB approval was procured. Departmental next generation sequencing (NGS) databases (with >4000 cases) were queried for hematopoietic malignancies with associated NGS data (Genoptix 5-gene panel, Genoptix 21-gene panel, FoundationOne, Illumina TruSeq Myeloid) and the data analyzed to identify cases of MDS and CMML with spliceosome mutations occurring singly or concurrently with other spliceosome gene mutations (SF3B1, SRSF2, U2AF1, ZRSR2, U2AF2). Overall survival was determined by chart review. Fischer's exact test and unpaired t-test was performed for statistical analysis. **Results:** The number of MDS or CMML patients with single mutations were as follows: SF3B1 only (35), SRSF2 (3), U2AF2 only (8). 1/46 (2%) of patients in this group transformed to acute myeloid leukemia (AML). Cases with more than one spliceosome mutation were as follows: SF3B1 SRSF2 (7), SF3B1 U2AF1 (1), SF3B1 ZRSR2 (3), SRSF2 U2AF1 (2), SRSF2 ZRSR2 (1), U2AF1 ZRSR2 (3). 2/13 (15%) of patients in this group progressed to AML ( $p=0.12$ ). Overall survival in the single mutation group was 40.3 months vs. 33.1 months in the concurrent mutations group ( $p=0.5$ ; 95% CI -14.09 - 28.63) based on available OS data. **Conclusions:** Although the single spliceosome mutation group showed a trend for better prognosis and lower probability for transformation to AML, the differences were not statistically significant. Our data suggest that a single spliceosome mutation obviates the detrimental effect of additional changes in the same pathogenic category. Our data set contributes toward a broader understanding of the effect of multiple concurrent driver mutations in MDS.

## Infectious Diseases

**ID001. Development of a Real-time PCR Assay for the Direct Detection of *Mucorales* Species**K.D. Tardif<sup>1</sup>, K.E. Hanson<sup>2</sup><sup>1</sup>ARUP Laboratories, Salt Lake City, UT; <sup>2</sup>University of Utah, Salt Lake City, UT.

**Introduction:** Mucormycosis (previously called zygomycosis) is a serious but rare fungal infection caused by molds belonging to the order *Mucorales*. These molds live throughout the environment especially in decaying organic matter. Mucormycosis mainly affects people with weakened immune systems or following trauma and can occur in nearly any part of the body. Culture and tissue histopathology are the diagnostic reference methods, but these approaches have significant limitations. We developed a real-time PCR assay for the detection of *Mucorales* in body fluid, respiratory, serum, and tissue specimens. **Methods:** DNA from serum specimen was extracted using the Chemagic platform (Perkin Elmer) and tissue, body fluid, and respiratory specimens were extracted using the Maxwell platform (Promega). To improve extraction efficiency, all specimens were subjected to vigorous beat beating using a FastPrep-24 instrument and Lysing E Matrix (MP Biomedicals) prior to extraction. A DNA internal control was co-extracted with each sample to monitor nucleic acid extraction and PCR inhibition. Real-time PCR amplification was performed on the QuantStudio (ThermoFisher) using Pleiades hybridization probes for *Mucorales* species and an internal control. The assay also uses 2 *Mucorales* specific primers (one limiting and one in excess). The *Mucorales* real-time PCR assay targets the 18S gene in the most (>90%) common genera associated with *Mucorales* infections (*Cokeromyces*, *Syncephalastrum*, *Rhizomucor*, *Lichtheimia*, *Apophysomyces*, *Cunninghamella*, *Mucor*, *Rhizopus*, and *Saksenaia*). **Results:** Analytical sensitivity of the real-time PCR assay was determined to be  $\leq 100$  copies per reaction using organism spiked into patient specimen. The assay accurately identified 40 serums, 37 body fluid, 40 respiratory and 37 tissue specimens spiked with *Mucorales* species for an agreement of 100%, 95%, 100%, and 93%, respectively. The specificity of the real-time PCR assay was tested with thirty-one different organisms and, in all cases, the results were negative. **Conclusions:** We developed a real-time PCR assay for *Mucorales* that is analytically accurate, sensitive, and specific. A potential benefit of a molecular diagnostics for *Mucorales* is the rapid turnaround time relative to standard methods. Shorter time to detection and identification is essential for guiding optimal antifungal therapy, which may in turn help reduce the high mortality rates associated with mucormycosis.

**ID002. Comparison of the Roche Cobas Ampliprep/Cobas Taqman v2.0 and Cobas 6800 for HIV, HCV, HBV and CMV Viral Load Determination**T.R. Sundin, D. Seidman, M. Aunchman, L. Grissom, J. Inman, P.A. Mollica, R. Post, A. Shean, K. Slaughter, S. Nasim  
Sentara Healthcare, Norfolk VA.

**Introduction:** Viral load testing is generally performed to monitor patients previously diagnosed with an infection to guide treatment and observe responses to treatments. As a result of the repetitive nature of the testing, experimental consistency to ascertain viral load is essential. The Roche cobas 6800 offers identical chemistry and a similar test menu as the cobas Ampliprep/cobas Taqman v2.0, including HCV, HIV, HBV and CMV viral load assays. However, unlike the cobas Ampliprep/cobas Taqman v2.0, the cobas 6800 offers primary tube-to-answer testing. This improvement decreases potential for preanalytical error inherent in sample transfer from the primary tube to a secondary container. **Methods:** Accuracy of the cobas 6800 instrument was evaluated using patients previously analyzed by the cobas Ampliprep/cobas Taqman v2.0 and commercially available standards (HCV n=99; HBV n=90; HIV n=103; CMV n=144). Linearity was assessed using commercially available standards with 5-7 panel members. Limit of detection (LOD) was challenged using 20 replicates of commercially available material diluted to near the stated LOD for each assay. Precision was evaluated using 2 levels of controls for 10 days for each assay. Lean assessment was performed to determine the benefits of

implementing cobas 6800 workflow. **Results:** All assays (HIV, HCV, HBV, and CMV) produced an average of less than 0.2 log difference between the cobas Ampliprep/cobas Taqman v2.0 and the cobas 6800 result (HBV 0.05 log<sub>10</sub> IU/mL; HCV -0.13 log<sub>10</sub> IU/mL; HIV -0.17 log<sub>10</sub> copies/mL; CMV -0.13 log<sub>10</sub> IU/mL). Differences in results exceeding 0.5 log<sub>10</sub> were rare, occurring in less than 4% of samples (CMV 3.5%; HIV 1.9%; HBV 1%, HCV 0%). All assays were determined to be linear ( $R^2 > 0.99$ ). LOD for all assays was successfully verified by producing a positive result 95% of the time. Each assay was determined to be precise, with percent coefficient of variation (CV) below 7% across both high and low precision standards. **Conclusions:** The cobas 6800 offers equivalent performance to the cobas Ampliprep/cobas Taqman v2.0 for HIV, HBV, HCV and CMV viral load monitoring. The cobas 6800 reduces the amount of technologist time utilized by 3.5 hours per day by eliminating 70% of the process steps required for the cobas Ampliprep/cobas Taqman v2.0 workflow including 4 steps prone to error.

**ID003. Evaluation of a Commercial Sample-to-Answer Assay for the Detection of Varicella-Zoster Virus Directly from Clinical Specimens**M.J. Espy, N. Jensen, M.J. Binnicker  
Mayo Clinic, Rochester, MN.

**Introduction:** The use of real-time PCR is a sensitive and specific method for the detection of varicella-zoster virus (VZV) in clinical specimens. To date, most laboratory workflows require that specimens first be processed to extract nucleic acids, followed by amplification and analysis by real-time PCR. These processes have traditionally involved separate steps with manual intervention by laboratory technologists, thereby increasing the risk for errors. Recently, Luminex (Luminex Corporation, Austin, TX) has developed VZV analyte-specific reagents (ASR) that can be used in conjunction with the automated ARIES platform (Luminex), which can perform extraction, amplification, and real-time PCR analysis on a single instrument. In this study, we compared VZV detection on the ARIES to routine testing for the qualitative detection of VZV in clinical specimens. **Methods:** One hundred sixty-six specimens (dermal [n=60], genital [n=35], CSF [n=15], ocular [n=16], anal/rectal [n=14], upper respiratory [n=15], and lower respiratory [n=11]) submitted for routine VZV real-time PCR by a laboratory developed test (LDT) were also tested by the ARIES VZV ASR. Testing by the LDT consists of 200  $\mu$ L of sample being extracted on the MagNA Pure (Roche Diagnostics) followed by testing of 5  $\mu$ L of extract using VZV ASR on the LightCycler 2.0 (Roche). An aliquot of each sample (200  $\mu$ L) was also tested by the ARIES VZV assay. Data were analyzed by comparing the ARIES results to those of routine testing, which was considered the reference standard. **Results:** Following testing of the one 166 specimens, the ARIES VZV assay demonstrated a sensitivity of 100% (61/61) and specificity of 99.1% (104/105) for VZV when compared to routine testing by real-time PCR. The one discordant sample was initially positive by ARIES and negative by the routine method; however, this specimen was positive for VZV by the routine test upon repeat testing. Additionally, four specimens were initially invalid by the ARIES system, but on repeat testing, all four generated a valid result by the ARIES assay. **Conclusions:** Testing of clinical specimens by the ARIES VZV assay showed comparable performance to routine testing using a workflow that requires separate nucleic acid extraction, test set-up and real-time PCR analysis. Turnaround time ranged from ~2 h by ARIES to ~3 h by the routine method. Additionally, ARIES requires less hands-on time compared to the reference method (~10 min vs. ~30 min, respectively). The results of this study suggest that the ARIES system may serve as an option for clinical laboratories that are seeking an automated platform that performs all current PCR functions on a single instrument.



#### ID004. Evaluation of a Novel Isothermal Amplification Assay for Detection and Genotyping of Human Papillomavirus in Formalin-fixed Paraffin-embedded Tissue of Oropharyngeal Carcinomas

L. Lozano<sup>1,4</sup>, X. Chen<sup>2</sup>, A. Moreno<sup>1</sup>, A. Torroba<sup>1</sup>, Y. Wang<sup>2</sup>, Y. Tang<sup>3,4</sup>  
<sup>1</sup>Hospital Universitario Virgen de la Arrixaca, Murcia, Spain; <sup>2</sup>Atila Biosystems Inc, Mountain View, CA; <sup>3</sup>Memorial Sloan Kettering Cancer Center, New York, NY; <sup>4</sup>Weill Medical College of Cornell University, New York, NY.

**Introduction:** Human papillomavirus (HPV)-related oropharyngeal squamous cell carcinomas (OPSCC) exhibit prognostic and therapeutic differences with non-HPV-related OPSCC that have prompted the development of new technologies to accurately determine the HPV status in OPSCC in the clinical setting. Atila AmpFire assay incorporates a novel real time fluorescent isothermal amplification technique to detect 15 high risk HPV types and simultaneously genotype HPV 16 and 18 in a single tube reaction. This presentation reports a validation of the AmpFire assay on OPSCC formalin fixed paraffin-embedded (FFPE) samples.

**Methods:** The AmpFire assay detects HPV virus directly from FFPE samples that were heat treated in an Atila lyse buffer for 90 minutes without DNA extraction. The entire procedure takes about two hours from sample to answer. Analytical sensitivity, specificity and reproducibility were determined for 78 FFPE blocks from OPSCC diagnosed at Hospital Universitario Virgen de la Arrixaca between 2005 and 2016. HPV detection and genotyping results were compared with those obtained by the INNO-LiPA HPV Genotyping Extra II (Fujirebio) test which was performed according to the manufacturer's instructions. Discrepant results were resolved by standard MY09/11 consensus PCR followed by sequencing. **Results:** The AmpFire assay's dynamic range of detection was 2 to 2x10<sup>8</sup> copies/reaction. The limit of detection was 20 copies/reaction for the 15 HPV types detected (100%) and 2 copies/reaction for 8 of those HPV types. The reproducibility study yielded concordant results for all replicates. HPV DNA was detected by the AmpFire assay in 17 (20.9%) of the 78 samples, yielding overall, positive and negative percent agreements (OPA, PPA, NPA) of 79.2%, 52.0% and 92.3%, respectively. Four HPV-positive samples which were negative by INNO-LiPA were confirmed positive by the MY09/11 consensus PCR/sequencing, resulting in a final PPA of 58.6% and NPA of 100%. HPV16 accounted for 90% of the genotypes detected. The positive samples yielded a single HPV 16 genotype in all cases except one sample with HPV 35 genotype, and with perfect type-specific agreement between assays. **Conclusions:** The Atila AmpFire assay provides another reliable tool for detection and genotyping of high-risk HPV in OPSCC in FFPE samples. Parameters of simple specimen processing, small sample size requirement, rapid turnaround time and near instrument-free render it well-suited for all kinds of laboratories.

#### ID005. Second Generation Next-generation Sequencing-based System for Detecting Drug Resistance Mutations in HIV-1 Combined with Isothermal Amplification

E.J. Wee<sup>1</sup>, Y. Ng<sup>1</sup>, H. Suhardi<sup>1</sup>, C. Villy<sup>1</sup>, P. Ariyaratne<sup>1</sup>, W. Lye<sup>1</sup>, G.S. Tan<sup>1</sup>, A. Fan<sup>1</sup>, N. Kng<sup>1</sup>, R. Luo<sup>1</sup>, I. Ng<sup>1</sup>, G. Michel<sup>2</sup>, C. Lee<sup>1</sup>  
<sup>1</sup>Vela Diagnostics Singapore Pte Ltd, Singapore; <sup>2</sup>Vela Diagnostics GmbH, Hamburg, Germany.

**Introduction:** Antiretroviral drug resistance testing has become an indispensable tool in the therapeutic management of HIV-infected individuals. Next Generation Sequencing (NGS) technology, which provides excellent accuracy and sensitivity in the detection of low frequency variants as well as mixed infections, is increasingly being used for HIV drug resistance testing in clinical practice. Herein, we developed and evaluated an improved automated NGS-based system for the detection of drug resistance mutations in HIV-1 infection. **Methods:** The Sentosa HIV NGS system comprised of 1) A robotic liquid handling system (Sentosa SX101) for viral RNA extraction, PCR setup and NGS library preparation; 2) Ion Torrent NGS platform; 3) kits for RNA extraction, library preparation, template preparation and sequencing and 4) Data analysis, reporting and interpretation software. While already demonstrated to be useful clinically, improvements have since been made to enhance assay performance. First, we replaced the classical Emulsion-PCR (emPCR)-based template preparation with a rapid Isothermal Amplification (IA)-based method performed on the SX101. Second, we

automated the chip loading on the SX101 aided by a custom fabricated chip holder via a novel "iso-volumetric" method. Third, we worked with SeraCare to develop a Positive Control (PC) using a recombinant non-infectious alpha-virus containing the target HIV nucleic acids and representative drug resistance mutations (DRMs) to control for extraction, amplification and detection. 87 retrospective EDTA plasma clinical HIV-1 samples were evaluated on the improved system. **Results:** By using an IA template preparation method, we reduced the hands-on time for templating/enrichment from 65 min to 20 minutes, and the reaction time from 6 hours to 2.5 hours. The improved Sentosa HIV NGS system now requires less than 1 hour of hands-on time with a total turnaround time of about 26 hours. The assay is able to process up to 22 samples with System Control and PC. The limit of detection (LoD) is 1000 copies/mL on the subtypes (A, B, C, D, F, G, H, J, K) of HIV-1 Group M tested. IA and emPCR had similar templating efficiency in terms of live sequencing beads, final usable sequencing reads, sequencing depth and identical mutation calls when evaluated on three libraries of 22-23 samples. The PC was robust at 5xLoD with all incorporated DRMs detected in all sequencing runs performed. The assay also detected all expected mutations in 87 pre-characterized samples in triplicates. **Conclusion:** The 2<sup>nd</sup> generation Sentosa HIV NGS system provides an automated "sample-to-result" solution for HIV drug resistance monitoring while warranting improved robustness, higher sample throughput and rapid turnaround with minimal hands-on time.

#### ID006. Comparison of Real-time PCR with Transcription Mediated Amplification for HPV Detection/Genotype and Correlation with Cytological and Histological Results

S. McClellan<sup>1</sup>, M. Carter<sup>2</sup>, W. Lebar<sup>2</sup>, D. Newton<sup>2</sup>, P. Lephart<sup>2</sup>, M. Bachman<sup>2</sup>

<sup>1</sup>Michigan Medicine, Sandusky, OH; <sup>2</sup>Michigan Medicine, Ann Arbor, MI.

**Introduction:** Human papilloma virus (HPV) is the most common sexually transmitted infection in the U.S. More than 40 of the 100+ genotypes can infect the genital, anal and oral areas of both males and females and several high-risk (HR) genotypes (16 and 18) are associated with carcinoma of those sites in persistent infection not cleared by the immune system. The most prevalent is cervical cancer which can be detected earlier with co-testing of cytology and molecular methods to improve patient outcomes. The current study sought to compare 2 FDA-cleared nucleic acid amplification assays using automated platforms for detection and genotyping of HPV: DNA using real-time PCR (Roche Cobas HPV) and mRNA using transcription mediated amplification (Hologic Aptima HPV). The results were examined for correlation with cytological and histological findings. **Methods:** The Cobas HPV identifies DNA of 14 HR types reported as 16, 18 or "other" (non 16/18) while Aptima HPV identifies viral messenger RNA (mRNA) reported as 16 and 18/45. Cervical specimens (N=447) in PreservCyt Solution submitted for cytology and HPV DNA testing were also analyzed for HPV mRNA. Negative (N=118) and positive (N=176) HPV DNA specimens were selected for mRNA testing. Additionally, clinical specimens were evaluated in parallel for HPV DNA and mRNA (N=153). Positive DNA specimens included 26 HPV16, 9 HPV18, 125 Other, 12 Other/16 and 4 Other/18. Chart review was performed to obtain concurrent cytology and histology results of colposcopy, where available. **Results:** Agreement of the 2 assays based on positive/negative results was assessed using Cohen's kappa (GraphPad) and found to be 'good' (kappa=0.746; 95% CI: 0.683-0.808). Similar analysis using genotype categories was also classified as 'good' (weighted kappa=0.782; 95% CI: 0.679-0.796). All nine discordant HR16/18 positive cases had NILM cytology with 3 HR16 cases also having negative colposcopy. Cytological diagnosis of 143 Cobas+/Aptima+ cases were: NILM (N=70), ASC-US/ASC-H (N=48), CIN1 (N=19), CIN2-3 (N=3) and unsatisfactory (N=3). The distribution of cytology diagnoses of 47 Cobas+/Aptima- cases were significantly different (chi-square, p=0.0006), showing: NILM (N=39, ASC-US/ASC-H (N=5), CIN1 (N=2), CIN2-3 (N=0) and unsatisfactory (N=1).

**Conclusions:** Good correlation was found between the Cobas and Aptima HPV assays with similar sensitivities, but increased specificity relative to cytological diagnosis. Cobas+/Aptima- cases were associated with significantly more "benign" cytology than concordant positives which is comparable to literature suggesting fewer false positives with

Aptima. Aptima testing may lead to fewer (negative) colposcopic exams, reducing healthcare costs and psychological burden.

#### ID007. The Diagnostic Yield of Universal Pathogen Detection by Next-generation Sequencing Compared to the Standard of Care in Patients with Pneumonia

B.A. Young<sup>1</sup>, H. Xie<sup>2</sup>, D.J. Thomas<sup>2</sup>, T.M. Snow<sup>3</sup>, K.E. Hanson<sup>1</sup>

<sup>1</sup>University of Utah, Salt Lake City, UT; <sup>2</sup>IDbyDNA, Inc., Salt Lake City, UT; <sup>3</sup>ARUP Laboratories, Inc., Salt Lake City, UT.

**Introduction:** Unbiased next-generation sequencing (NGS) can theoretically detect and identify all potential pathogens in a clinical sample, but test performance relative to standard laboratory practice is incompletely understood. NGS performance is determined by 1) the ability to generate sequencing data for pathogens of interest from complex biological matrices and 2) the ability of data analysis software to correctly identify those pathogens. We compared results from a validated NGS protocol to routine testing using bronchoalveolar lavage (BAL) fluid from immunocompromised adults with pneumonia. **Methods:** BAL specimens were tested as a part of usual clinical care by bacterial, mycobacterial, viral, and fungal culture, targeted PCR for *Pneumocystis jirovecii* and atypical bacteria, multiplex PCR for respiratory viruses, and *Aspergillus* Galactomannan. Bacterial culture was reported semi-quantitatively (i.e. 1+, 2+, 3+, 4+) and the growth of normal respiratory flora was recorded. Residual samples were analyzed retrospectively by NGS using the Illumina NextSeq. Each run contained 6 patient BALs and 2 external controls, all spiked with internal controls. A metagenomics analysis tool called Taxonomer was used to analyze DNA and RNA sequences. Results were compared to determine if NGS detected the same organisms identified by routine testing with a focus on possible respiratory pathogens. **Results:** BALs from 48 individual patients were analyzed; 18 (38%) were negative and 30 (62%) were positive by standard testing. NGS detected 16 (59%) of 27 possible pathogens including 5 (56%) of 9 bacteria, 7 (88%) of 8 viruses, and 4 (40%) of 10 fungi. The 4 missed bacteria included methicillin-resistant *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Enterobacter aerogenes*, and *Pseudomonas aeruginosa*. These bacteria, however, were detected by NGS in other samples. Cytomegalovirus (CMV) grew in culture but was negative by NGS. The missed fungi included 5 of 6 total cases of *P. jirovecii* and *Coccidioides immitis*. NGS detected all 3 culture-positive *Aspergillus* specimens. There was no correlation between culture quantity, mixed flora background, or PCR crossing threshold and discrepant results. **Conclusions:** Our preliminary findings suggest that current protocols require further optimization before NGS can replace routine respiratory testing. NGS may be most useful when the results of routine testing are negative or inconclusive and suspicion for infection remains high. Limitations of our study include the small number of samples and use of previously frozen BAL for NGS, which may have affected analytic sensitivity. Future work will evaluate NGS-only detections and adjudicate the clinical significance of organisms detected in culture-negative or PCR-negative samples.

#### ID008. Spectrum Profile of Respiratory Pathogens Detected by the BIOFIRE Plex Assay: Experience of a Major Tertiary Care Center in Lebanon

S. Halabi, S. Abou Daya, S. Damianos, A. Saeed, D. Ghizzawi, T. Chaaban, I. Bou Akl, R. Mahfouz

American University of Beirut Medical Center, Beirut, Lebanon.

**Introduction:** The FilmArray Respiratory Panel produced by BIOFIRE is a self-contained system that can detect simultaneously 10 pathogens: 7 viruses and 3 bacteria directly from respiratory samples. The pathogens are: Adenovirus, Coronavirus 229E, HKU1, NL63, OC43, Human Metapneumovirus, Human Rhinovirus/Enterovirus, Influenza virus A & B, Parainfluenza virus 1, 2, 3, 4, Respiratory Syncytial virus (RSV), *Bordetella pertussis*, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae*. Aim: Investigate the rate of positivity of respiratory pathogens tested by Biofire Plex assay with the co-infection rate among cases. **Methods:** This study will include all cases referred to the Molecular Diagnostics Laboratory at AUBMC for Respiratory Panel analysis from January 1, 2017 till May 25, 2018. It is a review of the laboratory records collected for CAP proficiency and

statistical analysis purposes without any patient clinical information collection, and only analysis of spectrum of positivity of the listed pathogens. **Results:** The spectrum of positivity in our results varies among organisms: Human Rhinovirus/Enterovirus (90 cases, 25.6%), Adenovirus (38 cases, 10.8%), Influenza virus A & B (27 cases, 7.7%), Respiratory Syncytial virus (RSV) (23 cases, 6.5%), Human Metapneumovirus (22 cases, 6.3%), Coronavirus (20 cases, 5.7%), Parainfluenza virus (11 cases, 3.1%), Mycoplasma Pneumonia (3 cases, 0.85%), *Bordetella pertussis* (1 case, 0.3%) and *Chlamydia pneumoniae* (0). There is a high co-infection rate among cases. 6 cases had Coronavirus with Human Rhinovirus/Enterovirus (1.7%), 5 cases had Adenovirus with Human Rhinovirus/Enterovirus (1.4%), 3 cases had Human Metapneumovirus with Human Rhinovirus/Enterovirus (0.85%), 3 cases had Influenza virus with Human Rhinovirus/Enterovirus (0.85%), 3 cases had RSV virus with Human Rhinovirus/Enterovirus (0.85%), 2 cases had Adenovirus with Parainfluenza virus (0.57%), 2 cases had Adenovirus with Influenza virus (0.57%), 1 case had Human Metapneumovirus with Adenovirus (0.3%), 1 case had Coronavirus with Adenovirus (0.3%), 1 case had RSV with Influenza virus (0.3%), 1 case had RSV with Adenovirus (0.3%), 1 case had RSV with Human Metapneumovirus (0.3%), 1 case had Human Rhinovirus/Enterovirus with *Mycoplasma pneumoniae* (0.3%), 1 case had Human Rhinovirus/Enterovirus with Parainfluenza virus (0.3%), 1 case had Coronavirus with Influenza virus (0.3%), 1 case had Coronavirus with Human Rhinovirus/Enterovirus and Adenovirus (0.3%), and 1 case had Human Rhinovirus/Enterovirus with Adenovirus and Human Metapneumovirus (0.3%). **Conclusion:** This study is of extreme importance in analyzing the rate of positivity of different respiratory pathogens, in addition to the rate of co-infection. It is the first as well from our country.

#### ID009. Evaluation of Cobas HBV, HCV, and HIV-1 Tests on the Cobas 6800 Platform

M.K. Leong, S. Capinpin, C. Chai, T. Png, C. Lee, M. Khoo, L. Chiu, N. Tee, B. Yan, H. Lee

National University Hospital, Singapore.

**Introduction:** Monitoring quantities of hepatitis B, C, and human immunodeficiency viral (HBV, HCV, and HIV) genomes in peripheral blood are essential in management of the diseases and selection of a suitable treatment. The quantitative polymerase chain reaction (qPCR) with a wide quantitative dynamic range has been the method of choice to quantitate the viral loads in the clinical setting. Here, the cobas HBV, HCV and HIV Tests (Roche Molecular Diagnostics) on the cobas 6800 platform were evaluated and compared against the existing methods at the National University Hospital (NUH). **Methods:** The cobas HBV, HCV, and HIV-1 Tests were tested using 128, 112, and 87 archived serum samples, respectively. All samples were received at the NUH between September 2015 and March 2018 for routine diagnosis using the existing methods. The existing methods were the artus HBV RG PCR Kit, the COBAS AmpliPrep/TaqMan HCV, and the COBAS AmpliPrep/TaqMan HIV-1 Tests. **Results:** Comparing the new methods with the existing methods, the kappa coefficient scores of 0.935 (95% CI 0.863-1.000), 0.844 (95% CI 0.740-0.947), and 0.472 (95% CI 0.252-0.693) were obtained from the respective sample testings. The percentages of positive and negative agreements were 99% (95% CI 94-100%) and 94% (95% CI 78-99%) for the HBV testing, 90% (95% CI 81-95%) and 100% (95% CI 90-100%) for the HCV testing, and 84% (95% CI 87-91%) and 77% (95% CI 46-95%) for the HIV testing. Intraclass correlation coefficients (ICCs) of the HBV, HCV, and HIV testings for absolute agreement for the average measures of viral loads (in IU/mL) produced by the matching methods were 0.986 (95% CI 0.978-0.991), 0.991 (95% CI 0.983-0.995), and 0.989 (95% CI 0.956-0.996), respectively. The absolute mean differences of the 3 matched testings were 0.100 log, 0.103 log, 0.190 log IU/mL, respectively. **Conclusions:** All new and existing methods have generally produced highly agreeable viral load results (ICCs of more than 0.986), based on samples with quantitative results available from both new and existing assays. The misses by the new methods could be largely attributed to a freeze-thaw cycle of the archived samples, leading to degradation of samples with low viral RNA genomes. Particularly, these phenomena were observed in HIV sample testings, whereby the misses

by the new methods were samples with low viral loads. Noteworthy, HBV and HIV old methods produced 2 and 3 misses (all with low viral load), respectively. This could be attributed to the stochastic error of pipetting on low viral load sample, sample quality, or random error. The absolute mean differences of all matching viral load testings were less than 0.190 log IU/mL. Such difference may not be clinically significant in the patient management.

#### ID010. Performance and Workflow Comparison of Simplexa Bordetella Direct (IUO) with illumigene Pertussis

E.M. Dault<sup>1,2</sup>, A.E. Prada<sup>1</sup>, B.L. Boyanton<sup>1,2</sup>

<sup>1</sup>Beaumont Health, Royal Oak, MI; <sup>2</sup>Oakland University William Beaumont School of Medicine, Rochester, MI.

**Introduction:** *Bordetella pertussis*, a fastidious Gram-negative bacterium, causes whooping cough. Infected individuals are highly communicable during the catarrhal and paroxysmal phases. Rapid, nucleic acid amplification tests are needed for expedited diagnostic and infection control purposes. Herein, we describe the performance and workflow characteristics of the Simplexa Bordetella Direct (IUO) Kit (DiaSorin Molecular) and the FDA-cleared *illumigene* Pertussis Test (Meridian BioScience). **Methods:** A total of 113 individual nasopharyngeal swabs specimens in 1-mL COPAN ESwab media were included. Prospective samples (n=43), collected from April 1 to May 31, 2018 were tested simultaneously by *illumigene* and Simplexa on the same day of service. Archived samples (n=70) were tested by *illumigene* in 2014 (n=30), 2015 (n=9) and 2017 (n=31); these were maintained at -70°C until Simplexa testing. *illumigene* was performed according to the manufacturer's instructions with the addition of a pretreatment step on all samples after March 3, 2016. Simplexa was performed according to the manufacturer's IUO instructions. Discrepant analysis was performed by DiaSorin Molecular in blinded manner using in-house validated PCR plus bidirectional sequencing assays for IS481 and IS1001. Workflow analysis determined the number of procedural steps, hands-on-time, walk-away-time and total assay time for each assay. **Results:** Thirty-two (n=32) and seventy-seven (n=77) samples were positive and negative for *B. pertussis* by both methods, respectively. Four (n=4) samples were incongruent. Three were *illumigene*-negative and Simplexa-positive; discrepant analysis confirmed the presence of *B. pertussis* - these were classified as *illumigene* false negatives. One sample was *illumigene*-positive and Simplexa-negative; discrepant analysis confirmed the absence of *B. pertussis* - this one was classified as an *illumigene* false positive. Sensitivity, specificity, PPV and NPV were: *illumigene* (91.4%, 98.7%, 96.9%, 96.3%); Simplexa (100%, 100%, 100%, 100%). *illumigene* workflow consisted of 22 procedural steps requiring 3.5 minutes (hands-on-time per sample), 77 minutes (total assay time) and 64.4 minutes (walk-away-time). Simplexa workflow consisted of 14 procedural steps requiring 1.5 minutes (hands-on-time per sample), 78 minutes (total assay time), and 71 minutes (walk-away time). The maximum number of samples per run was 8 and 10 for Simplexa and *illumigene*, respectively. **Conclusions:** Simplexa yielded superior test performance as compared to *illumigene* for the detection of *B. pertussis*. Both assays could be performed in about 1.25 hours; however, Simplexa workflow was more simplified and required 57% less hands-on-time. Additionally, Simplexa can also detect *B. parapertussis*.

#### ID011. Performance Evaluation of Two Commercial Molecular Assays for Genotyping Hepatitis C Virus

S.L. Mitchell<sup>1,2</sup>, S.B. Laus<sup>3</sup>, C.R. Rinaldo<sup>1</sup>

<sup>1</sup>University of Pittsburgh, Pittsburgh, PA; <sup>2</sup>Children's Hospital of Pittsburgh, Pittsburgh, PA; <sup>3</sup>University of Pittsburgh Medical Center, Pittsburgh, PA.

**Introduction:** Treatment of HCV has dramatically improved with the development of novel "direct acting" antivirals (DAA). DAAs prevent viral replication by inhibiting non-structural HCV proteins and are divided into 4 classes: 3/4A protease inhibitors, NS5A nucleoside polymerase inhibitors, NS5B non-nucleoside polymerase inhibitors, and NS5A inhibitors. Given the high genomic heterogeneity of HCV isolates, successful treatment of HCV infection with DAAs is highly dependent on the specific HCV genotype. Therefore, accurate genotyping of HCV isolates is critical to the proper treatment and management of HCV infection. **Methods:** This research study assessed genotyping results from the GenMark Dx

eSensor XT-8 Research Use Only (RUO) HCVg Direct Test (eSensor) compared to the Abbott m2000 real-time qualitative HCV Genotype II assay (Abbott), which served as a reference method. 48 residual clinical EDTA blood plasma samples were tested, comprised of 20 negative and 22 positive for HCV genotypes 1a, 1b, 2 and 3. Six samples that were untypable by the Abbott assay were included. Discrepant analysis was performed by a reference clinical laboratory. Performance characteristics of percent agreement, precision, specificity, limit of detection and turn-around-time (TAT) were evaluated. **Results:** 100% positive agreement and negative agreement were observed in samples that produced a genotype and 95% positive agreement and 100% negative agreement in samples that produced a subtype were observed for eSensor compared to Abbott, with an overall accuracy of 95% (41/43) for subtyping and 100% accuracy (43/43) for genotyping. Seven discrepant results were found, which included the five samples that were untypable by Abbott but were typed by eSensor as genotypes 1a, 2a/c, 2b and 3 (N=2). The other two samples were correctly genotyped by Abbott as 1 and 2, but eSensor was able to further subtype into 1b and 2b, respectively. The eSensor was 100% precise within and between runs at 500 IU/mL and 100% specific. The LOD of both assays were comparable, with a range of 100-500 IU/mL for Abbott compared to 125-500 IU/mL for eSensor, however, the LOD was variable by genotype. The TAT of eSensor was approximately 5 hours, which was comparable to Abbott (6 hours). **Conclusions:** The eSensor assay is a highly sensitive and accurate method for genotyping HCV and provides better typing and subtyping resolution of HCV isolates compared to the Abbott assay. While the TAT between the methods are similar, the additional flexibility of the eSensor to run non-batched samples allows for more rapid genotyping of HCV isolates, which can result in quicker administration of more appropriate treatment for patients with HCV infection.

#### ID012. Comprehensive Solid Tumor Microbiome Profiling via Analysis of Unmapped Reads in Large Panel, Hybridization Capture-based NGS Assay Data

C.M. Vanderbilt, S. Middha, A.S. Bowman, M.E. Arcila, E. Babady, S. Dogan, M. Ladanyi, J.F. Hechtman, A. Zehir

Memorial Sloan Kettering Cancer Center, New York, NY.

**Introduction:** Comprehensive characterization of the tumor microbiome has, to date, not been attempted on large-scale, next generation sequencing (NGS) data in conjunction with tumor molecular profiling. Many tumor-microorganism relationships have been described. Viruses are known to be important in both the prognosis and the management of cancer patients. Recently, experimental models have also shown bacterial colonization can play an important role in the treatment response of tumors. Here, we investigate an efficient and cost-effective pan-cancer technique for characterizing tumor microbiomes.

**Methods:** We used data generated from over 25,000 cancer samples using MSK-IMPACT, our institution's clinical genomic profiling assay for solid tumors. In this retrospective analysis, we use the sequencing reads that do not map to the human genome (hg19) during the bioinformatics analysis. We aligned these unmapped reads to the NCBI NT database using the BLAST algorithm to find known bacteria and virus DNA sequences in the tumor samples. We chose well-described, oncogenic microorganisms that have known clinical associations and validated orthogonal tests to elucidate the accuracy of the method. **Results:** Every tumor sample tested had a small component of reads that did not map to the human genome: approximately  $10^3$  to  $10^4$  reads out of  $10^6$  to  $10^7$  reads sequenced per sample were unmapped. These reads mapped to a diverse array of bacteria and viruses in all samples. Among viruses, hepatitis B virus, Merkel cell polyoma virus, and human papillomavirus (HPV) were enriched in the expected tumor types (hepatocellular carcinoma, Merkel cell carcinoma, and squamous cell carcinomas, respectively). Current clinical assays for high risk HPV commonly use in situ hybridization (ISH) on tissue sections. We retrospectively evaluated results of clinical HPV ISH testing and found 95% (119/125) concordance with our method; sensitivity and specificity were 90% and 99%, respectively. For bacteria, *Fusobacterium* sp. is known to be associated with colon cancer (published frequency of 13% by reference PCR methods). In the MSK-IMPACT cohort, 19% (314/1622) of colon cancer cases had *Fusobacterium* sp. reads, suggesting that our method



performed similarly to reference PCR methods. **Conclusions:** Our study suggests that unmapped read analysis using data generated by large panel, hybridization capture-based NGS assays may represent a cost and tissue efficient approach to tumor microbiome profiling. Further validation studies and exploratory analyses are ongoing.

#### ID013. Evaluation of the DiaSorin Molecular Simplexa Bordetella Real-time Sample-to-Result PCR Test on the LIAISON MDX System

T.E. Schutzbank<sup>1</sup>, T. Her<sup>2</sup>

<sup>1</sup>Quantigen, Fishers, IN; <sup>2</sup>St. John's Hospital and Medical Center, Grosse Pointe Woods, MI.

**Introduction:** *Bordetella pertussis* (*B. pertussis*), the etiologic agent for whooping cough, remains a public health issue despite widespread vaccination of most children in the U.S. This is due to the failure of some parents to vaccinate their children, as well as waning immunity in the vaccinated population typically between the ages of 11 to 18 years. The very contagious nature of *B. pertussis* is responsible for localized community outbreaks of whooping cough requiring the need for a rapid, highly specific and sensitive test. DiaSorin Molecular has developed a "sample to answer" test for *Bordetella pertussis* and *Bordetella parapertussis*, based on TaqMan PCR technology, for their LIAISON MDX platform. The instrument utilizes an 8 well direct amplification disc into which the patient sample is added. Once placed into the LIAISON MDX instrument, sample processing and PCR analysis are fully automated. The purpose of this study was to determine the suitability of the LIAISON MDX platform for detection of *B. pertussis*/*parapertussis* DNA directly from patient samples compared to our current method utilizing Luminex MultiCode B.

*pertussis*/*parapertussis* reagents on the Luminex ARIES platform.

**Methods:** Testing for *B. pertussis*/*parapertussis* on the LIAISON MDX platform using the DiaSorin Molecular Simplexa Bordetella Real-Time Sample-to-Result PCR Test was performed using 50 µL of patient sample (nasopharyngeal swabs in M4 transport media) as per the manufacturer's instructions. MultiCode primers for *B. pertussis* and *B. parapertussis* PCR and ARIES test cassettes were obtained from Luminex. Testing was performed using 200 µL of patient sample according to standard instrument settings supplied by Luminex using their proprietary SYNCT software. **Results:** We tested 90 clinical samples submitted to our laboratory for *B. pertussis*/*parapertussis*. All samples that tested positive with the ARIES platform agreed with the LIAISON MDX results. All samples resulting as negative on the ARIES agreed with the LIAISON MDX results with one exception; a sample that was negative on the ARIES for *B. pertussis* was positive on the LIAISON MDX. This sample was weakly positive with a Ct value of 39.7. Upon repeat the negative results were confirmed. **Conclusions:** 1) The LIAISON MDX system is a robust, simple to use, sample to result platform. 2) The performance of the Simplexa Bordetella assay on LIAISON MDX system compared very favorably to results obtained using the MultiCode *Bordetella* reagents on the ARIES platform. 3) A major advantage of the LIAISON MDX method is the elimination of all upfront sample processing steps, significantly decreasing hands-on time, and greatly enhanced workflow, resulting in decreased turnaround time to a final result.

#### ID014. Evaluation of Panther Fusion System for Respiratory Viral Detection in a Pediatric Hospital

A. Rector<sup>1</sup>, D. Cuevas<sup>1</sup>, R. Webb<sup>1</sup>, J. Dunn<sup>2</sup>

<sup>1</sup>Texas Children's Hospital, Houston, TX; <sup>2</sup>Baylor College of Medicine, Houston, TX.

**Introduction:** The Panther Fusion (Hologic, Inc., San Diego, CA) respiratory assays are modular, multiplex real-time PCR (RT-PCR) diagnostic tests used in conjunction with the Panther Fusion System for the differentiation of influenza A (FluA), influenza B (FluB), respiratory syncytial virus (RSV), parainfluenza 1-4 viruses (P1-4), human Metapneumovirus (hMPV), Adenovirus (ADV), and human Rhinovirus (hRV) in less than three hours. Within the fully automated system, samples are lysed, nucleic acid is captured and transferred to the reaction tube containing the assay reagents, and RT-PCR is performed using TaqMan RT-PCR. We evaluated the performance of the Panther Fusion to detect respiratory viruses in 5 different specimen types. **Methods:** A total of 284 respiratory samples including 105 nasal washes (NW), 80

nasal/nasopharyngeal swabs (NP), 55 bronchoalveolar lavages (BAL), 42 tracheal aspirates (TA), and 2 sputums (SP) were evaluated. Results were compared to those obtained with the ProFlu+, ProAdeno+, ProParaflu+, ProhMPV+ assays (Hologic, Inc.) and a lab-developed RT-PCR test (LDT) for hRV. A subset of 135 specimens (29 NW, 28 NP, 40 BAL, and 38 TA) were also tested using the ePlex Respiratory Pathogen (RP) Panel (GenMark Diagnostics, Inc., Carlsbad, CA). Discordants were tested on the FilmArray Respiratory Panel v1.7 (Biofire Diagnostics LLC, Salt Lake City, UT). **Results:** A total of 292 respiratory viruses were identified. The Panther Fusion showed an overall agreement of 99.7% (291/292). Percent positive agreement for each target was 100% for FluA (41/41), FluB (19/19), RSV (32/32), P1 (23/23), P2 (17/17), hMPV (54/54), ADV (39/39), and hRV (29/29), and was 96.9% (31/32) for P3. Four additional positives (3 hRV and 1 P3) were identified by the Panther Fusion but negative by ProParaflu+ or hRV LDT; all were confirmed by FilmArray. Compared to the ePlex RP, the Panther Fusion detected 2 additional P3, 1 ADV and 4 hMPV; all confirmed by in-house testing and/or FilmArray. Panther Fusion and ePlex assays correctly identified P4 in 6 specimens. **Conclusion:** The Panther Fusion System may be a viable alternative to the current batch-testing, real-time PCR assays for respiratory viruses offered at Texas Children's Hospital. The Panther Fusion is a relatively easy to perform, sample-to-answer platform with minimal hands-on time of ~10-15 minutes for approximately 30 samples. With a run time of <3 hours, this random and continuous access platform could enable same-day results not routinely available with our current high-complexity, batch-testing workflows. The flexibility of the 3 modular assays also allows for an algorithmic, reflexive approach to respiratory virus testing.

#### ID015. Adjusting the pH of Urine Samples at the Time of Collection May Provide a More Accurate Measurement of Cytomegalovirus (CMV) Viral Load

M. Galdzicka, J. Strong

Quest Diagnostics, Marlborough, MA.

**Introduction:** The use of molecular diagnostics methods to measure CMV viral load in urine samples is well established, but storing samples leads to degradation of nucleic acid and decreased accuracy. Degradation of nucleic acid is likely caused by nucleases, the activity of which depends on pH. In this study, we investigated the effect of pH on viral load and if changing the pH protects nucleic acid from degradation. **Method:** Three experiments are described. 1) To test the effect of pH on stability of CMV nucleic acid in urine samples, normal samples were divided into 7 aliquots and pH was adjusted to 3, 4, 5, 6, 7, 8, and 9. Three aliquots of each pH were spiked with CMV to obtain 3 concentrations. The samples were stored at -80°C before testing. 2) To test the effect of storing urine samples at room temperature (RT), urine samples with the same concentration of CMV at 7 pH values (3-9) were stored at RT for 1, 6, 12, and 24 hours before storing at -80°C and testing. 3) To test the effect of modifying pH during sample preparation (mimicking time of collection), 2 sets of samples were prepared at 7 pH values with the same viral load. One set was tested immediately after preparation, while a second set was spiked with Tris/EDTA buffer pH 8, stored at -80°C, and then tested.

**Results:** When samples were stored at -80°C immediately after preparation, the highest viral load was in samples with low pH (3 and 4) and high pH (8 and 9). The lowest viral load was in samples with pH 6. Storage at RT of urine samples with pH 6 for 1, 6, or 12 hours (and 24 hours) led to decreased viral load of at least 1, 2, or 3 log, respectively. Viral load measurements from samples at pH 3, 8, or 9 were stable when stored at RT for 24 hours. The average log<sub>10</sub> viral load was comparable for samples tested immediately after preparation and samples spiked with Tris/EDTA buffer during preparation but before storage at -80°C; the difference was only 0.02 (range -0.12 to 0.1). **Conclusion:** Urine samples with a low or high pH had better recovery than those with pH 6, suggesting that pH levels affect stability of the viral nucleic acid in urine. Addition of Tris/EDTA buffer to urine samples helped preserve the original viral load. DNase I, the main nuclease in human urine, is active in the presence of divalent ions; optimum pH is ~6.5. Our results suggest that by adjusting the pH to 8 and chelating divalent ions with EDTA, degradation of nucleic acid was stopped. Thus, adding Tris/EDTA during urine sample collection could provide more accurate viral load results.

**ID016. Detection of Herpes Simplex Virus (HSV) Types 1 and 2 and Varicella-Zoster Virus (VZV) From Cutaneous and Mucocutaneous Lesions Using the Quidel Solana HSV 1+2/VZV Assay**

E. Tam<sup>1</sup>, C. Webb<sup>1</sup>, J. Dunn<sup>1,2</sup>

<sup>1</sup>Texas Children's Hospital, Houston, TX; <sup>2</sup>Baylor College of Medicine, Houston, TX.

**Introduction:** The Solana HSV 1+2/VZV Assay (Quidel, San Diego, CA) is a moderately-complex, *in vitro* diagnostic test that detects and differentiates HSV-1, HSV-2, and VZV from active cutaneous or mucocutaneous lesions. The assay isolates and purifies viral DNA followed by isothermal, helicase-dependent amplification using target-specific primers and fluorescent probes. Up to 12 samples can be run simultaneously with results available in <1 hour. In this study, we evaluated the performance of the Solana HSV 1+2/VZV Assay at Texas Children's Hospital (TCH) using samples obtained from vesicular skin and genital lesions. **Methods:** A total of 67 unique patient specimens and 1 CAP ID1 survey challenge were evaluated, including 65 pediatric samples (57 retrospective and 8 prospective) from TCH and 2 prospective adult samples. Results of Solana testing were compared to culture using H&V Mix shell vials (Quidel, San Diego, Ca) stained for the presence of virus using Light Diagnostics SimulFluor HSV/VZV reagent (MilliporeSigma, Burlington, MA). Analytical sensitivity studies included replicate testing of quantified viral material of HSV-1, HSV-2 (Zeptomatrix, Buffalo, NY) and VZV (Qnostics, Shirley, NY) diluted to varying concentrations in M4RT transport media. Analytical specificity studies included 10 specimens contrived with other viral, bacterial and fungal targets. Precision/reproducibility studies were performed using a panel of 4 contrived specimens (1 each of HSV-1 positive, HSV-2 positive, VZV positive and negative samples) tested by 5 different technologists.

**Results:** The overall agreement of the Solana assay compared to culture was 100% (68/68), with a percent positive agreement of 100% for HSV-1 (12/12), HSV-2 (14/14) and VZV (6/6). The limit of detection with a 100% detection rate was 50 copies/reaction for HSV-1, 10 copies/reaction for HSV-2 and 100 copies/reaction for VZV. Analytical specificity and precision/reproducibility were both 100%. **Conclusion:** The Solana HSV 1+2/VZV Assay is an effective platform to qualitatively detect HSV-1, HSV-2, and VZV from vesicular skin and genital lesions in symptomatic patients. This moderately complex assay is easy to set up with approximately 20 minutes of hands-on time and a run time of <1 hour; thus enabling results in an actionable time frame without the need for highly specialized molecular testing capabilities.

**ID017. Efflux Gene Expression by Ofloxacin Stress in Multidrug-resistant *Mycobacterium tuberculosis* and Extensively Drug-resistant *M. tuberculosis* with/without *gyrA* Mutation using RNA-seq**

H. Lee, H. Kang, Y. Kim, Y. Han, S. Kim, S. Cho

Kyung Hee University School of Medicine, Seoul, South Korea.

**Introduction:** Tuberculosis remains leading cause of mortality. Drug resistance of *Mycobacterium tuberculosis* is a major problem in the world's healthcare and much of the mechanism of resistance in secondary drugs used in MDR and XDR tuberculosis remains unknown. An efflux pump could be an explanation for many drug resistances; however, the relationship between the expression of genes and phenotypic resistance is complicated due to the post-translational regulatory network. In this study, we investigated differences in gene expression levels according to ofloxacin stress in MDR and XDR *M. tuberculosis* with/without associated mutations. **Methods:** Two ofloxacin resistant *M. tuberculosis* strains with (MDR strain no. M137) or without (XDR strain no. X39) *gyrA* mutation were prepared after single colony isolation. The MIC for ofloxacin of two strains were 8 µg/mL and 2 µg/mL, respectively. *M. tuberculosis* H37Rv was also prepared as reference. McFarland No.1 suspension of MTB strains were incubated in 7H9 broth for 24 hours with 0%, 25% and 50% of MIC of ofloxacin on the shaker. RNA extraction was performed on the culture medium and RNA seq was performed with Illumina HiSeq 4000 through purification and library preparation using the Illumina TruSeq RNA Sample Preparation Kit. RNA-Seq reads were mapped to the genomic DNA reference (GCF\_000195955.2) using a Bowtie aligner and the expression level of each gene was calculated using the reads per kilobase per million mapped reads (RPKM) method. Significantly differentially expressed genes (fold change ≥2) were screened based on their

normalized value after Log2 transformation. KEGG PATHWAY Database and volume plot using the R program were used for comparison.

**Results:** A total of 3,946 genes were analyzed in this study. In the comparisons between X39 without ofloxacin stress and H37Rv, Rv2936 (*drdA*), Rv2937 (*drdB*) and Rv2938 (*drdC*), all showed more than 2 fold change. The top 12 genes showing significant fold change were selected. When comparing X39 with drug stress (25% and 50% of MIC) with H37Rv, down-regulation (fc<-2) was showed in Rv2846c (*efpA*), whereas up-regulation (fc≥2) was showed when comparing M137 with drug stress (0%, 25% and 50% of MIC) with H37Rv. **Conclusions:** Our study showed significant changes in Rv2846c (*efpA*) gene expression according to ofloxacin stress in *M. tuberculosis* with/without *gyrA* mutation. Rv2936 (*drdA*), Rv2937 (*drdB*), Rv2938 (*drdC*) expression changes observed in X39 without mutation. Compared with X39 without mutation showed more changes than M137 with *gyrA* mutation. It suggests when there is no chromosomal resistance gene, an efflux pump is more deeply related to the resistance mechanism. It may be a helpful finding to identify a new resistance mechanism.

**ID018. Triplex Assay for Zika, Dengue, and Chikungunya Viruses by Sentosa SA Real-time RT-PCR Assay**

J. Wong<sup>1</sup>, A. Soh<sup>1</sup>, G. Tan<sup>1</sup>, Y. Ng<sup>1</sup>, H. Suhardi<sup>1</sup>, M. Jansen<sup>2</sup>, P.

Rooney<sup>2</sup>, W. Lye<sup>1</sup>, R. Luo<sup>1</sup>, E. J. Wee<sup>1</sup>, G. Michel<sup>3</sup>, C. Lee<sup>1</sup>

<sup>1</sup>Vela Diagnostics Singapore Pte Ltd, Singapore; <sup>2</sup>Vela Diagnostics USA Inc., Fairfield, NJ; <sup>3</sup>Vela Diagnostics GmbH, Hamburg, Germany.

**Introduction:** Zika (ZIKV), Dengue (DENV) and Chikungunya (CHIKV) are *Aedes* mosquito-borne viruses that are predominantly endemic in tropical regions of the world, having similar epidemiology and clinical symptoms at onset. Their increased incidence, expanding geographical range and serious health threats has raised global concerns. Here, we developed a real-time RT-PCR assay for the simultaneous qualitative detection and differentiation of ZIKV, DENV and CHIKV RNA in clinical specimens from EDTA plasma, serum and urine (only for ZIKV) during the acute phase of infection. **Method:** The Sentosa ZIKV-DENV-CHIKV assay runs on an automated workflow for nucleic acid extraction, PCR set-up, detection on the ABI 7500 Fast Dx qPCR platform (Sentosa SA201) and reporting (Sentosa SA201 Reporter). The assay contains primers/probes for the amplification and detection of specific targets in ZIKV (FAM), DENV1-4 (ROX) and CHIKV (VIC). To establish analytical performance, virus stocks of 2 ZIKV strains, 4 DENV subtypes and 1 CHIKV strain were spiked into respective plasma, serum and urine matrices at concentrations. To assess competitive inhibition of target extraction and detection in cases of co-infections, combinations of low and high viral loads with 250 copies/mL up to 1,000,000 copies/mL were spiked into samples. For clinical validation, clinical samples (225 ZIKV, 141 DENV and 100 CHIKV samples in various matrices) are to be tested with the assay, and compared with the RealStar Zika Virus RT-PCR Test (for ZIKV) and FTD Zika/Dengue/Chik assay (for DENV and CHIKV). **Results:** We established that the 95% analytical limit of detection (LoD) for ZIKV PRVABC59, ZIKV MR766, CHIKV, DENV1, DENV2, DENV3 and DENV4 is 500cp/mL, 980cp/mL, 169cp/mL, 59cp/mL, 83cp/mL, 70cp/mL and 250cp/mL, respectively. The assay also had no cross-reactivity with other vector-borne pathogens such as West Nile virus, Yellow Fever virus, Parvovirus, *Plasmodium*, among others. Results of the co-infection study showed that the assay was able to detect co-infections efficiently, comparable to single target specimens. The assay was also 100% reproducible when tested with contrived samples at 3xLoD. Clinical validation is in progress. **Conclusion:** We have developed a robust and highly sensitive automated assay to detect ZIKV, CHIKV and DENV in a variety of clinical specimens in a single test run.

**ID019. A Host Gene Signature for Diagnosis and Risk Stratification of Acute Infection and Sepsis at Hospital Admission: HostDx Sepsis**

A. Liesenfeld<sup>1</sup>, J. Romanowsky<sup>1</sup>, L. Buturovic<sup>1</sup>, W. Nie<sup>1</sup>, M. Eshoo<sup>1</sup>, P.

Khatir<sup>2</sup>, T.E. Sweeney<sup>1</sup>

<sup>1</sup>Inflammatix Inc., Burlingame, CA; <sup>2</sup>Stanford University, Palo Alto, CA.

**Introduction:** Acute infections and sepsis, as leading causes of morbidity and mortality, represent a major burden to healthcare systems. In the United States, an estimated 15 million patients are assessed each year for acute infections and sepsis in emergency departments (EDs). Diagnostic

procedures to evaluate patients with suspected acute infections or sepsis in the ED are inaccurate or slow. Analysis of host-response signatures using RNA expression has been described for both diagnosis and risk stratification of patients with acute infections or sepsis. We here describe the development of HostDx Sepsis, a 30-host-gene PCR test that identifies i) the presence of an infection, ii) the type of infection (viral or bacterial), and iii) the severity of the infection using whole blood collected in PAXgene RNA tubes. The HostDx Sepsis test is being developed as a cartridge-based, sample-to-answer, quantitative assay with turn-around time of less than 60 minutes. **Methods:** To identify gene signatures specific for the presence, type and severity of infection, we analyzed publicly available microarray and next generation sequencing (NGS) gene expression data sets from cohorts of children and adults with community- and hospital-acquired infection and sepsis. We discovered gene sets that can distinguish between infections and non-infectious inflammation, between viral and bacterial infections, and that can predict the severity of infection. Gene signatures were validated in 38 independent cohorts (total N=2,452) to establish clinical performance. **Results:** Validation performance for the presence of any bacterial infection in a hospital population showed a 94% sensitivity and 60% specificity (99% negative predictive value at 15% prevalence). The mean area under the receiver operator characteristics curve (AUROC) was 0.88 for prediction of 30-day mortality, markedly improving AUROCs for laboratory parameters and/or clinical scores including lactate and SOFA. To demonstrate proof of feasibility, a 7-gene subset signature distinguishing between viral and bacterial infections was successfully converted to a rapid multiplex PCR assay format, with correlation of 0.95 to a NanoString standard based on digital detection and quantification of unique transcripts. **Conclusions:** Emergency physicians currently rely on a battery of tests with low accuracy to diagnose acute infections and sepsis. The host-response signatures described has demonstrated high diagnostic and prognostic accuracy in numerous independent cohorts. As a rapid triage assay HostDx Sepsis could allow for improved decision making for antibiotics, downstream testing, and level-of-care decisions.

#### ID020. Development and Validation of a Quantitative Multiplex Real-time PCR Assay for Identification of Bacterial Pathogens From Respiratory Specimens

A. Seth, R. Chandra

Accureference Medical Laboratory, Linden, NJ.

**Introduction:** Development of timely and accurate methods for diagnosing bacterial respiratory tract infections has been a challenge for the clinical microbiology laboratory. Currently available commercial respiratory panels do not identify some important bacterial pathogens responsible for lower respiratory tract infections. The objective of this study was to validate a laboratory developed sample to answer multiplex PCR for rapid detection of *Streptococcus pneumoniae*, *Haemophilus influenza* and *Moraxella catarrhalis* from respiratory specimens on the Luminex ARIES platform. The assay was evaluated for its analytical performance characteristics and its utility for patient testing.

**Methods:** Bacterial cultures obtained commercially were used for assay validation. Previously tested positive specimens were obtained through laboratory exchange. The assay target genes *lytA* for *S. pneumoniae*, *fucK* for *H. influenza* and *copB* for *M. catarrhalis* were chosen from previously published literature. The primers and probes were manufactured by IDT. Samples were pretreated with proteinase K followed by extraction and a multiplex PCR on the ARIES instrument using an early access ARIES Exo+ Ready Mix master mix with a user defined program. Analysis of results was performed using the SYNCNT software. **Results:** Standard curves from serial dilutions of pure cultures of *Streptococcus pneumoniae*, *Haemophilus influenza* and *Moraxella catarrhalis* were used for comparison with colony forming units (CFU). Concordance with culture was 100% and the bacterial load was quantified accurately when all three bacterial targets were mixed together in varying concentrations. The crossing threshold values (Ct) were similar in bacterial mixtures when compared to amplifying the target alone. Analytical sensitivity and limit of detection was determined with at least 6 replicates of all dilutions. The assay was linear between 100- 80,000 CFU's per reaction and the limit of detection observed was between 30-100 CFU's/reaction depending on the bacterial target. The assay is highly

reproducible with variation coefficients for replicates in both intra and inter run reproducibility assays being less than 5% for all bacterial targets. Previously determined positive clinical specimens (n=30) obtained from external laboratories were re tested using our lab developed assay. The overall concordance obtained for detection of bacterial pathogens present individually or as mixed infections was 99%. **Conclusions:** This pilot study shows that a quantitative molecular assay developed on the ARIES platform for identification of bacterial respiratory infections has the potential to provide fast and accurate results to enable use of targeted antibiotics and improve patient management.

#### ID021. Evaluation and Time-motion Analysis of the GenePOC Rapid *C. difficile* Assay compared to the Meridian Illumigene Assay

H. Webber, G. Royal, M. Ianosi-Irimie

NorDx, Scarborough, ME.

**Introduction:** *C. difficile* is the causative agent of mild to severe diarrhea and pseudomembranous colitis. It is also the most common nosocomial pathogen in hospitals, leading to about 500,000 infections and nearly \$5 billion per year in hospitalization costs in the United States. *C. difficile* was on the rise from 1990-2000, but the rate of infection has plateaued over the past decade, possibly due to the widespread use of NAA clinical tests. Molecular assays have surpassed immunoassays as the test of choice and are now used in 70% of laboratories. Faster and more reliable detection of the *C. difficile* toxin from soft and liquid stool samples is imperative for treatment of the patient as well as for infection control and prevention. **Methods:** In an effort to reduce hands-on-time and improve performance, we compared our current methodology, the Meridian Illumigene assay (Meridian Bioscience Inc., Cincinnati, OH), to the newly FDA-approved GenePOC CDiff (GenePOC Inc., Quebec, Canada) real-time PCR test. For the GenePOC CDiff assay, preparation and loading of the 8 samples per batch involves a minimal hands-on time of 14 minutes. With the use of microfluidic cartridges (PIEs), specimen processing including lysis, amplification and detection are completely automated on the revogene instrument and results are available after 70 minutes. The Illumigene assay requires 26 minutes of hands-on-time and 50 minutes of instrument time to run 8 samples. **Results:** Four hundred twenty-two stool samples were tested on both the GenePOC CDiff assay (*tcdB*) and our current methodology, the Meridian Illumigene assay (*tcdA*). Discrepant samples were tested on the Quidel Solana assay (*tcdA*). There were 355 total negative samples and 67 total positives. 14 results were found to be discrepant between the GenePOC and Illumigene assays. 5 of the discrepant results were found to be contraindicated for *C. difficile* testing and were thrown out after review of the patient's clinical scenario. Of the remaining 9 discrepant samples, 4 were determined to be false positives on the GenePOC, 2 were false positives on the Illumigene, and 3 were false negatives on the Illumigene (based on repeat testing across 3 platforms and clinical correlation). **Conclusions:** The performance of the GenePOC CDiff assay was equivalent to the Illumigene assay with approximately half the hands-on-time.

#### ID022. Evaluation of the Galileo Pathogen Solution Next-generation Sequencing Pipeline for the Identification and Quantification of DNA Viruses in Transplant Patients

M.L. Carpenter<sup>1</sup>, L.G. Bentley<sup>1</sup>, Q. Dong<sup>1</sup>, V. Nagesh<sup>1</sup>, T. Watson<sup>2</sup>, G. Walz<sup>2</sup>, S.K. Tan<sup>3</sup>, J. Weber<sup>3</sup>, M.K. Sahoo<sup>3</sup>, B.A. Pinsky<sup>3</sup>

<sup>1</sup>Arc Bio, LLC, Menlo Park, CA; <sup>2</sup>Arc Bio, LLC, Cambridge, MA; <sup>3</sup>Stanford University School of Medicine, Stanford, CA.

**Introduction:** Arc Bio is developing the Galileo Pathogen Solution (Galileo), a sample-to-report next-generation sequencing pipeline to enable comprehensive testing for infectious disease and eliminate the need to query specific pathogens independently. The assay incorporates a proprietary step to enrich for pathogen-specific sequence reads, as well as an automated bioinformatics pipeline. In this proof of principal study, we demonstrate the first use of this pipeline for the identification and quantification of DNA viruses in transplant patients. **Methods:** 12 transplant virus linearity panels (11 individual viruses and 9 viruses combined) were prepared to determine linear range using donor EDTA plasma spiked with 10-fold dilutions of standard material (Exact Diagnostics) from 10<sup>2</sup> to 10<sup>5</sup> cp or IU/mL. In brief, total nucleic acid was extracted from 0.4 mL of plasma using the EZ1 platform (QIAGEN),



followed by DNA library preparation with pathogen enrichment/human background depletion, sequencing (NextSeq 500, Illumina), and automated data analysis. Sequencing reads were filtered based on sequence quality and queried against a curated selection of references. In addition, 24 residual EDTA plasma samples previously tested with multiplex qPCR assays (CMV=4, EBV=4, hAdV=4, BKV=4, HHV-6=4, negative=4) were sequenced. **Results:** All panels were linear from  $10^2$  to  $10^5$  cp or IU/mL. At an average sequencing depth of 20 million paired-end (PE) reads for the linearity panels, sensitivity at  $10^2$  cp or IU/mL ranged from 67% to 100% depending on the virus, and at  $10^3$  cp or IU/mL sensitivity ranged from 83% to 100%. For the 24 residual clinical samples at an average of 15 million PE reads, we obtained a positive percent agreement of 100%, with viral loads ranging from <135 (CMV) to 112,813 IU/mL (BKV). An additional 27 potential co-infections were detected by Galileo, and 5 CMV co-infections were confirmed by reviewing patient medical records. Furthermore, the residual clinical samples were accurately quantitated using the standard curve generated from the multiplexed linearity panel. **Conclusions:** This proof of concept dataset suggests the Galileo assay may be a promising solution for the comprehensive detection and quantification of transplant DNA viruses at similar levels of sensitivity to standard-of-care qPCR methods. Further work includes testing additional samples at or around the qPCR lower limit of quantitation, as well as confirming any identified co-infections using orthogonal methods.

#### ID023. Clinical Implications of the Increased Sensitivity of the FDA Roche 6800 CMV Viral Load Assay

J. Pettersson, L. Du, T.I. Long, E. Blodgett, L. Dubeau, P.M. Ward  
University of Southern California, Los Angeles, CA.

**Introduction:** Cytomegalovirus (CMV) infection is a major health issue following organ transplantation. While there is no standardized approach, treatment is stopped at our Institution when a patient's circulating viral load is <137 IU/mL based on the Ampliprep TaqMan test, the lower limit of detection for this assay. The new Roche Cobas 6800 test introduced two modifications that increase its analytical sensitivity compared to the Ampliprep TaqMan assay, one being a lower limit of detection (35 IU/mL) and the second being a shorter amplicon (150bp compared to 350bp with the Ampliprep TaqMan assay). The latter modification raises concerns about capturing fragmented genomic CMV DNA not representative of viable viral particles. We sought to compare test results from both assays in all post-transplant CMV-infected patients over a 5-month period at University of Southern California to better understand the clinical significance of these modifications and better guide therapeutic decisions following our switch from the existing Ampliprep assay to the new Cobas 6800 test. **Methods:** Approximately 100 patients were screened with the Ampliprep TaqMan assay to capture those with active CMV infection and reanalyzed with the Cobas 6800 CMV assay. We compared absolute viral titers in all positive patients and time at which individual patients became negative based on each assay. **Results:** A total of 84 samples from 8 patients with active disease were tested, 48 of which showed results within quantitative range with both tests. There was a significant proportional bias with the Cobas 6800 assay, where CMV results were 67% higher than with the Ampliprep in 80% of the cases (P-value <0.001, Bland-Altman test). Patients remained positive for an average of 10 more days (range of 5-28 days) when tested with the Cobas 6800 assay based on respective lower limit of detection values for each assay. Using 137 IU/mL as stop rule the 6800 assay still extended the positive period by 4 days (median: range 0-21). Using an adjusted 229 IU/mL as cutoff for active disease to account for differences in analytical sensitivity, the 6800 assay extended the remarkable period by a single day. **Conclusion:** The Roche Cobas 6800 test generated higher titer values and increased the period during which patients undergoing treatment remained positive, potentially resulting in longer treatment periods for some patients. The increase in treatment length could be decreased by applying a correction factor accounting for increased sensitivity.

#### ID024. Performance Evaluation of AdvanSure RV-plus Real-time PCR Assays for the Detection of Respiratory Viruses

J. Sohn, J. Cho, E. Lee, G. Oh

Eone Laboratories, Incheon, South Korea.

**Introduction:** The AdvanSure RV real-time PCR kit (AdvanSure; LG Life Sciences, Korea) is based on multiplex real-time PCR and can simultaneously detect 14 respiratory viruses. Newly introduced AdvanSure RV-plus real-time PCR kit (AdvanSure; LG Life Sciences, Korea) can detect 15 viruses including enterovirus, and requires fewer tubes for reaction. We compared the performance of the AdvanSure RV-plus assay with the RV kit (AdvanSure; LG Life Sciences, Korea). **Methods:** The performance of the AdvanSure RV-plus real-time PCR kit was compared with those of the AdvanSure RV using 214 clinical respiratory specimens. Specimens with discordant results between the 2 assays were confirmed by Sanger sequencing. Analytical sensitivity of the RV-plus kit was determined using coronavirus OC43 (CoV OC43), influenza virus A (FluA), parainfluenza virus 3 (PIV3) and adenovirus (ADV) extract. **Results:** AdvanSure RV detected 180 (84.1%) positive cases and RV-plus detected 178 (83.2%) positive cases. The ranges of positive percent agreement, negative percent agreement, and kappa values between the AdvanSure RV and RV-plus assays for each virus were 82.8-100%, 98.1-100%, and 0.82-1.00, respectively. Sequencing for samples with discrepant results demonstrated that the majority of results were concordant with those from the RV-plus assay. The analytical limits of detection of CoV OC43, FluA, PIV3 and ADV were 9.7, 4.0, 8.4 and 1.3 PFU/mL TCID<sub>50</sub>, respectively. **Conclusions:** In conclusion, the AdvanSure RV-plus assay demonstrated comparable performance to AdvanSure RV assay. Thus, the AdvanSure RV-plus assay is a potentially useful tool for detecting respiratory viruses in clinical laboratories where high-throughput batch testing is required.

#### ID025. Quantitative Detection of HCV Using the NeuMoDx Molecular Diagnostic System

J. Zhu, E. Craig, M. Carey, C. Couture, M. Mastronardi, B. Wu, S.

Brahmasandra

NeuMoDx Molecular Inc, Ann Arbor, MI.

**Introduction:** Determining Hepatitis C Virus (HCV) RNA levels in plasma and/or serum is an important tool to characterize viral loads in infected patients to monitor disease progression, efficacy of antiviral therapies, as well as to detect drug resistant mutants and identify relapse upon discontinuation of an antiviral therapy. The NeuMoDx HCV Test is an in-vitro diagnostic assay incorporating a universal nucleic acid isolation chemistry enabling extraction of qPCR ready RNA from serum and plasma specimens, combined with a sensitive quantitative rt-PCR assay to deliver highly accurate results in a completely automated, "random access" manner on the NeuMoDx Molecular System. In addition, all reagents and disposables are room temperature stable and are intended to remain on-board the system to provide a seamless, on-demand testing workflow. **Methods:** The NeuMoDx Molecular System automates and integrates the extraction, purification, quantification, and results interpretation of infectious disease nucleic acid targets using quantitative RT-PCR. The objective of this study was to test and report performance of the NeuMoDx HCV Test in key analytical performance metrics. Internal pre-analytical studies were performed to characterize the analytical sensitivity, linearity, precision, inclusivity, turnaround time, as well as characterizing quantitative correlation to a reference test using split samples. The results of these studies are presented here. **Results:** The NeuMoDx HCV Test showed a detection limit of 7.4 IU/mL (95% CI of (6.3, 9.2)) and lower limit of quantification of 9.4 IU/mL using the 5<sup>th</sup> International WHO HCV Standard. A master calibration curve traceable to the 5<sup>th</sup> WHO HCV Standard as well as external calibrators (based on secondary standards traceable to the 5<sup>th</sup> WHO HCV Standard) were developed to provide accurate quantitative results across multiple systems and reagent lots. The NeuMoDx HCV Test demonstrated excellent linearity across an 8 Log dynamic range ( $R^2 > 0.99$ ). The NeuMoDx HCV Test showed equivalent detection performance across all relevant HCV genotypes and a time to first results of ~80 min. No cross-reactivity or interference was observed against any of the pathogens or agents tested. The method correlation study performed with the NeuMoDx HCV test and using split samples demonstrated excellent concordance with a reference

test. An  $R^2 > 0.95$  and bias of HCV viral load output from the two tests of less than 0.5 Log IU/mL was obtained. **Conclusions:** The performance data presented here clearly demonstrate that the NeuMoDx HCV Test is an extremely easy to use, rapid, automated test for the sensitive and accurate monitoring of HCV viral load.

#### ID026. Quantitative Detection of Epstein-Barr Virus (EBV) in Plasma and Whole Blood Matrices

L. Gong, M. Carey, K. Krach, J. Zhu, M. Mastronardi, B. Wu, S. Brahmasandra

NeuMoDx Molecular Inc., Ann Arbor, MI.

**Introduction:** Immuno-compromised individuals lacking EBV antibodies, are especially at risk for acute EBV infection that may cause significant mortality and morbidity in organ transplant recipients because of posttransplant lymphoproliferative disorders (PTLD). EBV DNA can be detected in the blood of infected patients and increasing levels of EBV DNA has been shown to correlate significantly with subsequent development of PTLD in susceptible patients. As a result, EBV viral load is important tool for monitoring patients at risk for PTLD and provides prognostic information for effective treatment and management of such patients. The NeuMoDx EBV Test is a "sample to result" type in-vitro diagnostic assay incorporating automated extraction of qPCR ready DNA from whole blood and plasma specimens, combined with a sensitive real-time PCR assay to deliver highly accurate results in a completely automated manner. **Methods:** Performance of the NeuMoDx EBV Test was characterized in both plasma and whole blood matrices. The objective of this study was to test and report performance of the NeuMoDx EBV Test across key analytical performance metrics. Studies were performed to characterize the analytical sensitivity, limits of quantitation, linearity, precision, turnaround time, and equivalency across specimen types. Testing was performed using either a 550 uL sample of plasma or a 220uL sample volume of whole blood specimen. **Results:** Evaluation of the analytical sensitivity using a Probit style analysis was performed using the 1<sup>st</sup> WHO International EBV Standard, and the lower limit of quantitation (LLOQ) was determined using the Total Analytic Error  $<1.0$  criterion. In plasma specimens, the NeuMoDx EBV Test was able to demonstrate a limit of detection (LoD) of 40 IU/mL and an LLOQ of 60 IU/mL. In whole blood specimens, the NeuMoDx EBV Test demonstrated an LoD of 80 IU/mL with an LLOQ of ~120 IU/mL. The NeuMoDx EBV Test demonstrated excellent linearity across typical, clinically relevant measurement dynamic ranges ( $R^2 > 0.99$  across 6 logs), as well as precision across systems, days, and reagent lots. Turnaround time (TAT) for the NeuMoDx EBV Test was ~65 min and the results interpretation module incorporated for automated processing of data provided extremely accurate results. No cross-reactivity was observed against any of the non-target pathogens tested and the test performed efficaciously in the presence of interfering moieties. Finally, equivalent performance was demonstrated across both plasma and whole blood specimen matrices. **Conclusions:** The NeuMoDx EBV Test demonstrated excellent performance and is well suited for implementing viral load monitoring using both plasma and whole blood specimens.

#### ID027. WITHDRAWN

#### ID028. Evaluation of the GenMark ePlex Respiratory Pathogen Panel for the Detection of Respiratory Pathogens

A.M. Carlin<sup>1</sup>, C. Gentile<sup>1</sup>, M. Elkan<sup>2</sup>, A. Cárdenas<sup>1</sup>, V. Van Deerlin<sup>1</sup>

<sup>1</sup>Hospital of the University of Pennsylvania, Philadelphia, PA; <sup>2</sup>Children's Hospital of Philadelphia, Philadelphia, PA.

**Introduction:** Respiratory infections present with similar symptoms making fast and accurate identification of causative pathogens necessary for patient treatment and management. The ePlex Respiratory Pathogen (RP) Panel (GenMark Diagnostics, Carlsbad, CA) is an FDA-cleared sample to answer platform for the detection of 15 viral and two bacterial pathogens from nasopharyngeal swabs (NPS). Although NPS are the most common specimen for respiratory testing in adults, bronchial lavages (BAL) are used to diagnosis lower respiratory tract infections and nasopharyngeal aspirates (NPA) are used for testing children and neonates. The goal of this study was to assess performance of the ePlex RP Panel in NPS, BAL, and NPA specimens and to assess quality metrics

after implementation of the ePlex RP Panel. **Methods:** NPS (n=87), BAL (n=43) and NPA (n=31) specimens were used to evaluate the performance. All samples were evaluated using 200  $\mu$ L of specimen according to manufacturer's recommendations for NPS in viral transport medium (VTM). In addition, a subset of BALs and NPAs were tested with a pre-processing step which included extraction of viral nucleic acid using the QIAamp DSP Virus Spin Kit (Qiagen, Valencia, CA) followed by a 1:1 dilution in VTM. Concordance of the ePlex RP Panel results was assessed by comparison to a laboratory developed test (LDT). Quality metrics were assessed pre and post implementation. **Results:** The concordance rate between the ePlex RP Panel and the LDT for NPS was 97% (84/87). Three discordant results were due to the ePlex RP Panel not detecting a pathogen which was detected by the LDT. For BAL samples evaluated using a pre-processing step, the concordance rate increased from 69% (11/16) to 100% (15/15). For NPA, the concordance rate was 90% and remained unchanged with addition of the pre-processing step. Implementation of the ePlex RP Panel in the 2017-18 flu season resulted in a 4 hour reduction in turnaround time despite a 73% increase in testing volume compared to the 2016-17 flu season. Inconclusive rate decreased from 1.5% to 0.5% and repeat rates remained steady at approximately 6%. During the 2017-18 season the detection rate increased from 17% to 31%, corresponding to approximately 1000 additional positive results, due to the additional targets in the ePlex RP Panel compared to the LDT. **Conclusion:** This study demonstrates the ability of the ePlex RP Panel to quickly and accurately assess NPS, BAL and NPA specimens for respiratory pathogens. Due to the variability in volume and cellularity of BALs, we found that a pre-processing step improved concordance rates for this sample type. Implementation of the ePlex RP Panel at our institution allowed the processing of an increased number of specimens while reducing turnaround time.

#### ID029. Development of a New Diagnostic System Based on Real-time LAMP PCR for Specific Detection of 10 Species of Arboviruses

E. Choi, S. Kang, K. Lim, S. Lee, H. Park

SD Biosensor, Suwon Gyeonggi-do, South Korea.

**Introduction:** Arboviruses (arthropod borne virus) are a major cause of fever in human, and are known for Zika virus, Dengue virus, Chikungunya virus, Yellow fever virus, Japanese encephalitis virus, West Nile virus, and Mayaro virus. Arboviruses are mainly transmitted by mosquito mediated infection. Person-to-person transmission is not common, but usually occurs by mother-to-child and blood transfusions. The diagnosis of arbovirus is mainly used by Immunochromatography rapid tests for detection of antigen and antibody (IgM/IgG). Rapid tests have the advantage of being short in time, but have lower sensitivity and specificity than nucleic acid amplification methods. **Methods:** In this study, we developed a Real-Time LAMP (Loop-mediated isothermal AMPlification) method using IB-DFO (Isothermal-based dual function oligo) probe. The method can diagnose 10 species of arboviruses (Zika, Dengue type 1 ~ 4, Chikungunya, Japanese encephalitis virus, Yellow fever virus, West Nile virus, Mayaro virus). Using Real-Time LAMP (Loop-mediated isothermal AMPlification), cDNA synthesis and isothermal amplification reaction were done at 65°C. Multiplexing technology with FAM, HEX and Cy5 IB-DFO probes were used to detect three different infectious diseases in the same tube and the result of the amplification reaction can be obtained within 20 minutes. **Results:** It was confirmed that all 10 species of arbovirus were detected with a sensitivity of less than 100 copies per test. Even if patients were infected simultaneously with several arbovirus infectious diseases, it was confirmed that they could be detected normally without any non-specific reaction by other target. **Conclusions:** The SDB standard M Arbovirus panel will allow accurate diagnosis quickly in less than 20 minutes and contribute to early detection of Arbovirus infection with similar symptoms.

**ID030. Analytical Validation of a Sample-to-Sequence Pipeline for Non-targeted Pathogen Detection in Clinically Relevant Matrices**

K. Parker<sup>1</sup>, B. Campos<sup>1</sup>, H. Wood<sup>1</sup>, D. Yarmosh<sup>2</sup>, J. Russell<sup>2</sup>, J. Aspinwall<sup>1</sup>, K. Werking<sup>1</sup>, P. Chain<sup>3</sup>, P. Li<sup>3</sup>, K. Davenport<sup>3</sup>, J. Jacobs<sup>2</sup>, R. Winegar<sup>1</sup>

<sup>1</sup>MRIGlobal, Palm Bay, FL; <sup>2</sup>MRIGlobal, Gaithersburg, MD; <sup>3</sup>Los Alamos National Lab, Los Alamos, NM.

**Introduction:** The ability to identify an unknown infectious agent in a clinical sample is often limited by the tools available to the clinician. Current microbial and molecular methods are complicated by factors such as fastidious growth conditions, the need to perform a series of differential growth tests, and the challenges of designing large panels of molecular assays that are both sensitive and specific over a broad range of organisms. Next generation sequencing (NGS) provides a means for unbiased detection of pathogens from a variety of clinical matrices. With a non-targeted approach, NGS has the potential to identify any pathogen within a sample, providing a more comprehensive assessment of the infectious agents and any commensal organisms. **Methods:** MRIGlobal has developed PanGIA (Pan-Genomics for Infectious Agents), a sample to sequence workflow capable of detecting pathogens from clinical samples. This fully integrated workflow includes all steps required for sample preparation through library preparation, next-generation sequencing on the Illumina MiSeq and bioinformatics analysis for unbiased pathogen identification. Data analysis was based on reads normalized to reference, linear coverage and confidence scores. **Results:** Here we present the results from our analytical validation of the PanGIA system with clinically relevant matrices, demonstrating the limit of detection and repeatability of the system with a wide range of pathogen types. We observed an approximate LOD of  $10^3 - 10^4$  PFU/CFU per mL for our pathogen surrogates *V. cholerae* (Gram negative bacteria), *S. aureus* (Gram positive bacteria), Modified Vaccinia Ankara virus (DNA virus) and Venezuelan Equine Encephalitis virus (RNA virus). **Conclusions:** PanGIA allows for processing, sequencing and analysis of samples within approximately 24 hours, providing the end user with a metagenomics analysis of the clinical sample. The results presented here demonstrate the ability to use the PanGIA pipeline for the detection of pathogens from clinically relevant matrices.

**ID031. Identification of *M. tuberculosis* and *M. bovis* in Clinical Respiratory Specimens Using the VELA Diagnostics Sentosa SA MTC PCR Assay**

H. Webber, A. Vachon, D. Thayer, C. Dragoni, M. Ianosi-Irimie NorDx, Scarborough, ME.

**Introduction:** Twenty-five percent of the population worldwide is infected with *Mycobacterium tuberculosis* (TB). In 2016, 1.7 million people died from TB infection, making it of great public concern. In the United States, the majority of TB cases are caused by the bacterium *Mycobacterium tuberculosis*, but *Mycobacterium bovis* can also cause TB infection in people. TB is a contagious, airborne, respiratory disease that can be fatal without proper, timely, treatment. Infection occurs when someone inhales droplet nuclei containing tubercle bacilli that reach the lungs. At present, patients admitted to the hospital with suspected TB are kept in isolation until laboratory testing procedures can confirm or rule-out active TB infection. Removing a patient from isolation requires either three negative acid-fast smears or two negative TB PCR results. To improve the accuracy and TAT of TB diagnosis and reduce the time in isolation for high-risk patients, we developed a rapid molecular assay to detect the *Mycobacterium tuberculosis* Complex (MTC), *M. tuberculosis*, *M. bovis*, or *M. bovis* bacilli Calmette-Guérin (BCG) using the VELA Diagnostics Sentosa SA MTC PCR reagents (Fairfield, NJ). **Methods:** 46 unique respiratory sample sediments were pooled in this study, including sputum, pleural fluid, BAL's, and lung tissue, to prepare the total 140 specimens tested using the MTC PCR assay. Positive *M. tuberculosis* culture from patients and BCG was spiked into negative specimens to determine assay sensitivity and specificity as well as identify potential inhibitors. Specimens were lysed and heat-inactivated in a BSL-3 room. DNA extraction, followed by detection of *M. tuberculosis* or *M. bovis* by real time PCR was carried out in general lab space.

**Results:** Agreement between PCR and acid-fast smear/culture was 100%. Assay precision was established, indicating no significant

difference in detection for either organism on different days, or by different technologists. The limit of detection was determined to be  $1.8 \times 10^2$  copies/reaction for both *M. tuberculosis* and *M. bovis*/BCG. No cross-reactivity was observed with other *Mycobacterium* species: *M. fortuitum*, and *M. kansasii*. **Conclusion:** The development of an MTC PCR assay using the VELA Diagnostics Sentosa SA MTC PCR reagents promises faster turn-around-time, high sensitivity and specificity, all of which will lead to shorter patient stays in hospital isolation and decreased time between suspected infection and treatment. Widespread use of NAAT assays in clinical laboratories rather than depending solely on acid-fast smear/culture is a step towards improving patient care and infection control.

**ID032. Pre-market Evaluation of Hologic's Group B *Streptococcus* PCR Assay on the Panther Fusion System**

F. Zhang, R.H. Manji, M. Olszewski, S. Juretschko Northwell Health Laboratories, Lake Success, NY.

**Introduction:** Screening vaginal/rectal swabs for the presence of beta hemolytic Group B *Streptococcus* (GBS) is recommended for pregnant women at 35-37 weeks gestation. A combination of Lim broth culture enrichment and PCR has been shown to be sensitive and accurate for detecting GBS colonization. The Panther Fusion GBS assay (pre-market) is an automated *in vitro* diagnostic test utilizing real-time polymerase chain reaction (PCR) for detection of GBS from enriched LIM broth. This study compared performance of the Panther Fusion GBS assay with the BD MAX GBS Assay (FDA-cleared). Assay sensitivity, specificity and accuracy were recorded to evaluate clinical performance. **Methods:** Prospectively collected vaginal/rectal swabs (n=510) for routine GBS PCR were placed in Lim broth and incubated for 18-24 hours at 35°C. After incubation, Lim broth samples were tested with the GBS assay on Panther Fusion and the GBS assay on BD MAX according to the manufacturer's instructions. Invalid tests were repeated according to manufacturer's recommendations. Discordant results were arbitrated by culture from LIM broth enrichment. **Results:** Among the 510 prospective specimens tested, 123 samples tested positive for GBS and 482 tested negative on both platforms. 6 invalid specimens on BD Max generated valid results upon repeat testing. No invalid results observed on Panther Fusion. Of the 5 discordant specimens, 4 initially tested positive on BD MAX and negative on Panther Fusion, however these 4 specimens were negative upon retesting on BD MAX. One of the discordant specimens was negative on BD MAX and positive on Panther Fusion and remained same upon retesting from a new aliquot. All 5 discordant specimens were negative for GBS by culture. **Conclusions:** Results for both assays were highly concordant, indicating similar clinical sensitivity and specificity of GBS detection when testing from enriched Lim broth. PPA/Sensitivity was 100%; NPA/Specificity was 99.79% and assay accuracy 99.80%. There were no invalid GBS results on the Panther Fusion system while the BD MAX GBS assay showed a 1.2% invalid rate. Technical hands-on-time was approximately 1.5 min/sample for both assays, but significant benefits of the Panther Fusion GBS assay are full automation, random access and reduced labor compared to the BD MAX GBS assay.

**ID033. Multicenter Evaluation of the Sentosa SA HSV1/2 Qualitative PCR Test**

D. Kohn, L. Doyle, G.W. Procop Cleveland Clinic, Cleveland, OH.

**Introduction:** Herpes Simplex virus type 1 (HSV1) and type 2 (HSV2) are ubiquitous double stranded DNA viruses. HSV1/2 are human pathogens, causing chronic, recurrent infections and viral meningitis in adults and children. Traditionally, cell-based culture methods have been used to detect HSV however, the enhanced sensitivity, robustness, and rapid time to result of commercially-available amplification methods for the detection of viral DNA are leading to their increasing use in the clinical laboratory. We evaluated the Vela Sentosa SA HSV1/2 Qualitative PCR Assay (Sentosa) (Vela Diagnostics Inc., Fairfield, NJ). **Methods:** The Sentosa is a multiplex, real-time PCR-based qualitative *in vitro* diagnostic test for detection and typing of HSV1 and HSV2 DNA from internal and external anogenital and oral lesions. The clinical evaluation compared the Sentosa to culture by the ELVIS HSV ID and D3 Typing Test System (ELVIS) (Quidel Corporation, San Diego). A total of 2,295 residual clinical samples



(317 oral lesions and 1,978 anogenital lesions) collected from 8 sites were tested by Sentosa (4 sites) and ELVIS (3 sites). Discordant results were resolved with bi-directional sequencing (1 site). Panels spiked with commercially available strains of HSV1/2 were used to determine analytical sensitivity, precision, reproducibility, cross-reactivity, the effect of interfering substances and cross contamination. **Results:** For HSV from anogenital lesions the Sentosa compared to ELVIS with sensitivity and specificity of 96.9% and 95.8% for HSV1 and 98.5% and 90.7% for HSV2. For oral lesions sensitivity and specificity was 100.0% and 86.4% for HSV1 and 66.7% and 99.7% for HSV2. For analytical sensitivity the limit of detection (LOD) is defined as the HSV titer detected with a probability of  $\geq 95\%$ . The LOD for HSV1 and 2 was 40 TCID<sub>50</sub>/mL and 4 TCID<sub>50</sub>/mL respectively. Precision analysis of controls and panel members at 3xLoD and 1.5xLoD for HSV1/2 demonstrated 100% agreement. Reproducibility with spiked samples across multiple sites was excellent with 100% agreement and % CV of 1.01-5.25. No cross-reactivity was shown for the detection of HSV1/2 in the presence of other organisms (N=55) related to HSV1/2 or present in oral or genital swab samples at high concentrations. No interfering effects were observed with 31 substances commonly found in genital and oral specimens. The overall contamination rate of was 0% at HSV1 sample concentration of  $1 \times 10^5$  TCID<sub>50</sub>/mL. **Conclusion:** The Vela Sentosa SA HSV1/2 Qualitative PCR Assay is a sensitive and specific test to detect HSV1/2 in anogenital and oral samples. The Sentosa compares favorably with existing cell culture methods and has the advantage of an automated workflow (sample prep, PCR set up, amplification and reporting system).

#### ID034. A Rapid Host Gene Expression Assay to Discriminate Bacterial from Viral Infections

W. Nie<sup>1</sup>, N. Huynh<sup>1</sup>, D. Rawling<sup>1</sup>, M.W. Eshoo<sup>1</sup>, P. Khatri<sup>2</sup>, J. Romanowsky<sup>1</sup>, R. Howard<sup>3</sup>, G. Sanders<sup>3</sup>, P. Harding<sup>3</sup>, M. Twisk<sup>3</sup>, O. Liesenfeld<sup>1</sup>, O. Liesenfeld<sup>1</sup>, T.E. Sweeney<sup>1</sup>

<sup>1</sup>Inflammatix Inc., Burlingame, CA; <sup>2</sup>Stanford University, Palo Alto, CA; <sup>3</sup>TTP Plc, Cambridgeshire, United Kingdom.

**Introduction:** Acute infections are among the most frequent diagnoses in outpatient care settings. Early, accurate and rapid differentiation between viral and bacterial infections is critical to guide the choice of antimicrobial treatment, improve patient outcome, and to ensure antimicrobial stewardship. Current molecular diagnostics that rely on direct pathogen detection are limited by their breadth and sensitivity. Recently, host response-based molecular diagnostics have been considered as a novel alternative or complementary approach. We have developed and validated a 7-gene expression panel that accurately discriminated between viral and bacterial infections (in 6 validation cohorts, summary ROC AUC of 0.91 (95% CI, 0.82 to 0.96)). In the present study we describe the development of a rapid 7-gene host response biomarker PCR assay that discriminates bacterial from viral infections, called HostDx Fever. **Methods:** The original microarray-derived 7-gene panel was developed into a rapid multiplex quantitative reverse transcription real-time PCR assay (RT-qPCR) that can be performed on a variety of quantitative PCR platforms. The assay was designed to be multiplexed, specific for mRNA and specific for each of the seven host mRNA targets. Data from the RT-qPCR was then compared with quantitative gene expression data generated on the NanoString and an ultrafast qPCR platforms, (TTP Puckdx), respectively. **Results:** The seven gene real-time RT-qPCR assays were divided into two multiplex reactions, one 5-plex and one 4-plex. A housekeeping control was included in each of the two multiplexes. Ten clinical samples from healthy subjects (3) or patients with confirmed viral (4) or bacterial (3) infections were tested in parallel on three platforms: regular qPCR, an ultrafast qPCR and NanoString platform. We found a high degree of concordance with R values of  $>0.95$  between our assay and NanoString platforms, and R values of  $>0.94$  between our assay and the ultrafast qPCR platform. Ultrafast qPCR results were obtained in 12 minutes from the TTP Puckdx prototype system. **Conclusions:** The 7-gene panel was validated and allows for robust discrimination between bacterial and viral infections. Multiplexing permits a more cost-effective method of testing. As a rapid test, HostDx Fever could assist in improved decision making for outpatients with suspected acute infections.

#### ID035. High Throughput FluA/B/RSV Testing May Complement Existing Methods During the Peak of Flu Season

R. Hein, S. McCune, J. Engstrom-Melnik

Roche Diagnostics Corporation, Indianapolis, IN.

**Introduction:** Viral respiratory illnesses that are common during flu season can cause severe complications, especially in young children and older adults with weakened immune systems. Thus, it is critical that timely results are provided to ensure appropriate patient management. During peak period of the flu season, there may exist a need to adjust laboratory workflow in order to accommodate bursts of high-volume testing to meet the clinical testing demands and to maintain the rapid standard of resulting. An efficient testing workflow may utilize a combination of a point-of-care and high-throughput systems, such as the cobas 6800 System, which is a fully automated, sample-to-result system for routine or high-volume molecular testing. The cobas omni Utility Channel (UC), the open channel functionality of the cobas 6800 System, supports a complete, automated Lab Developed Test (LDT) workflow and allows for the routine performance of user-defined real-time PCR tests. With commercially available primers and probes, opportunities exist to incorporate LDTs onto the automated platform and into clinical laboratory workflows without the need for full assay development, while also allowing laboratories to continue to provide timely patient results when test volumes reach a critical mass. Here, we demonstrate the adoption of a Flu A/B and RSV primer/probe cocktail on the cobas 6800 System. **Methods:** Commercially available control material was used to assess the performance of an available FluA/B/RSV primer/probe set. Adoption for use on the UC followed vendor provided protocol recommendations. Serial dilution of the 6-member panel was performed using the BD universal viral transport system (UTM); two swabs were dipped directly into the respective dilution vial and transferred to individual transport tubes. Each dilution was run twice on the cobas 6800 System using the FluA/B/RSV primer/probe set on the UC. Concordance with the cobas Influenza A/B and RSV, performed on the cobas Liat System (Liat), was determined by transferring 200uL from each UTM vial directly to the test tubes. Additional workflow and process timings were captured for both systems. **Results:** The FluA/B/RSV primer/probe set was able to accurately detect all control panel members at variable dilution levels. Similar detection efficiency was observed for both systems across the 6-member panel. Both workflows on the UC and Liat demonstrated limited hands-on time and similar sample-in and result-out processes. **Conclusions:** Influenza seasons are marked by spurts of both high- and low-volume testing requests; incorporating automated solutions into current workflows may complement clinical testing practices and alleviate some of the burdens associated with this ebb and flow.

#### ID036. WITHDRAWN

#### ID037. A High Throughput System for Profiling Respiratory Tract Microbiota

J. Li<sup>1</sup>, K. Li<sup>1</sup>, N.M. Fantin<sup>1</sup>, S.N. Patel<sup>1</sup>, B.J. Moy<sup>1</sup>, T.A. Hartshorne<sup>1</sup>, B. Huang<sup>1</sup>, H. Ji<sup>2</sup>, I. Pagani<sup>1</sup>, E.E. Zeringer<sup>3</sup>, N. Puri<sup>1</sup>, L. Nguyen<sup>1</sup>, K.H. Varma<sup>1</sup>

<sup>1</sup>Thermo Fisher Scientific, South San Francisco, CA; <sup>2</sup>Thermo Fisher Scientific, Pleasanton, CA; <sup>3</sup>Thermo Fisher Scientific, Austin, TX.

**Introduction:** As one of the leading causes of death globally, respiratory infections could be caused by viral, bacterial or fungal pathogens that present in the upper and lower respiratory tract. Panel based testing using molecular methods to identify multiple pathogens simultaneously can contribute to better understanding of respiratory infections. **Methods:** To develop a comprehensive and flexible panel, we chose TaqMan OpenArray platform to identify common respiratory tract organisms via real-time PCR. Target organism sequences were acquired from IMG and NCBI database. Divergent gene targets were chosen to design assays with high specificity (ANI  $>96\%$ ) and wide strain coverage. For each target, multiple assays were designed *in silico* and then assay performance was evaluated using various controls including synthetic and genomic DNA and RNA, as well as human respiratory specimen. Assays were selected to build up the TaqMan OpenArray panel. To achieve better Limit of Detection, a pre-amplification step is introduced into the workflow. **Results:** A qPCR solution was developed using TaqMan

OpenArray Plates nanofluidics platform to detect a panel of 12 bacteria, 31 viruses and 1 fungus of common respiratory microbes. The performance of each assay has been verified with several levels of control templates including DNA plasmid, in vitro transcripts of RNA, genomic DNA and RNA, and nucleic acids extracted from human respiratory tract specimen. Optimized components and thermal cycling condition for reverse transcription and PCR pre-amplification was achieved by extensive DOE studies. With pre-amplification, this system demonstrates high sensitivity as well as specificity. High R-squared value ( $>0.99$ ) and good PCR efficiency ( $>90\%$ ) was observed in linearity studies in all assays. Furthermore, 144 repository samples were tested to compare with the results from other platforms and high accordance was observed. Additional orthogonal verification using Sanger sequencing confirmed detected results that were discordant from others. **Conclusions:** A novel research application has been developed for respiratory tract microbiota profiling. Assays in the panel demonstrate desirable performance in terms of sensitivity, specificity and linearity range. The application enables both customizable and high-throughput panels for respiratory infection research and provides a cost-effective tool for researchers to understand pathogenicity in respiratory tract infections.

#### ID038. WITHDRAWN

#### ID039. Cost Effectiveness Model Describing Emergency Department Use of a Novel Multi-mRNA Test for Diagnosis and Risk Assessment of Acute Respiratory Tract Infections and Sepsis

I. Stojanovic<sup>1</sup>, J. Romanowsky<sup>2</sup>, P. Schuetz<sup>3</sup>, J. Schneider<sup>1</sup>, O. Liesenfeld<sup>2</sup>, T.E. Sweeney<sup>2</sup>

<sup>1</sup>Avalon Health Economics, LLC, Morristown, NJ; <sup>2</sup>Inflammatix Inc., Burlingame, CA; <sup>3</sup>University Department of Medicine, Kantonsspital, Aarau, Switzerland.

**Introduction:** Emergency physicians currently rely on multiple imperfect tests to diagnose acute respiratory tract infections (ARTIs) and sepsis, leading to excess morbidity and mortality. A novel 30-host-gene PCR test (HostDx Sepsis by Inflammatix) identifies i) the likelihood of a bacterial infection, ii) the likelihood of a viral infection, and iii) the likelihood of sepsis and disease progression, in  $<60$  minutes. This work examines the cost effectiveness of the HostDx Sepsis test for use in US healthcare system emergency departments (ED) for the assessment of ARTIs and sepsis. **Methods:** A decision-analytic simulation model compares the standard of care for patients assessed in the ED for ARTIs and suspected sepsis to a hypothetical care pathway with the HostDx Sepsis test. Analysis informed on expected costs and outcomes occurring during an ED visit for a hypothetical cohort of patients. Selected input outcomes were based on review of peer-review articles. The 30-day outcomes considered in the study were expected cost per person presenting with ARTI or suspected sepsis to an ED, incremental cost per life-year saved, antibiotics days and hospital length of stay (HLOS). Sensitivity analysis of key inputs was also conducted. **Results:** In the base-case scenario, across a cohort of 1,000, the HostDx Sepsis arm saves 10 lives/1,000 person-years and results in expected costs of \$1,404/person less than the standard of care arm, reduced total antibiotics exposure by 1,097 days and 341 days of hospital stays comparing to the standard of care arm. Standard of care relied on empirical treatment, clinical judgment and multiple testing for bacterial and viral infections, with accuracy for bacterial, viral and mortality risk differentiation lower than that of HostDx Sepsis. In one- and two-way sensitivity analysis, model results were most sensitive to the HLOS, followed by the HostDx Sepsis test price, and length of antibiotics treatment. **Conclusions:** The HostDx Sepsis arm demonstrated clinical utility and cost effectiveness versus the current standard of care arm. Improved care is reflected by fewer unnecessary antibiotic prescriptions and side effects, fewer admissions and shorter HLOS. Results are likely generalizable as HostDx Sepsis arm remained cost effective in rigorous sensitivity analysis. Interventional studies are necessary to compare actual clinical and costs outcomes between current practice and that which incorporates HostDx Sepsis.

#### ID040. Quantitative Detection of Cytomegalovirus on NeuMoDx Molecular Systems

M. Mastronardi, C. Couture, L. Gong, H.L. Lee, A. Narwold, B. Wu  
Neumodx Molecular, Inc., Ann Arbor, MI.

**Introduction:** Cytomegalovirus (CMV) is one of the major causes of opportunistic infection, and associated morbidity and mortality, in immunocompromised patients such as organ transplant recipients. Quantitative PCR methods are among the most useful diagnostic tests available for measuring CMV replication in such patients. Such viral load measurements are crucial to monitoring disease progression, efficacy of antiviral therapies, as well detecting drug resistant mutants and identifying relapse upon discontinuation of antiviral therapy. The NeuMoDx CMV Test is a "sample to result" in-vitro diagnostic assay offered on two NeuMoDx Molecular Systems – the NeuMoDx 288 and the NeuMoDx 96 - which automate and integrate the extraction, amplification, and results interpretation using quantitative PCR. Performance of the NeuMoDx CMV Test on both systems was characterized and compared to an existing reference test. **Methods:** The objective of this study was to characterize performance of the NeuMoDx CMV Test across key performance metrics. Analytical studies – sensitivity, cross-reactivity, inclusivity, interfering substances, and a pilot method comparison study – were performed. Analytical sensitivity was determined via a Probit-style analysis using the 1<sup>st</sup> WHO International CMV Standard, and the lower limit of quantitation (LLoQ) was determined using the Total Analytic Error  $<1.0$  criterion. Specificity was tested by screening  $>30$  phylogenetically similar or cohabitating organisms while typical interfering moieties were tested to screen for interfering effects. Finally, a small-scale method correlation study involving 100 specimens was performed to assess concordance with a reference test. **Results:** Evaluation of analytical sensitivity shows the NeuMoDx CMV Test was able to detect  $\sim 40$  IU/mL with at least 95% sensitivity, and LLoQ was also determined to be 40 IU/mL. The NeuMoDx CMV Test demonstrated excellent linearity across clinically relevant dynamic range ( $R^2 > 0.99$  across 6 logs), as well as precision across systems, days, and reagent lots. Turnaround time (TAT) for the NeuMoDx CMV Test was  $\sim 65$  min and the automated processing of data provided extremely accurate results. No cross-reactivity was observed against any of the non-target pathogens tested and the test performed efficaciously in the presence of interfering moieties. The comparison study conducted between the NeuMoDx CMV Test and the reference test showed excellent linear correlation and a bias of  $< 0.5 \text{ Log}_{10} \text{ IU/mL}$ . **Conclusions:** The NeuMoDx CMV Test provides an excellent workflow for implementing viral load monitoring for immunocompromised patients. Performance across analytical metrics was superior to existing tests while demonstrating excellent correlation to a current reference test.

#### ID041. Performance Evaluation of Unpreserved Stool and Stool in Transport Medium with a Multiplex Gastrointestinal Pathogen Panel with an Automated, High Throughput System

C. Knoth, K. Jeevaparakash, J. Kirchner, A. Pham, M. Aye  
Applied BioCode, Inc., Santa Fe Springs, CA.

**Introduction:** Gastroenteritis is a leading cause of death worldwide across all age groups. It is estimated that 1.31 million people died from diarrheal disease in 2015. High-throughput multiplex assays can aid in rapid identification of pathogens that can cause outbreaks of diarrhea and for infection control in healthcare settings. Despite recent introduction of molecular multiplex pathogen detection platforms, there is a limited choice of systems for clinical labs with medium to high-throughput automation. To address this need, Applied BioCode has developed an automated high-throughput molecular diagnostic assay system in a 96-well format. The BioCode GI Pathogen Panel is an multiplex molecular assay for detection of gastrointestinal pathogens which include: bacteria (*Campylobacter*, *C. difficile* toxin A/B, *Salmonella*, *Shigella*/EIEC, EAEC, ETEC, STEC, STEC, *E. coli* O157, *Vibrio*, *Yersinia enterocolitica*), viruses (Norovirus I/II, Adenovirus F, Rotavirus A), and parasites (*Cryptosporidium*, *Entamoeba histolytica*, *Giardia lamblia*). **Methods:** The BioCode MDx 3000 platform integrates and automates PCR, post-PCR sample handling and detection steps in a 96-well format. Following extraction of nucleic acids targets are amplified by one-step RT-PCR. PCR products are captured by target-specific probes coupled to Barcoded Magnetic Beads (BMBs), and the presence of target sequence(s) is detected by a fluorescent conjugate.

Qualitative results are determined by a median fluorescent index (MFI) value relative to assay cutoff. In this study, 397 raw stool specimens were assayed with the BioCode GPP then stored at -80°C. The specimens were then thawed and inoculated in Cary-Blair transport media for testing with the BioCode GPP. Raw stool and inoculated Cary-Blair samples with discordant results were freshly prepared, extracted and tested in parallel. **Results:** 2/397 (0.5%) raw stool specimens were invalid for failure to detect internal control. There were no invalid Cary-Blair specimens. Overall positive agreement between the raw and Cary-Blair inoculated specimens was 89.3% (133/149). Upon repeat testing of discordant specimens in parallel, overall positive agreement was: 99.3% (137/138). Positive agreements for representative targets after discordant analysis were: 8/8 for *Campylobacter* spp., 42/42 for *C. difficile*, 12/13 for EAEC, 30/30 for Norovirus GI/GII and 13/13 for Rotavirus A (see poster for all analytes). **Conclusions:** On-board run time for MDx 3000 was about 3.5 hours for 96 samples. The invalid rate was <1%; resulting in minimal repeats or unresolved samples. The results of this evaluation indicated that the BioCode GPP produced equivalent results with either Cary-Blair or unpreserved stool specimens.

#### ID042. Detection of Group A *Streptococcus* Using a Laboratory Developed Test on the Fully Automated Panther Fusion Open Access System

L. Vang, B. Wilkins, S. Panuganti, V. Srinivasan, A. Worlock  
Hologic, Inc., San Diego, CA.

**Introduction:** *Streptococcus pyogenes*, or Group A streptococcus (GAS), is a Gram-positive bacterium associated with mild infections of the throat and skin. If left untreated without antibiotics GAS can cause severe invasive infections including rheumatic fever and glomerulonephritis. Rapid Antigen Detection tests (RADTs) and culture are commonly used for GAS diagnosis. RADTs show variable sensitivity and culture takes ≥48 hours for results. Clinical laboratories use Laboratory Developed Tests (LDTs) to detect emerging pathogens or due to lack of high throughput options. But LDTs commonly require excessive labor and time. Here, we show the development of Analyte Specific Reagents (ASRs) for detection of GAS from throat swabs using Panther Fusion Open Access. Panther Fusion is a fully automated system that extracts nucleic acid from clinical specimens, detects targets by Real-time Polymerase Chain Reaction (RT-PCR) and has Open Access functionality. Panther Fusion Open Access system enables the laboratories to develop LDTs using ASRs and ready to use reagents requiring minimal handling and delivers short processing times. **Methods:** Panther Fusion Open Access System allows the user to load a user-defined oligonucleotide/salt mixture to reconstitute a pre-formulated, lyophilized pellet containing components necessary for a PCR or reverse transcription PCR. Here, a primer probe containing ASR to detect GAS was characterized for analytical sensitivity, specificity, PCR efficiency and % agreement using clinical specimens on the Panther Fusion Open Access. Analytical sensitivity was evaluated by testing 6 different GAS strains at concentrations ranging from 3.16 to 1000 Colony Forming Units/mL (CFU/mL). Reactivity was evaluated by testing 15 different GAS strains. Analytical specificity was evaluated by testing non-GAS organisms potentially found in clinical specimens. PCR efficiency was evaluated by testing serial dilutions of GAS plasmid. Percent agreement with culture results was determined by testing 36 clinical specimens. **Results:** The analytical sensitivity for the GAS-ASR ranged between 31.6 and 1000 CFU/mL with a PCR efficiency ≥100%. A 100% reactivity was observed for all GAS strains. Cross reactivity/interference was not observed with any of the non-GAS organisms. Clinical specimen testing yielded a ≥ 92% agreement with culture. **Conclusion:** The performance evaluation studies demonstrated robust analytical sensitivity and specificity for the GAS-ASR assay on Panther Fusion Open Access System. These results suggest that Hologic's GAS-ASR can be used as an LDT with minimal hands on time, efficient workflow and high throughput.

#### ID043. Development and Evaluation of a High Throughput Multiplex Molecular Panel that Detects 20 Respiratory Pathogens in Clinical Specimens

M. Aye, S. Mir, L. Hodaie, K. Jeevaprakash  
Applied BioCode, Inc., Santa Fe Springs, CA.

**Introduction:** Respiratory diseases pose a serious public health problem, with high social and economic impact and contribute to morbidity and mortality worldwide (Williams *et al.*, *Lancet Infect. Dis.* 2002). Therefore, timely and accurate diagnosis of respiratory infections is important for patient management. Using proprietary barcoded magnetic bead (BMB) technology, we have developed MDx 3000, an automated PCR, post-PCR sample handling and detection system in a 96-well format. To expand applications for this automated system, we are developing a Respiratory Pathogen Panel (RPP) for simultaneous detection of Influenza A (subtypes H1, H1N1 2009pdm, H3), Influenza B, Parainfluenza virus (type 1, 2, 3, 4), Metapneumovirus, RSV, Rhinovirus/Enterovirus, Coronavirus (OC43, NL63, 229E, HKU1), Adenovirus and bacteria (*Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Bordetella pertussis*). **Methods:** Nucleic acids were extracted with an off-board automated system and were amplified by one-step RT-PCR. PCR products were captured by target-specific probes coupled to barcoded magnetic beads (BMBs), and signal was generated by incubation with a fluorescent conjugate. Qualitative results were determined by Median Fluorescent Index (MFI) threshold from analyte-specific BMBs on BioCode MDx 3000 system. **Results:** BioCode RPP detected intended targets and did not cross-react with organisms tested in this study. Negative controls and no template controls gave clean background signals, demonstrating no false positive results. Quality Control for RPP is accomplished by using commercially available positive controls, either for individual targets or in pathogen pools. Reproducibility studies using 4 different pathogen pools on two instruments with 3 runs on each (6 total runs) gave 100% positive detection rate with 16 of 20 targets giving MFI CV <20% (range: 4-57%). Clinical performance with 307 nasopharyngeal swabs showed 96% positive agreement and 100% negative agreement for all targets with a FDA-cleared multiplex assay. **Conclusions:** BioCode Respiratory Pathogen panel (RPP) is a rapid, highly specific, high throughput test, and offers high sensitivity that can be used for detection and identification of 20 common respiratory pathogens (viruses and bacteria) in clinical laboratories.

#### ID044. Hepatitis C Virus Genotyping by Next-generation Sequencing: an Accurate and Cost-effective Alternative

B.G. Nezami, N. Sadri  
University Hospitals Cleveland Medical Center/Case Western Reserve University, Cleveland, OH.

**Introduction:** Chronic hepatitis C virus (HCV) is a major cause of end-stage liver disease and hepatocellular carcinoma. One of the strongest predictors of response to antiviral therapy is HCV genotype. Patients with genotype 1 infection have lower response rates to interferon and ribavirin, and may benefit from longer course of therapy or higher dosage. We aimed to develop a robust and affordable next generation sequencing (NGS) assay with an expedited workflow to enable us the capability to bring HCV genotype testing in-house. **Methods:** A cohort of 52 frozen plasma samples with viral loads ranging from  $2.0 \times 10^2$  to  $2.7 \times 10^7$  IU/mL that had orthogonal testing (Versant HCV Genotype 2.0 Assay, LiPA, Siemens) performed by Quest Diagnostics were used to test the performance of the assay. RNA was extracted from plasma using QIAmp Viral RNA Isolation kit (Qiagen). Multiplex RT-PCR with barcoded primers was used to amplify the 5' untranslated region (UTR) and core regions of the HCV genome. The PCR products were then purified and sequenced using Ion Torrent Sequencer (ThermoFisher). A custom in-house pipeline was used to provide genotypes based on 5'UTR and core sequences, independently. **Results:** Genotypes were called with 100% concordance to LiPA method. On a per sample basis, there was complete concordance between genotypes derived from 5'UTR and core sequences. Overall, 49 patient samples and 4 commercial controls were accurately identified with following genotypes: 1a (n=34), 1b (n=11), 2b (n=5), 3a (n=2), and 4b (n=1). RT-PCR failed for three low viral samples (<350 IU/mL), which were also unsuccessful using the LiPA method. The assay performed best for samples with viral load >1,000 IU/mL, although a sample with viral load



of 20 IU/mL was able to be successfully genotyped in the routine workflow. The complete workflow from plasma to results requires approximately 2.5 hours of hands-on time, 24 hours total time, and total reagents cost of \$40 per sample. **Conclusion:** Here we present an accurate and cost-effective alternative to current HCV genotyping assays (many that require capital investments) that can be easily be incorporated into molecular pathology and microbiology labs that have NGS capabilities. This assay can easily be scaled for smaller batches or much larger throughput as needed.

#### ID045. Technology to Produce Non-infectious Recombinant Virus as Reference Materials for Unculturable or Highly Dangerous Viral Pathogens

B. Anekella<sup>1</sup>, R. Vemula<sup>1</sup>, V. MacKeen<sup>2</sup>, J. Leach<sup>2</sup>, C.E. Huang<sup>1</sup>

<sup>1</sup>SeraCare Life Sciences, Gaithersburg, MD; <sup>2</sup>SeraCare Life Sciences, Milford, MA.

**Introduction:** Diagnostic laboratories and test developers need to design, manufacture and validate assays for pathogenic viruses and this requires stable, reproducibly manufactured positive reference materials. SeraCare has developed AccuPlex recombinant viral technology using Sindbis virus to meet this need. Recombinant viral technology has many advantages as a Nucleic Acid Testing (NAT) quality control material: it mimics clinical samples because it undergoes the entire extraction procedure; it is non-infectious; and it has extended stability at 2-8 °C. This technology is particularly needed for evaluation of the upper end of the reportable range of an assay, as very high titer patient specimens are often not available. AccuPlex materials also allow for evaluation of assays for select agents or highly dangerous pathogens to ensure preparedness for outbreaks.

**Methods:** A recombinant Sindbis virus was produced bearing 1,014 bp of HCV sequence from the 5' UTR region. High titer viral supernatant was produced using BHK-21 cells. The virus was diluted into pooled, filtered, human plasma and then serial 10-fold dilutions were made to generate a linearity panel. Testing was performed using Roche COBAS AmpliPrep/COBAS TaqMan HCV Test v2.0 as well as the Abbott RealTime HCV assay run on the m2000 system. Recombinant Sindbis viruses were also produced for Zika, Dengue, Chikungunya and Ebola viruses and were tested on either the Hologic Aptima Zika Virus Assay, Roche cobas Zika test, or Cepheid Xpert Ebola Assay and inhouse developed digital PCR assays for Chikungunya and Dengue. **Results:** HCV linearity panel member 1 had greater than 8E+07 IU/mL and member 7 (the lowest positive member) had concentration of less than 100 IU/mL, so this is the only linearity panel in the market that spans the entire reportable range of the Roche COBAS AmpliPrep/COBAS TaqMan HCV Test and Abbott m2000 assay. The R-squared value, which is a measure of how close the data are to the fitted regression line, was 0.999. AccuPlex Zika and AccuPlex Ebola reference materials were positively detected on the evaluated Emergency Use Authorization assays. Furthermore, using a laboratory developed real time RT-PCR assay, no changes in AccuPlex Ebola reference material titer were detected after 22 days at 37 °C or after 25 months at the recommended storage temperature of 2-8 °C. **Conclusions:** These studies demonstrate how AccuPlex recombinant Sindbis virus technology can be used for stable, well-characterized linearity panels and reference materials. This technology allows production of non-infectious reference materials for viruses that are difficult to source or propagate, additionally these materials will help laboratories to validate molecular tests and ensure preparedness.

#### ID046. Detecting *Helicobacter pylori* and Predicting Antibiotic Resistance from Formalin-fixed Paraffin Embedded Gastric Biopsies Using Targeted Next-generation Sequencing

B.G. Nezami, D. Rhoads, N. Sadi

University Hospitals Cleveland Medical Center/Case Western Reserve University, Cleveland, OH.

**Introduction:** *Helicobacter pylori* antibiotic resistance is increasing worldwide leading to increased treatment failures. Personalizing antimicrobial therapy based upon the infecting strain's antimicrobial resistance is needed; however, characterization of *H. pylori* using culture techniques is challenging due to the fastidious nature of the organism. We developed a multiplex PCR assay and sequenced the amplicons with

next-generation sequencing (NGS) to detect *H. pylori* and to characterize common resistance-associated mutations from formalin-fixed paraffin embedded (FFPE) gastric biopsy samples. **Methods:** Records of FFPE gastric biopsies processed at University Hospitals Cleveland Medical Center in 2017 were searched for *H. pylori* positive cases identified histopathologically. One hundred forty-two samples were identified and curls were cut from corresponding blocks. DNA was extracted using FFPE DNA isolation kit (Qiagen). Multiplex PCR was designed to detect common mutations in 23S rDNA, 16S rDNA, and *gyrA* genes to infer potential resistance to clarithromycin, tetracycline, or quinolones, respectively; as well as served for identification of *H. pylori* within the samples. PCR products were sequenced with Ion Torrent Sequencing (ThermoFisher) and in-house pipeline was used to identify *H. pylori* and call variants aligned to strain 26695 (ATCC 700392). Electronic medical records were searched for treatment details and response to therapy for retrospective clinical correlation. Follow-up data was found on 64 patients: 18 that failed therapy and 46 that had successful eradications.

**Results:** The assay was able to detect *H. pylori* in 140 of 142 cases. Fifty-one percent of cases had a mutation in *gyrA* (n=71), 38% in 23S (n=53), and 5% in 16S (n=7). More than one mutation was seen in 23% of all cases (n=32 of 140). The presence of more than one mutation correlated with therapy failure (p < .0001; greater than one mutation in 13 of 18 in the failure group vs 8 of 46 in the success group). Furthermore, a high degree of correlation between the presence of an A2142G or A2413G mutation in 23S and clarithromycin treatment failure (p < .0001) was observed. In the cohort treated with clarithromycin, these two mutations were found in 16 of 18 in the failure group vs 4 of 43 in the success group. Other 23S mutations (T2182C, C2195T, and T2190C) did not correlate with treatment response to clarithromycin. **Conclusions:** *H. pylori* mutation rates in our northern Ohio population is comparable to other reports in the USA. This assay is reliable for detecting *H. pylori* (sensitivity = 98.6%), for characterizing antimicrobial resistance mutations from FFPE tissue, and may be considered a reasonable alternative to culture-based testing for guiding *H. pylori* antimicrobial therapy.

#### ID047. Clinical Evaluation of the Aptima *Mycoplasma genitalium* Assay Reveals the Prevalence of *Mycoplasma genitalium* Infection among Patients Tested for other Sexually Transmitted Pathogens in Indiana

R.F. Relich, D.A. Warren, J.A. Koehlinger, C.L. Emery, T.E. Davis  
Indiana University Health, Indianapolis, IN.

**Introduction:** *Mycoplasma genitalium* is a leading cause of sexually transmitted urogenital tract infections, especially urethritis. In the U.S., infections caused by this bacterium are frequently undiagnosed due to a lack of readily available diagnostics. In Europe, the Aptima *Mycoplasma genitalium* Assay (MG; Hologic) has received regulatory clearance, broadening the availability of *M. genitalium* diagnostic testing on that continent. In this study, we evaluated the MG assay and, as a consequence, determined the prevalence of *M. genitalium* among a cohort of clinical specimens from Indiana that were originally submitted for non-*M. genitalium* sexually transmitted pathogen testing. **Methods:** A total of 945 remnant clinical specimens, including cervical (n = 157), endocervical (n = 67), penile (n = 2), rectal (n = 1), throat (n = 4), urethral (n = 3), urine (n = 668), and vaginal (n = 43) specimens, submitted over a three-week period for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and/or *Trichomonas vaginalis* testing, were analyzed by the MG assay, which was performed on the Panther instrument. The overall prevalence, as well as the incidence of co-infection, were subsequently calculated.

**Results:** Eighty-two specimens (8.7%) tested positive for *M. genitalium*. Of these, 70 (85.4%) were from females (age range, 15 – 45 years; median, 21 years) and 14 (14.6%) were from males (age range, 17 – 40 years; median, 26 years). In females, the specimen type that yielded the highest number of *M. genitalium*-positive results was urine (44/70, 62.9%) and in males, urine was the only specimen type positive for *M. genitalium*. Co-infections with other pathogens occurred infrequently; *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis* were detected in 3 (4.3%), 1 (1.4%), and 3 (4.3%) of the *M. genitalium*-positive specimens, respectively. With the exception of a single *T. vaginalis*-positive specimen, all co-infections were detected in specimens from females.

**Conclusions:** Various surveillance studies have demonstrated a

prevalence of *M. genitalium* infection to be as high as 10% among symptomatic patients, while other studies, especially those conducted in the Eastern Hemisphere, have indicated a much higher prevalence. To that end, following a clinical evaluation of the MG assay, we determined the prevalence of *M. genitalium* among our patient population to be closer to that observed previously in the U.S. (approximately 10%). The diagnosis of infections caused by this bacterium is crucial for the effective treatment of infection caused by this bacterium; however, until a test such as the MG assay is commercially available in the U.S., *M. genitalium* infections will continue to be undiagnosed.

#### ID048. Evaluation of DiaSorin Molecular Simplexa Bordetella Direct Kit for the Detection and Differentiation of *Bordetella pertussis* and *Bordetella parapertussis*

T. Ton<sup>1</sup>, M. Tran<sup>1</sup>, E. Tam<sup>1</sup>, K. Newton<sup>1</sup>, R. Webb<sup>1</sup>, J. Dunn<sup>1,2</sup>

<sup>1</sup>Texas Children's Hospital, Houston, TX; <sup>2</sup>Baylor College of Medicine, Houston, TX.

**Introduction:** Whooping cough is a highly contagious respiratory tract infection caused mainly by *Bordetella pertussis* and, less frequently, by *Bordetella parapertussis*. The Simplexa Bordetella Direct assay (Diasorin Molecular, Cypress, CA) is a qualitative, real-time PCR assay that combines direct amplification, detection and differentiation of *B. pertussis* (Bp) and *B. parapertussis* (Bpp) DNA from nasopharyngeal (NP) swabs. This study compares the performance of the Simplexa Bordetella Direct assay to the currently used lab-developed real-time PCR assay (LDT) at Texas Children's Hospital (TCH). **Methods:** A total of 49 NP swab samples collected using the ESwab Mini-tip Collection & Transport device (BD, Franklin Lakes, NJ) were tested: 41 prospective and retrospective patient specimens and 8 previously negative patient samples contrived by adding dilutions of Bpp from 1x10<sup>3</sup> to 2.5x10<sup>5</sup> cells/mL. For the LDT, 200µL of sample was extracted using the EZ1 DNA Tissue Kit on the EZ1 Advanced XL (Qiagen, Germantown, MD) with the Bacteria Card and eluted in 50µL. Real-time PCR targeting IS481 (Bp) and IS1001 (Bpp) was performed on the LightCycler 2.0 (Roche, Indianapolis, IN) using 5µL input DNA. ESwab samples were archived at -80°C after initial testing and were retrieved and retested with the Simplexa Bordetella Direct kit on the LIAISON MDX instrument. Automated analysis was performed with the LIAISON MDX Studio Software. Serial dilutions of bacterial DNA (ATCC, Manassas, VA) at defined concentrations were tested by both assays to compare the analytical sensitivity for both targets. **Results:** The clinical sensitivity for the Simplexa assay was 100% for Bp (21/21) and Bpp (9/9) compared to the LDT. Clinical specificity was also 100% (19/19) for both targets. The Simplexa assay demonstrated higher limits of detection (LOD) by 2 Log<sub>10</sub> for Bp and 1 Log<sub>10</sub> for Bpp compared to the LDT. Specifically, the Simplexa LOD was 3 fg/µL for Bp and 10 fg/µL for Bpp, while the LDT detected 30 ag/µL and 1 fg/µL, respectively. Crossing thresholds for patient samples tested on the Simplexa assay were on average 9.8 and 7.7 cycles higher than those on the LDT for Bp and Bpp, respectively. **Conclusion:** Despite the difference in LOD between Simplexa and the LDT, the overall agreement for patient samples was 100%. With a total test time of 1.25 hours (compared to 2.5 hours for the LDT) and reduction in hands-on time by eliminating extraction and testing NP specimens directly, the Simplexa assay could be run on-demand multiple times per day; thus impacting the time to detection of organisms with public health implications and institutional infection control concerns for healthcare exposures.

#### ID049. A Cross-sectional Study of Swab versus Tissue Sampling of Wounds for the Detection of Microbes by PCR

E. Baum-Jones<sup>1</sup>, K. Kaur<sup>1</sup>, Y. Wang<sup>1</sup>, M. Frasco<sup>1</sup>, S. Slowik<sup>2</sup>, R. Schechter<sup>3</sup>, A.L. Del Tredici<sup>1</sup>, T.A. Moreno<sup>1</sup>

<sup>1</sup>Millennium Health, LLC, San Diego, CA; <sup>2</sup>Tri-City Medical Center, Oceanside, CA; <sup>3</sup>Palomar Health, Escondido, CA.

**Introduction:** Chronic wound infections are traditionally diagnosed using microbial cultures. IDSA guidelines recommend sampling tissue from the wound bed, which is believed to capture microbes from deeper wound layers. An alternative to this is swabbing the wound, which is less invasive but believed to be less sensitive than tissue as a culture starting material. Molecular methods such as PCR are agnostic to organism growth requirements and can provide a snapshot of the wound microbiota

regardless of the source material. The objective of this study was to evaluate the extent of agreement in PCR results between different sample types (tissue and swabs) collected from the same wound. **Methods:** An observational clinical study designed to evaluate swab and tissue collections from open wounds was performed under an IRB-approved protocol. Three samples were obtained from consenting patients seeking treatment at two outpatient wound care centers: a swab collected before (SB) and after debridement (SA), and a tissue specimen (TC). Samples were transported in an inactivating solution that stabilizes DNA to preserve the original microbiota, and when received in the laboratory, DNA was isolated using commercially available extraction kits. TaqMan PCR amplification assays targeting 27 clinically relevant microbial species in wounds were performed on the Fluidigm Biomark HD platform. Results for the 27 PCR tests were aggregated and compared across sample types using McNemar's test for differences in paired proportions. **Results:** A total of 373 subjects with chronic wound specimens were sampled, and the results for 10,071 PCR tests (373 specimens x 27 tests) were compared in a pairwise analysis between sample types. There was a high overall agreement (>98%) in the results between all sample types compared. The rate of positive PCR results was 3.9% for TC, 4.5% for SA and 4.8% for SB. There was no significant difference for results between the swab samples (SAxSB, p = 0.08). However, swab samples had a higher rate of positive PCR results compared to tissue (SAxTC and SBxTC, p < 0.0001). **Conclusions:** This large cohort of patient specimens collected from open wounds shows a high level of agreement of PCR results between swab and tissue samples from the same wound. Further analysis will help elucidate the role of multiple sampling and debridement in molecular detection of pathogens in wound samples.

#### ID050. Evaluation of a Next-generation Sequencing Assay: The Sentosa SQ HIV Genotyping Assay for HIV Genotype and Drug Resistance Mutation Analysis

D. Kohn, L. Doyle, B. Yen-Lieberman, G.W. Procop  
Cleveland Clinic, Cleveland, OH.

**Introduction:** Detection of drug resistance mutations (DRMs) is critical in determining therapeutic antiviral drug regimes in patients infected with HIV. Resistance assays are used to select antiviral drugs before therapy is initiated, in cases of antiretroviral regimen failure or suboptimal viral suppression, and in pregnant women with HIV-1 infection. We evaluated the Vela Sentosa SQ HIV Genotyping Assay (Sentosa) (Vela Diagnostics Inc., Fairfield, NJ.) by testing validation panels and clinical samples from HIV positive patients. **Methods:** The Sentosa assay is a next generation sequencing (NGS) platform for the detection of mutations in Protease (PR), Reverse Transcriptase (RT) and Integrase (IN) regions from plasma in patients with HIV infection. It is comprised of a liquid handling system which enables automated RNA extraction, library construction, template preparation, sequencing and data analysis. Panels of wild type and mutant HIV plasmids were used to identify DRMs. Commercial panels were used to determine precision, analytical sensitivity and specificity, effect of interfering substances and specimen stability. Cross-reactivity and mixed genotype detection were also tested. The accuracy of the Sentosa assay was measured by testing frozen plasma from HIV-1 samples that were previously tested by a reference laboratory. Reference methods were RT-PCR followed by sequencing of the HIV-1 polymerase gene for RT and PR. For IN mutations a separate RT-PCR followed by sequencing of the HIV-1 IN gene is used. **Results:** The Sentosa identified the HIV-1 subtype B in 92% (22/24) of samples with ≥ 1,000 copies/mL. The assay detected 122/128 (97%) DRM's across the three target regions (RT, PR, IN). Of the 128 targeted mutations, the following DRM's for RT (M184V, M41L, M230L), PR (L10F, I50L, L90M), and IN (T66K, Y143C, Q148H) were detected at levels of ≥ 5% of the virus population within a sample. Interfering substances (EDTA, Heparin, hemolysis, lipemic and icteric plasma) had no effect on the detection of HIV-1 B subtype and there was no cross reactivity with HCV, HBV, EBV, CMV. Of 28 clinical samples tested for RT and PR, the reference method detected 80 DRMs and the Sentosa detected 89 DRMs. Only 11 of the clinical samples had IN resistance ordered and 2 DRMs were detected by both methods however, the Sentosa assay detected 4 DRM in samples that did not have IN ordered. In one instance the Sentosa was able to determine genotyping results when the reference method could not. **Conclusion:** The Sentosa

assay performed well with commercial panels and clinical samples. The Sentosa is a robust, clinically applicable assay with a turnaround time of 3.5 days and can determine DRMs in all 3 genes (RT, PR and IN). Further testing with clinical samples is planned.

#### ID051. Rapid and Accurate Cross-kingdom Human Pathogen Identification and Detection Using Hyb & Seq Technology

D. Bezdan<sup>1</sup>, M. Gregory<sup>2</sup>, C. Forsberg<sup>2</sup>, R. Fropf<sup>2</sup>, D. Lyakhov<sup>2</sup>, C. Chou<sup>1</sup>, G. Williams<sup>3</sup>, G. Meredith<sup>2</sup>, M. Rhodes<sup>2</sup>, D. Dunaway<sup>2</sup>, D. Kim<sup>2</sup>, E. Afshinnekoo<sup>1</sup>, J. Beechem<sup>2</sup>, L. Westblade<sup>1</sup>, S. Horner<sup>3</sup>, C.E. Mason<sup>1</sup>

<sup>1</sup>Weill Cornell Medicine, New York, NY; <sup>2</sup>NanoString Technologies, Inc., Seattle, WA; <sup>3</sup>Duke University School of Medicine, Durham, NC.

**Introduction:** Cross-kingdom pathogen identification has a number of challenges including: An effective, universal sample preparation workflow for both easily lysed and recalcitrant organisms. Ability to maintain DNA and RNA integrity for detection of both DNA and RNA viruses. Standard culture-based techniques are slow to diagnose fastidious organisms. Shotgun sequencing methods suffer from the vast excess of human DNA in clinical samples, limiting the number of useful reads or necessitating steps to deplete human DNA prior to sequencing. We investigated the ability of a novel hybridization based sequencing system, Hyb & Seq technology to overcome these challenges. **Method:** We initially designed a cross kingdom panel against 10 different species including yeast, gram-negative and gram-positive bacteria, and an RNA virus. 18-20 genomic sites were interrogated per species. Additionally, ribosomal RNA probes were designed for 5 of the bacterial species to allow simultaneous DNA and RNA detection. Initial testing was carried out using a commercially available panel (ZymoBIOMICS) to determine Limits of Detection (LOD) and linearity of response. This panel was tested on clinical samples from various sites including lung and wound tissue. This initial panel was then further refined by the addition of more pathogenic targets, the RNA probes were expanded to cover all possible pathogens in the panel. The protocol was optimized to give a sample to answer run time of 6 hours on a breadboard instrument. **Results:** Using the mock panel, a single lysis protocol allowed simultaneous detection of 10 species with LOD as low as a single genomic equivalent. To test the ability to detect pathogens against a background of human genomic material, 5 Million human cells were spiked into samples containing 100-10,000 pathogen cells. Our targeted panel sequencing revealed no discernible effect of an excess of human DNA on sensitivity or signal-to-noise ratio. The clinical samples were tested in a clinical pathology lab using standard culturing techniques and compared to results obtained using the Hyb & Seq technology and the expanded panel. In all cases the Hyb and Seq system agreed with the pathology findings as well as qPCR tests run in parallel. **Conclusions:** A single tube assay for cross-kingdom pathogen detection was constructed and successfully recapitulated previous analyses of clinically-relevant samples using Hyb & Seq. The highlights of Hyb & Seq include an amplification-free universal workflow, simultaneous detection of DNA and RNA in clinical samples and a workflow that will be capable of achieving an under 4 hour sample to answer workflow on a production system.

#### ID052. Validation of a Novel Qualitative Real-time PCR Assay Versus Direct Fluorescent Antibody Testing for the Detection of *Pneumocystis jirovecii* Pathogen

A. Spohn

Penn State Health Milton S Hershey Medical Center, Hershey, PA.

**Introduction:** *Pneumocystis jirovecii* is an opportunistic pathogen that causes pneumonia not only in HIV positive patients but also in HIV negative patients that are immunocompromised. A rapid clinical diagnosis of *Pneumocystis jirovecii* pneumonia infection is important since the organisms can infiltrate lung tissue. Early detection of *Pneumocystis jirovecii* pneumonia can facilitate initiation of appropriate treatment options and may improve chances of patient survival. Direct fluorescent antibody (DFA) is the gold standard testing method to detect *Pneumocystis jirovecii* in respiratory specimens. The use of DFA with respiratory specimens provides a simple, highly specific test for the detection of *Pneumocystis jirovecii* with sensitivity at 100% and specificity at 95.8%. With the introduction of commercially available PCR primers for *Pneumocystis jirovecii*, we wanted to validate the performance of a

direct amplification PCR assay against the performance of the DFA assay.

**Methods:** BAL specimens were tested using BioRad's Monofluo *Pneumocystis jirovecii* IFA Test Kit and direct amplification reactions were performed using DiaSorin Molecular's *Pneumocystis jirovecii* primer pair and Universal Master Mix on the 96-well Universal Disc using the LIAISON MDX instrument. Comparison studies were performed by testing 20 frozen BAL specimens that were positive by DFA and by testing 21 random fresh BAL specimens first by DFA then followed by PCR. LOD was performed by diluting a positive specimen 10-fold and testing both by DFA and PCR. **Results:** Of the 20 frozen BAL specimens that were positive by DFA, all tested positive by PCR with Ct values that ranged from 17 to 35. The 21 random fresh BAL specimens all upon initial review of the DFA slides were negative. When tested by the PCR assay, one specimen was positive with a Ct value of 31.1. After a more thorough slide examination of the PCR positive specimen, 2 small areas of *Pneumocystis jirovecii* cysts were found. For the LOD study, *Pneumocystis jirovecii* cysts were detected at a dilution of 1:100,000 with 1 cyst found on the DFA slide. The PCR assay detected *Pneumocystis jirovecii* cysts at a dilution of 1:10,000,000 with a Ct value of 36.1. **Conclusions:** The novel DiaSorin Molecular PCR assay for the detection of *Pneumocystis jirovecii* had sensitivity that was equal to or greater than the BioRad MonoFluo *Pneumocystis jirovecii* IFA assay. Both assays do provide a rapid turnaround time, are simple to use and are very specific for *Pneumocystis jirovecii*. DFA does, however, have multiple steps to perform the test and can have false negative results due to low titers of *Pneumocystis jirovecii* cysts that PCR can detect.

#### ID053. Automating Hydrolysis Probe Real-time PCR Applications on a Sample to Answer Real-time PCR System.

A.P. Benfield, M. Phillips

Luminex, Austin, TX.

**Introduction:** Traditional, non-automated, real-time PCR workflows can be complex and time-consuming. This typically consists of nucleic acid extraction, master mix formulation, combining master mix and template, running on a specialized instrument, and finally analyzing data. Luminex's ARIES Systems are capable of performing automated real-time PCR assays in a sample to answer format. Assays are performed within a closed cassette where the specimen is lysed and nucleic acids are extracted and then transferred to PCR reagents contained within a tube attached to the cassette. Results are available in approximately 2 hours. In this research study, we evaluated the potential for ARIES Systems to perform hydrolysis probe chemistry and investigated the impact of proteinase K and carrier RNA sample pre-treatment on CT, CT standard deviation, and failure rates. **Methods:** To test the feasibility of transferring a non-automated hydrolysis probe assay onto an ARIES System, we designed a single well assay to detect *H. pylori* and clarithromycin resistance based on previously published literature. An internal control hydrolysis assay targeting MHV was run within the same well as described in published literature. All experiments were performed with commercially available control materials in a stool matrix. The impact of proteinase K and carrier RNA sample pre-treatment was also evaluated by testing their effect on the CT of a pan-adenovirus hydrolysis probe assay, employing the same MHV control as described above. These experiments were conducted with commercially-available control materials in UTM. **Results:** We observed that overall, pre-treatment with proteinase K and carrier RNA reduced CTs by ~3 (1 log), with no significant impact to CT standard deviation. Standard deviation was found to be less than 0.5 logs. Positive and negative controls for *H. pylori*, as well as clarithromycin sensitive and resistant strains, gave the expected results when the hydrolysis probe chemistry was performed on the ARIES System. **Conclusions:** We demonstrated that the ARIES System could be used to automate hydrolysis probe real-time PCR workflows by applying published assay conditions to the ARIES platform. We also showed that CTs can be reduced by approximately 1 log by incorporating a sample pre-treatment such as adding carrier RNA and/or proteinase K to the sample prior to addition to the ARIES cassette. Sample types that may benefit from the pre-treatment procedure include those that are low in nucleic acid content (e.g., UTM, CSF, plasma, Amies) and/or high in protein content (e.g., serum, whole blood). Automation of real-time PCR workflows as



demonstrated in this research study may help laboratories reduce hands-on time and turnaround time for molecular tests.

#### ID054. Simultaneous Detection of Tick-borne Pathogens Using a High Definition Multiplexed PCR Assay

M.W. Mashock, M.L. Faron, N.A. Ledebor, B.W. Buchan  
The Medical College of Wisconsin, Milwaukee, WI.

**Introduction:** Effective diagnosis of tick-borne illness remains a challenge, in part, because of a lack of readily available diagnostic tests and the array of pathogens transmitted by various tick species. Limited singleplex PCR tests are available through reference laboratories; however, no multiplexed syndromic options are currently available. The purpose of this study is to assess the performance of the Research Use Only (RUO) Tick-borne Panel (TBP) (ChromaCode, Carlsbad, CA). **Methods:** The TBP utilizes a novel high definition PCR technology (HDPCR) to detect 12 tickborne pathogens [*A. phagocytophilum*, *B. microti*, *B. miyamotoi*, *Borrelia* Group 1 (*B. burgdorferi*, *B. mayonii*), *Borrelia* Group 2 (*B. hermsii*, *B. parkeri*, *B. turicatae*), *E. chaffeensis*, *E. ewingii*, *E. muris eauclarensis*, and *Rickettsia* spp.] using only 3 channels of a standard RT-PCR instrument. HDPCR identifies up to 3 different genetic targets in a single fluorescent channel by using a different probe concentration for each target. Differentiation of targets is based on signal intensity (brightness), which is correlated with the concentration of each probe. The presence of multiple targets in a single channel results in an additive fluorescent signal, indicating a dual positive result. This is different from traditional qPCR, which achieves multiplexing by color differentiation only. Performance of the TBP and HDPCR was evaluated using synthetic DNA samples spiked into TE buffer at approximate concentrations of 1,000-100,000 copies/mL. Positive and negative samples were de-identified and tested using the ABI 7500 Fast Dx instrument. Raw data was uploaded to a cloud-based program for analysis and interpretation. **Results:** A total of 91 specimens were tested including 57 single target, 21 dual target, and 13 negatives. All targets had a sensitivity greater than 90.0% except *A. phagocytophilum* (80%), *B. microti* (80%), and *E. ewingii* (85.7%). All targets had a specificity greater than 97.0% except *B. microti* (95.1%), *Borrelia* Group 2 (94.9%) and *E. chaffeensis* (96.5%). Overall test sensitivity and specificity was 91.9% and 97.8%, respectively. All 16 false positive and 8 false negative results were attributable to synthetic DNA template contamination at time of specimen preparation. Amplification signal was observed in all 8 false negative specimens; however, the additional signal generated by contaminant in the same fluorescence channel resulted in an incorrect call, i.e. a false positive and false negative result simultaneously. **Conclusions:** The TBP is capable of accurately identifying 12 tick-borne pathogens using a standard 4 channel RT-PCR instrument. Additional studies using prospective clinical specimens (blood, serum, lesion swab) are underway support these preliminary results.

#### ID055. Clinical Performance Study Results of the Hologic GBS Assay on the Fully Automated Panther Fusion System

B. Eaton<sup>1</sup>, C. Emery<sup>2</sup>, G. Capraro<sup>3</sup>, J. Longshore<sup>3</sup>, S. Young<sup>4</sup>, B. Grobarczyk<sup>5</sup>, L. Franzil<sup>5</sup>, D. Kolk<sup>1</sup>, M. Exner<sup>1</sup>

<sup>1</sup>Hologic, Inc., San Diego, CA; <sup>2</sup>Indiana University, Indianapolis, IN; <sup>3</sup>Carolinas Pathology Group, Charlotte, NC; <sup>4</sup>Tricare Reference Laboratories, Albuquerque, NM; <sup>5</sup>Diagenode, Seraign, Belgium.

**Introduction:** *Streptococcus agalactiae*, or Group B *Streptococcus* (GBS), is a common bacterium often carried in the intestines or lower genital tract. GBS is usually harmless in immunocompetent adults, however, in newborns it can cause severe complications. This problem has resulted in Centers for Disease Control and Prevention (CDC) recommended screening guidelines as a routine part of prenatal care in the United States. The Hologic Panther Fusion GBS Assay is a fully-automated *in vitro* diagnostic test utilizing real-time PCR for the rapid, qualitative detection of GBS from clinical specimens. The assay is intended to aid in determining the GBS colonization status of antepartum women using vaginal/rectal swabs collected, inoculated and incubated in enrichment broth. In this prospective clinical study, the sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the assay were evaluated using vaginal/rectal swabs incubated in either Lim or Carrot enrichment broth.

**Methods:** Residual de-identified Lim Broth or Carrot Broth from GBS screening samples were evaluated at three geographically distinct sites, Indiana University (IU), Tricare Reference Laboratories (TRL) and Carolinas Pathology Group (CPG). Each sample was tested with Hologic's Panther Fusion assay and the reference culture method following the CDC guideline. The sensitivity and specificity versus reference culture was determined for the Panther Fusion assay by site and overall. Lim Broth samples with discrepant results were tested with the BD Max GBS assay. **Results:** In total, 947 unique patient samples were collected and included in the study. IU and TRL used Lim Broth samples (n of 343 and 300, respectively) and CPG used Carrot Broth samples (n of 304). The clinical sensitivity for each site was 100% with all 203 combined reference positive samples detected with the Panther Fusion assay. The clinical specificity for each site was 97.9%, 96.1%, and 95.5% for IU, TRL and CPG, respectively. The combined study specificity was 96.5% with 718 of 744 reference negative samples not detected with the Panther Fusion assay. The combined study PPV and NPV were 88.7% and 100%, respectively. Of the 16 discrepant Lim Broth samples with culture negative and Panther Fusion assay positive results, 14 were confirmed positive with the BD Max GBS assay. **Conclusions:** The prospective clinical study results demonstrated excellent sensitivity and specificity for both Lim and Carrot Broth enrichment samples. The robust performance of the Hologic Panther Fusion GBS Assay in combination with the system's throughput, random access, full automation, and ease of use offers a novel molecular diagnostic approach to GBS testing.

#### ID056. Quantification of CMV Using the m2000 RealTime CMV Assay

M.A. Johnston, S.F. Allen, H.B. Steinmetz, G.J. Tsongalis, J.A. Lefferts  
Dartmouth-Hitchcock Medical Center, Lebanon NH.

**Introduction:** Reactivation of latent cytomegalovirus (CMV) is a common complication in immunocompromised transplant patients. The virus may originate in seropositive donors or in allografts from seropositive donors. Monitoring CMV viremia at regular time points following transplantation can help guide antiviral therapy and reduce the risk for serious morbidity in the transplant population. The Roche COBAS AmpliPrep/COBAS TaqMan CMV Test (Roche Molecular) was the first commercial FDA-IVD assay for quantitative measurement of CMV DNA in plasma of transplant patients. Recently, the Abbott RealTime CMV Assay, with automated extraction and PCR set-up, was also approved by the FDA. Prior to clinical use of the RealTime CMV Assay, a series of verification studies were carried out to confirm performance characteristics and to compare patient results with those obtained using the COBAS CMV assay.

**Methods:** Plasma samples from 56 patient samples previously tested with COBAS CMV assay were assessed with the RealTime CMV assay according to the package insert (0.75 mL input volume). Additionally, kit controls and a linearity panel (Acrometrix, ThermoFisher) were tested. All results were log transformed to log IU/mL concentrations for statistical analysis using linear regression plots and bias visualized using a Bland-Altman plot. **Results:** Qualitative concordance was observed among all 56 patient samples. CMV DNA was within the analytical measuring range of both assays for 49 patient samples. Results from the RealTime CMV assay were consistently higher than the COBAS assay by an average of 0.5 log IU/mL. An R<sup>2</sup> value of 0.9882 was observed when comparing RealTime CMV results of the linearity panel with expected values, demonstrating excellent linearity. Precision or reproducibility was demonstrated using calibrator material run as unknown samples with average values of 3.43 log IU/mL (SD 0.4, CV 1.14%) and 6.20 log IU/mL (SD 0.04, CV 0.61%). **Conclusion:** The Abbott RealTime CMV assay performed well in terms of accuracy, precision and linearity and was found to be suitable for clinical testing. A bias of approximately 0.5 log IU/mL was observed between the two assays. This difference has been previously reported and may be due to differences in target sequences and sizes of PCR products.

#### ID057. High-definition PCR (HDPCR): a Novel, Instrument Agnostic qPCR Multiplexing Technology Applied to Tick-borne Pathogen Testing

B. Amro, H. Carolan, R. Abanes, J. Nayak, J. Hill, C. Smith, B. Leatham, L. Jacky, S. Powell, A. Schroeder, K. Menge  
ChromaCode, Carlsbad, CA.

**Introduction:** The U.S. is seeing an increase in disease from infected ticks, tick species that carry multiple diseases, and an expanding geography of tick-borne cases. There are limited singleplex PCR options and no multiplex PCR options for detecting tick-borne pathogens. This study describes the development and performance of a research use only Tick-borne Panel (TBP RUO) using ChromaCode's novel HDPCR qPCR multiplexing technology. **Methods:** *HDPCR Overview:* HDPCR is based on Caltech IP and is a novel way to enable multiplex testing (12-20 targets) on 4-6 channel qPCR instruments with no hardware changes. While traditional qPCR relies on differentiation of targets by color, HDPCR uses probe concentration as the limiting reagent, allowing multiple targets in the same color channel to be distinguished by signal intensity (brightness). *Test Design:* TBP RUO is a 9 target, single well assay: Channel 1 - *Borrelia miyamotoi*, *Ehrlichia chaffeensis*, *Borrelia* Group 1 (*B. burgdorferi*, *B. mayonii*); Channel 2 - *Anaplasma phagocytophilum*, *Ehrlichia muris euclarensis*, *Rickettsia* spp.; Channel 3 - *Ehrlichia ewingii*, *Babesia microti*, *Borrelia* Group 2 (*B. hermsii*, *B. parkeri*, *B. turicatae*); Channel 4 - Internal control. *Analytical Studies:* A series of analytical studies are being performed to characterize the performance of TBP RUO using synthetic and archived whole blood (EDTA) specimens. These studies include limit of detection (LOD), single/dual positive, analytical sensitivity (inclusivity), and analytical specificity (exclusivity) studies. *Testing and Analysis:* Testing is being performed on the ABI 7500 Fast, QuantStudio 7, and Viia 7. Results are being analyzed by uploading raw data from the qPCR instrument to ChromaCode's cloud-based software. **Results:** The LOD study was completed using double stranded synthetic DNA. All targets had an LOD of 10 copies/mL except for *A. phagocytophilum*, *E. chaffeensis*, *E. muris euclarensis*, and *Borrelia* Group 2, which had an LOD of 10-100 copies/mL. The single/dual positive study consisted of testing 57 single target specimens at 1K, 10K and 100K copies/mL and 21 dual positive specimens with 1K vs 10K copies/mL concentration of the two targets. Sensitivity was 100% for all targets except for *B. microti* (88.9%) and *Borrelia* Group 2 (76.9%). Specificity for all targets was 100% except for *B. microti* (98.7%), *E. ewingii* (97.4%), and *Rickettsia* spp. (98.4%). Overall sensitivity and specificity across the entire panel were 96.0% and 99.4%, respectively. **Conclusions:** HDPCR is a new technology that enables higher multiplexing on existing qPCR instruments. TBP RUO provides broad coverage of tick-borne pathogens in a single well, multiplex qPCR test. Additional studies are underway to characterize analytical sensitivity and specificity.

#### ID058. Evaluation of a Completely Automated BKV Viral Load Assay on the Abbott m2000 Platform

F. Nolte, A.M. Kegl

Medical University of South Carolina, Charleston, SC.

**Introduction:** Routine screening of renal transplant recipients for BKV viruria and/or viremia is standard of care for early recognition and management of BKV-associated nephropathy at many transplant centers. Currently, there are no FDA-approved BKV viral load assays and a variety of laboratory-developed assays (LDA) are employed. In order to increase productivity and keep pace with increasing demand for this test we validated a completely automated BKV viral load assay on the m2000 system. **Methods:** The MGB Alert BKV analyte-specific reagent (ASR; EiiTech Group) was run on the m2000 *sp/rt* platform. The real-time PCR assay amplifies a conserved 72-nt sequence from the VP-1 gene and contains synthetic DNA internal control. The amplified products are detected with probes designed with a minor groove binder, modified bases, and a dark quencher. DNA was extracted using a total nucleic acid protocol where ethanol was added to the standard mSystem DNA lysis and wash reagents. The comparator method was a previously validated LDA using the same EiiTech ASR, but with DNA extraction performed on the Qiacube (Qiagen) using the mini elute virus spin kit and manual PCR reaction set up. Both assays were run on the

Abbott *rt* instrument using the same thermal cycle parameters and calibrated with a 6 member BKV panel (Exact Dx) with concentrations ranging from 2.79 to 7.79 log<sub>10</sub> copies/mL. Correlation and agreement between the two LDAs was assessed using 83 plasma and 81 urine samples. Dynamic range and reproducibility of the completely automated LDA was assessed with Exact Dx panel members and controls. We also conducted a workflow analysis of automated and manual methods to determine the reduction in labor time. **Results:** BKV DNA was detected by the automated and manual assays in 54 and 52 plasma samples, respectively, and in 24 and 22 urine samples, respectively ( $p > 0.05$ ). Forty-eight plasma and 18 urine samples gave values in both assays that were highly correlated ( $R^2 = 0.91$ ). Bland and Altman analysis showed mean bias of -0.27 log<sub>10</sub> with plasma for the automated assay with 95% lower and upper limits of agreement between the assays of -1.25 and 0.71 log<sub>10</sub>, respectively. The mean bias for urine samples with the automated assays was -0.31 log<sub>10</sub> with 95% lower and upper limits of agreement between the assays of -1.59 and 0.97 log<sub>10</sub>, respectively. The average CV% of the crossing point values over the measurement range of the assay was 3.98%. We estimated that the labor time was reduced by 7 hours/week by switching to the completely automated method. **Conclusions:** The completely automated assay on the m2000 platform performed as well as our manual assay for quantitation of BKV DNA in both plasma and urine with a significant decrease in labor time.

#### ID059. Validation of Qualitative HIV Detection of HIV in Whole Blood with the Hologic Aptima HIV Assay

K. Tardif<sup>1</sup>, P. Kharidia<sup>1</sup>, D.R. Hillyard<sup>2</sup>

<sup>1</sup>ARUP Laboratories, Salt Lake City, UT; <sup>2</sup>University of Utah, Salt Lake City, UT

**Introduction:** Qualitative HIV testing is used to assess HIV infection status for infants with exposure risk. Assays that detect HIV RNA and DNA from whole blood samples as well as HIV RNA only tests using plasma are each used for this application. In this study, we examined specimens submitted for HIV whole blood qualitative testing on the Roche COBAS AmpliPrep/COBAS Taqman assay and compared results with testing of whole blood on the Hologic Aptima RNA assay.

**Methods:** Whole blood was diluted either 3-fold in Aptima Blood Processing Media (350  $\mu$ L whole blood added to 700  $\mu$ L Aptima Blood Processing Media) or 5-fold in Aptima Specimen Diluent (200  $\mu$ L whole blood added to 800  $\mu$ L Aptima Specimen Diluent) prior to testing 500  $\mu$ L in the Aptima assay. For testing in the Roche assay, 100  $\mu$ L of whole blood was added to 1000  $\mu$ L Specimen Pre-extraction Reagent (SPEX) before incubating at 56 °C for 10 minutes with shaking at 1000 rpm. Plasma was prepared from whole blood by centrifugation for 10 minutes at 1,000-2,000 xg. Analytical sensitivity of the Aptima assay was determined using serial dilutions of whole blood spiked with the 2<sup>nd</sup> WHO HIV RNA International Standard (NIBSC code 97/650). **Results:** When 35 whole blood samples with 'detected' results in the Roche assay were diluted in Aptima Specimen Diluent, 89% (31/35) generated a 'detected' result in the Aptima assay. Comparatively, for 28 whole blood samples with "detected" results in the Roche assay, 96% (27/28) had detectable HIV RNA levels in the Aptima assay when diluted in Aptima Blood Processing Media. Plasma prepared from eleven whole blood samples with 'detected' results in the Roche assay generated 'detected' results in the Aptima assay in only 73% (8/11) of samples. Analytical sensitivity of whole blood spiked with the WHO RNA Standard and diluted in Aptima Blood Processing Buffer was 105 copies/mL. **Conclusions:** Performance of the Aptima test was highest with whole blood diluted in Aptima Blood Processing Buffer compared to dilution in Aptima Specimen Diluent and plasma prepared from whole blood. The higher number of discordant results with plasma samples may be the result of HIV RNA lost in peripheral blood mononuclear cells (PBMCs) when cells are removed during centrifugation. Additional testing of a larger number of plasma samples is underway to confirm these differences.

#### ID060. The Prevalence of Clarithromycin-resistant *Helicobacter pylori* in Utah; a Laboratory-based Survey

K.N. Carter<sup>1</sup>, B.K. Lopansri<sup>2</sup>, W.B. Sinclair<sup>1</sup>, N. Spencer<sup>1</sup>, J. Wallentine<sup>3</sup>, J.F. Carlquist<sup>4</sup>

<sup>1</sup>Intermountain Healthcare, Murray, UT; <sup>2</sup>Intermountain Healthcare/Intermountain Medical Center, Murray UT; <sup>3</sup>Intermountain Healthcare/Intermountain Central Laboratory, Murray UT; <sup>4</sup>Intermountain Healthcare/LDS Hospital, Salt Lake City UT.

**Introduction:** *Helicobacter pylori* infection is a major cause of peptic ulcer disease, dyspepsia, and atrophic gastritis in humans. Approximately ninety-six million people in the United States and nearly two-thirds of the world's population are infected with *H. pylori*. First line therapy is a Clarithromycin-based triple therapy. Recent studies conducted outside the United States suggest a significant rise in Clarithromycin resistance (Hospital de la Princesa Universidad Autónoma de Madrid, Madrid, Spain) with United States surveys showing resistance rates of 35-40% (Centers for Disease Control and Prevention). We conducted a prevalence study to determine the frequency of Clarithromycin-resistant *H. pylori* within our patient population. **Methods:** From October 2017 to April 2018, 136 raw stool specimens were acquired retrospectively from a population of patient samples previously tested at the Intermountain Central Laboratory (Murray, UT) using *H. pylori* stool antigen assay (Meridian Bioscience) and found to be positive for *H. pylori*. Samples were collected and extracted for total DNA using the QIAamp Fast DNA Stool Mini Kit (Qiagen). Real-time PCR was performed on the Qiagen Rotor-Gene Q instrument. Analyte Specific Reagents utilized included: *H. pylori*/Clarithromycin Resistance Primer Set, *H. pylori* Probe, and *H. pylori* Clarithromycin Resistance Probe (Meridian Bioscience, Inc.). This method was utilized to detect the presence of *H. pylori* as well as mutations correlated with resistance within the *H. pylori* 23S rRNA region. Clarithromycin resistance was determined by Melt Curve Analysis and compared to sequencing results provided by Meridian Bioscience, Inc. **Results:** Of 2,664 stool samples screened, 185 (6.9%) tested positive for *H. pylori*. One hundred and two positive samples were randomly selected for molecular testing. Of these, 23.5% were positive for the point mutations that correlated with Clarithromycin resistance (A2143G, A2142G). **Conclusions:** Despite being lower than the national average, the high prevalence of resistance in our patient population suggests that a substantial number of patients with *H. pylori* infection are at risk for treatment failure. Knowledge of Clarithromycin resistance at the time of diagnosis can aid in selecting a treatment regimen to improve treatment success with *H. pylori* eradication.

#### ID061. Is There a Need for HCV Resistance Testing in Routine Diagnostics and Patient Treatment? Routine HCV Genotyping and Resistance Testing and Performance of the Sentosa SQ HCV Genotyping v2.0 Assay

M. Obermeier, A. Moritz, S. Breuer, R. Ehret  
Medical Center for Infectious Diseases, Berlin, Germany.

**Introduction:** The need for HCV resistance testing in routine diagnostics is still a subject of debate. We evaluated the recently updated Sentosa SQ HCV Genotyping Assay v2.0 from Vela diagnostics, a next generation sequencing (NGS)-based assay for identification of HCV genotypes 1 to 6 and detection of resistance associated substitutions (RAS). In comparison to the previous version now a nearly complete NS5B sequence can be obtained (v1: Amino Acid (aa) 339 – 566; v2: aa 1 – 566) with more robust RAS detection in all HCV genotypes. Prevalence of RAS in samples from our cohort in Berlin is reported here. **Methods:** Two hundred and seventy-four samples were tested with the Sentosa SQ HCV Genotyping Assay v2.0 for genotyping and resistance detection in the NS3, NS5A and NS5B genes. Beside the included sequence analysis in the Vela system we interpreted the raw data using our own NGS analysis pipeline. Resistance interpretation was performed with the geno2pheno [hcv] 0.92 online tool (<http://hcv.geno2pheno.org>). Samples were collected within the PEPSI project, a German wide initiative to collect data on HCV RAS prevalence coordinated by the University of Cologne. **Results:** Sequences for 274/276 samples could be generated, only missing two samples with low viral loads (<1,000 IU/mL) and a low number of drop outs for single genes (NS3 25/274, NS5A 10/274 and NS5B 3/274) mainly in non-1, non-3 genotypes. Genotype distribution was 1a (100), 3a (80), 1b (51), 4d (16),

2b (10), 4a (8), 6a (3), 2a (2) and one of 1g, 2c, 2k, 4o and 4r each. Overall NS3, NS5A and NS5B genes showed RAS in 21.9%, 16.8% and 9.9%, respectively. 33 of 51 1b genotype samples showed resistance, two samples were resistant to three classes. 50/100 genotype 1a samples had resistance mutations, mainly NS3 Q80K (35x) but also NS5A (12x 28VT and 7x 93HN) and NS5B (3x556g; 2x553v, 316Y and 556R). Twelve out of 80 samples with genotype 3a had NS5A mutations (6x 30K, 2x 30V and 6x 93H), except one 80K the genotype 3a samples had no NS3 or NS5B mutations. Overall 167 RAS were detected. (71, 60, 36 for NS3, NS5A and NS5B) including 35 RAS in minorities with a cut-off below 10% and greater than 2% of population. **Conclusions:** The Sentosa SQ HCV Genotyping Assay v2.0 performed excellent in the tested samples. A relative high proportion of investigated sequences showed RAS at a minority cut-off of 10%. Lowering this cut-off didn't significantly increase the number of detected RAS. Those RAS led to restrictions for single drugs or whole drug classes. The relatively high rates of RAS clearly indicate the necessity of resistance analyses before starting an HCV-therapy to avoid therapy failures and unnecessary given drugs avoiding side-effects and costs.

#### ID062. Evaluation of the ARIES *Bordetella* Assay for Detection and Identification of *Bordetella pertussis* in Nasopharyngeal Swab Specimens

T. McMillen, H. Chow, E. Babady  
Memorial Sloan Kettering Cancer Center, New York, NY.

**Introduction:** Pertussis is an acute, contagious respiratory disease caused by the bacterium *Bordetella pertussis*. *B. pertussis* can lead to life-threatening complications in infants and young children. *Bordetella parapertussis* similarly causes a pertussis-like syndrome, mainly in young children. Early and accurate diagnosis is critical for positive patient outcome as treatment is most effective within the first two weeks of infection. The ARIES *Bordetella* Assay (Luminex Corporation) has recently received FDA-clearance for the detection and differentiation of *B. pertussis* and *B. parapertussis* from nucleic acid in nasopharyngeal swab (NPS) specimens. The objective of this study was to evaluate the performance of the new ARIES *Bordetella* Assay (ARIES BA) in comparison to the BioFire FilmArray Respiratory Panel (FA RP). **Materials and Methods:** The ARIES BA was evaluated using retrospective, remnant, de-identified specimens, previously tested by FA RP. Remnant specimens were held at room temperature (15-30°C) for up to 8 hours, refrigerated at 2-8°C for up to 7 days, or frozen at ≤ -80°C prior to ARIES BA testing. Performance characteristics evaluated included positive (PPA) and negative percent agreement (NPA) with the FA RP. **Results:** A total of 300 NPS samples were included in the study, including 17 samples positive for *B. pertussis* by FA RP and 283 samples negative for *B. pertussis* by FA RP. Of the 283 samples negative for *B. pertussis*, 207/281 were negative for all pathogens detected by the FA RP and 74/281 samples were positive for pathogens others than *B. pertussis* including Rhinoviruses, Influenza B, Influenza A, Human metapneumovirus, Parainfluenza viruses, RSV and Adenovirus. There were no specimens positive for *B. parapertussis*. The ARIES BA was positive for 11/19 samples for a PPA of 64.7% (95% CI: 38.3-85.8%) with the FA RP. All NPS negative for the *B. pertussis* by FA RP were concordantly negative by the ARIES AB for a NPA of 100% (95% CI: 98.7-100%). The overall agreement between the ARIES BA and FA RP for the detection of *B. pertussis* was considered good at 98% with a kappa value of 0.78 (95% CI: 60.3-94.9%). **Conclusions:** Given the relatively well-known epidemiology of pertussis, an FDA-cleared *B. pertussis*/parapertussis molecular test may be more appropriate and cost-effective than a large multiplexed panel when *B. pertussis* infection is suspected. The ARIES BA offers a targeted approach to the rapid diagnosis of pertussis. The specificity of the ARIES BA was excellent, showing no cross-reactivity with common pathogens identified in NPS. On-going analysis of the discordant results by bi-directional sequencing will provide further information on the sensitivity of the ARIES BA.



#### ID063. A Quantitative, Multiplexed RNA Detection Platform for Rapid Pathogen Identification and Phenotypic Antibiotic Susceptibility Testing (AST) using NanoString Technology

R.P. Bhattacharyya<sup>1</sup>, R. Khafizov<sup>2</sup>, R. Boykin<sup>2</sup>, D. Dunaway<sup>2</sup>, L. Wu<sup>2</sup>, C.J. Liu<sup>3</sup>, S.S. Son<sup>1</sup>, N. Bandyopadhyay<sup>1</sup>, M. Walker<sup>1</sup>, N. Shores<sup>1</sup>, J. Livny<sup>1</sup>, J. Beechem<sup>2</sup>, D.T. Hung<sup>1</sup>

<sup>1</sup>Broad Institute, Cambridge, MA; <sup>2</sup>NanoString Technologies, Inc., Seattle, WA; <sup>3</sup>University of California, Berkeley, CA.

**Introduction:** Traditional culture-based assays, though accurate, are too slow to guide early antibiotic selection, while newer genotypic methods require more knowledge of antibiotic resistance mechanisms to predict phenotype. RNA detection offers a single approach for universal pathogen identification, and quantitative measurement of key antibiotic-responsive transcripts allows a rapid, phenotypic AST assay, agnostic to the genetic basis for resistance. **Methods:** For bacterial identification, we designed 190 probes that bind to regions of the 16S and 23S rRNA from 100 different bacterial pathogens, compatible with the NanoString nCounter for multiplexed, quantitative RNA detection. We tested this probeset against 150 isolates across 70 species. For AST, we defined a set of antibiotic-responsive genes using RNA-Seq in *Klebsiella pneumoniae*, *Escherichia coli*, and *Acinetobacter baumannii* whose expression level after exposure to one of three antibiotic classes (carbapenems, fluoroquinolones, and aminoglycosides) best distinguishes between susceptible and resistant isolates. We then designed probes to detect these antibiotic-responsive genes using crude lysate. We developed a classifier using machine learning algorithms to classify ~100 clinical isolates, including a “test set” of multidrug-resistant strains from the CDC, in a NanoString assay that took 8 hours for AST. We also designed probes to detect 5 common carbapenemase gene families (*KPC*, *NDM*, *IMP*, *VIM*, *OXA-48*).

**Results:** Using a 190-probe NanoString probeset, we identified 150 isolates representing 70 different bacterial species with >97% accuracy to the species level. All errors involved misidentifications to closely related species with similar clinical behavior. For AST, we tested 192 pathogen-antibiotic pairs across these 3 species and 3 antibiotics, with >98% accuracy and no resistant organisms misclassified as susceptible. We also detected carbapenemase genes in >75 CRE strains across 8 species, with 100% sensitivity and specificity, and no false positives in >30 non-carbapenemase containing strains. This phenotypic AST assay was performed in 8 hours from a positive blood culture bottle with minimal sample processing. **Conclusions:** We demonstrate accurate, sensitive broad-range bacterial identification and phylogenetic classification in a single multiplexed assay. The same RNA detection platform can also provide rapid, accurate phenotypic AST, with simultaneous detection of key genetic resistance determinants in a single assay, enhancing accuracy of carbapenem resistance detection and flagging discordant results. Finally, we are adapting the assay to Hyb & Seq technology for faster turnaround time.

#### ID064. The Galileo Pathogen Solution Next-Generation Sequencing Pipeline Detects and Identifies RNA Respiratory Viruses in Haematopoietic Stem Cell Transplant Patients

M.L. Carpenter<sup>1</sup>, G. Wang<sup>1</sup>, S. Kaplan<sup>2</sup>, V. Nagesh<sup>1</sup>, T. McMillen<sup>2</sup>, G. Wall<sup>3</sup>, Y. Tang<sup>2</sup>

<sup>1</sup>Arc Bio, LLC, Menlo Park, CA; <sup>2</sup>Memorial Sloan Kettering Cancer Center, New York, NY; <sup>3</sup>Arc Bio, LLC, Cambridge, MA.

**Introduction:** Arc Bio is developing the Galileo Pathogen Solution (Galileo), a sample-to-report next-generation sequencing pipeline for comprehensive pathogen detection that will eliminate the need to query specific pathogens independently. The assay incorporates a proprietary step to enrich for pathogen-specific sequence reads, as well as an automated bioinformatics pipeline. In this proof of principal study, we demonstrate its utility for the detection of RNA respiratory viruses in haematopoietic stem cell transplant patients. **Methods:** Seventy-five residual nasopharyngeal swabs (NPS) previously tested with the FilmArray assay (bioMérieux) were re-tested with the Galileo assay (15 RNA viruses in total). In addition, Respiratory Syncytial Virus (RSV) A, RSV B, and Influenza A H1N1 (Exact Diagnostics) were spiked into negative NPS samples to test the computational discrimination of these strains. In brief, total nucleic acid was extracted using the EZ1 platform (QIAGEN), followed by RNA library preparation with pathogen

enrichment/human background depletion, sequencing (Nextseq 500, Illumina), and automated data analysis. Approximately 5 million paired-end reads were sequenced per sample. Reads were filtered based on sequence quality and queried against a curated selection of references. **Results:** In the spike-in samples, all three pathogens were correctly discriminated. For the 75 NPS samples (70 positives, 5 negatives) compared to FilmArray, an overall positive percent agreement (PPA) of 92% at the species level was obtained (two samples failed QC prior to sequencing). At the individual virus level, the PPAs were as follows: Influenza 93.3%, Coronavirus 95.5%, Parainfluenza 81.8%, Human Metapneumovirus 84.6%, and RSV 100%. FilmArray and Galileo agreed on 21 Coronavirus strain calls and differed on one; FilmArray and Galileo agreed on Influenza A H1 or H3 in 10 calls and differed in 6. Parainfluenza 1 & 3, and Influenza B were correctly discriminated by Galileo. RSV A vs. B were discriminated by Galileo, but not by FilmArray. Furthermore, an additional 43 potential co-infections were detected with the Galileo assay. **Conclusions:** This proof of concept dataset suggests that the Galileo assay may be a promising solution for the comprehensive detection of respiratory RNA viruses at similar levels of sensitivity to an FDA-cleared method. Further work includes testing additional NPS samples and expanding the set of target pathogens. Additional studies are planned to fully determine performance characteristics with a design-locked kit.

#### ID065. Molecular Screening for *Trichomonas vaginalis* and *Mycoplasma genitalium* in the RADAR Longitudinal Cohort Study of Young Transgender Women and Young Men who Have Sex with Men

E. Munson<sup>1</sup>, D. Ryan<sup>2</sup>, A. Clifford<sup>2</sup>, B. Mustanski<sup>2</sup>  
<sup>1</sup>Marquette University, Milwaukee, WI; <sup>2</sup>Northwestern University, Chicago, IL.

**Introduction:** RADAR is a longitudinal cohort study of young transgender women (YTW) and young men who have sex with men (YMSM) that aims to understand the multilevel influences on HIV risk and substance use. Among factors investigated within these populations to better understand and prevent HIV infection is detection of sexually-transmitted infection (STI) agents. Increased sensitivity and specificity inherent to certain molecular diagnostics has facilitated off-label application of these assays to extra-urogenital specimens. The purpose of this investigation was to characterize detection rates of several STI agents and associated risk behaviors within this cohort. **Methods:** Study participants were required to be assigned male at birth, be between 16 and 29 years of age, and have had a sexual encounter with a male in the previous year or identify as gay, bisexual, or transgender. Within a three-month interval, 322 participants (92% YMSM) submitted first-void urine and rectal swabs that were assessed by commercial transcription-mediated amplification (TMA)-based assays for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* and by off-label TMA-based *Trichomonas vaginalis* and *Mycoplasma genitalium* testing. All positive results were verified by repeat testing. **Results:** Via routine *C. trachomatis* and *N. gonorrhoeae* screening, 51 participants (15.8%) were infected with either (23 with *C. trachomatis*; 22 with *N. gonorrhoeae*) or both (6 participants) agents. Overall detection rates ranged from 0.6-1.5% (urine) and 7.7-8.6% (rectal) for these two agents. Off-label screening identified 71 additional participants with STI (3 *T. vaginalis*; 68 *M. genitalium*). 90.1% of these novel identifications were generated from analysis of rectal specimens (3 participants with *T. vaginalis*; 61 participants with *M. genitalium*). Overall detection rates of *T. vaginalis* (0.3% urine; 1.5% rectal) and *M. genitalium* (9.2% urine; 23.9% rectal) were variable. Black participants yielded more rectal detection of *M. genitalium* (33.6%) than non-black participants (17.4%;  $\chi^2 = 11.03$ ;  $P < 0.001$ ). Fifty-two percent of HIV-positive participants yielded rectal detection of *M. genitalium* in contrast to 14.5% of HIV-negative participants ( $\chi^2 = 45.35$ ;  $P < 0.0001$ ). Participant age, cisgender/non-cisgender status, condomless insertive anal/vaginal sexual practice, and condomless receptive anal sexual practice were not associated with rectal *T. vaginalis* ( $P \geq 0.38$ ) or rectal *M. genitalium* ( $P \geq 0.25$ ) detection. **Conclusions:** Additive analyte testing, including that performed on extra-urogenital specimens, contributes to comprehensive STI screening. Additional studies may be warranted to determine the significance of rectal *T. vaginalis* and *M. genitalium* detection in the context of HIV infection.

# **ID066. Evaluation of Performance and Workflow using the GenePOC Strep A, C/G Assay for Detection of Group A, C, and G *Streptococcus* from Patients Presenting with Pharyngitis to the Emergency Department**

D. Mastandrea, A.T. Harrington

Loyola University Medical Center, Maywood, IL.

**Introduction:** Although Group A *Streptococcus* (GAS) is the most common etiology of bacterial pharyngitis, Group C and G (primarily *Streptococcus dysgalactiae* subspecies *equisimilis* (SDSE)) also cause bacterial pharyngitis that is clinically indistinguishable from GAS. GAS can be detected using a rapid antigen detection test (RADT), but there are currently no RADTs that detect SDSE. Culture can take 48-72 h, which impacts effective antimicrobial stewardship efforts. Molecular methods may offer alternatives to improve speed and accuracy in the diagnosis. The purpose of this study was to evaluate the performance and workflow of the GenePOC Strep A, C/G Assay as compared to standard diagnostic testing for throat swabs from our emergency department.

**Methods:** We tested residual throat swab specimens obtained from patients with signs and symptoms of pharyngitis collected for standard clinical care using the GenePOC Strep A, C/G Assay (GPACG) with the revogene instrument and compared the results to RADT for GAS and bacterial culture. GPACG is a qualitative real time PCR assay that can be run in batches from one to eight. Culture was performed using Strep Selective Agar plates incubated in CO<sub>2</sub> for 48 hours. Beta hemolytic colonies were identified using latex agglutination and MALDI-TOF MS based identification. Patient demographics and data points relevant to workflow analysis, including time of specimen collection, were also collected. **Results:** 96 consecutive swabs from unique patients collected in the emergency department were evaluated. 27 (28%) samples were positive by culture for GAS, 3 (3%) were positive by culture for SDSE, and 66 were negative. Three samples were positive for GAS by RADT but negative by culture and GPACG. One sample that was negative by RADT and culture was positive for GAS by GPACG. Overall the GPACG Assay demonstrated 99% agreement with culture based methods. In this evaluation the sensitivity of RADT for GAS was 77% and the specificity was 96%. Based on volume, all samples could be processed using one batched run per day. However, runs with fewer samples could be run at several time points during the day to achieve a more rapid turn around time. **Conclusion:** The GPACG provided comparable results for the detection of GAS and SDSE when compared to culture and significantly improved detection for GAS when compared to RADT. The limited 'hands-on' time in the workflow makes the assay advantageous for evaluating specimens on all shifts, which has the potential to optimize patient care and throughput in a busy emergency department.

# **ID067. Evaluation of the BD MAX Vaginal Panel for the Detection of Vaginitis in Women**

K. Culbreath<sup>1,2</sup>, K. Timm<sup>2</sup>, A. Thompson<sup>1</sup>

<sup>1</sup>University of New Mexico School of Medicine, Albuquerque, NM; <sup>2</sup>TriCore Reference Laboratories, Albuquerque, NM.

**Introduction:** The three main causes for vaginitis are bacterial vaginosis (BV), vulvovaginal candidiasis (VVC) and trichomoniasis (TV). Two multiplex assays are FDA-cleared for molecular detection of vaginitis: BD Affirm VPIII (VPIII) and BD MAX Vaginal Panel (MVP). The VPIII is a nucleic acid probe-based detection of *G. vaginalis*, *Candida* species and TV. The MVP assay is an NAAT detecting the DNA of multiple pathogens associated with BV, *Candida* (*Candida* group, *C. glabrata*, *C. krusei*), and TV. The purpose of this study is to evaluate the performance of the MVP assay compared to VPIII. **Methods:** We conducted a prospective study in which women with symptoms of vaginitis were recruited at 4 clinical sites between January and May 2018. Each patient had 4 vaginal swabs collected with these devices: BD Affirm VPIII Ambient Temperature Transport System, BD MAX UVE Specimen Collection Kit, Aptima Vaginal Swab Specimen Collection Kit and BD Liquid Amies Elution Swab (ESwab). The VPIII and MVP were performed according to manufacturer's instructions. Discordant results were resolved by: the Aptima Trichomonas vaginalis Assay, culture using BBL CHROMagar Candida and BBL Sabouraud Dextrose Agar Emmons, and Gram stain for Nugent scoring. Results were considered a true positive if there were at least 2 tests positive for the target. The study was approved and performed in

accordance with the Institutional Review Board. **Results:** A total of 117 women were enrolled in the study. The prevalence of BV, candidiasis and TV was 44.9%, 22%, and 5.3% respectively. The sensitivity and specificity of the MVP for BV was 93.8% (95% CI 82.1-98.4) and 96.4% (95% CI 86.4-99.4). The sensitivity and specificity of the VPIII for BV was 95.9% (95% CI 84.6-99.3) and 83.6% (95% CI 70.6 – 91.8). Five samples were intermediate by Nugent score, 4/5 and 1/5 were negative by MVP and VPIII, respectively. These were not included in the final analysis. The sensitivity and specificity of the MVP for *Candida* was 94.1% (95% CI 78.6-99.0) and 100% (95% CI 93.9-100). The sensitivity and specificity of the VPIII for *Candida* was 75% (95% CI 56.2-87.9) and 100% (95% CI 94.0-100). For TV, the sensitivity and specificity for both MVP and VPIII were 100% and 100%. The overall sensitivity and specificity of the MVP was 96.5% (95% CI 89.5 – 99.1) and 98.3% (95% CI 95.4-99.4) compared to 88.5% (95% CI 79.4 – 94.1) and 96.2% (95% CI 92.7 – 98.1) for the VPIII. **Conclusions:** In this study evaluating the performance of the BD MAX Vaginal Panel, we demonstrated improved accuracy for MVP for the detection of bacterial vaginosis and *Candida* compared with BD Affirm VPIII and no difference for detection of TV.

# **ID068. Evaluation of ELITech HSV 1&2 ELITe MGB for the Detection and Differentiation of Herpes Simplex Virus 1 and 2 from Lesions**

V.P. Maceira<sup>1,3</sup>, L.M. Tomaszek<sup>2</sup>, S.A. Butt<sup>1</sup>, K.A. Stelrecht<sup>1,3</sup>

<sup>1</sup>Albany Medical Center Hospital, Albany, NY; <sup>2</sup>University at Albany, State University of New York, Albany, NY; <sup>3</sup>Albany Medical College, Albany, NY.

**Introduction:** Nucleic Acid Amplification Technology (NAAT) is the standard of care for the diagnosis of a wide variety of infectious diseases including Herpes Simplex Virus 1 and 2 (HSV 1 and HSV 2). Herpes simplex viruses are host-adapted pathogens that are traditionally associated with orofacial (HSV 1) and genital (HSV 2) disease; however, lesion location is not always indicative of viral presence. Current HSV assays on the market offer higher sensitivity with less complexity/turn-around time (TAT) than viral culture. **Methods:** We evaluated the performance of the ELITech HSV 1&2 ELITe MGB assay on the ELITe InGenius system (ELITe) as compared to our laboratory-developed test (LDT) for HSV detection from sterile fluids using ELITech's MGB Alert HSV ASR primers and probe (ASR) on Cepheid SmartCyclers using residual extracts from ELITe. These assays were also compared to our laboratory test of record (TOR) for lesion specimens, a conventional LDA PCR targeting gB with an enzyme-labeled oligo sorbent assay (ELOS) for amplicon detection. The evaluation of the clinical performance involved a retrospective analysis of 138 consecutive lesion swabs in viral transport, received in the laboratory for HSV testing by TOR (HSV incidence = 33%), as well as 43 similar specimens, previously determined to be positive for HSV by TOR. **Results:** Of the 181 clinical specimens, 86 were positive for HSV by TOR. No specimens were invalid and all 86 were positive with both ELITe and ASR. ELITe has the added benefit of viral typing in which 35 specimens were HSV-1, 50 were HSV-2 and 1 was positive for both HSV-1 and HSV-2. Of the 95 specimens negative by TOR, 5 were positive by both ELITe and ASR (HSV-2) and 1 specimen was positive by ASR only. Based on definition of true positives as positive by two or more tests, the sensitivities of ELITe and ASR were 100%, while the sensitivity for TOR was only 95% for all HSV and 100% and 91% for HSV-1 and HSV-2, respectively. **Conclusions:** In summary, ELITe and ASR demonstrated excellent performance in detecting HSV. ELITe also differentiates HSV 1 & HSV 2. In addition, the kit is optimized for use on the ELITech ELITe InGenius sample to result system, which is an excellent option for laboratories seeking an automated molecular platform.

# **ID069. Different CMV Strains for Quality Controls and its Impact on Assay Calibration**

J. Boonyaratankornkit, W. Zuberi

Exact Diagnostics, Fort Worth, TX.

**Introduction:** Currently for CMV assay calibration, three different strains are utilized including the Merlin, AD169, and Towne strain. With the prevalence of different strains in primary and secondary standards as well as different gene targets from the assay manufacturers it is important to understand its impact on assay calibration. For BKV and JCV standards, recent studies have shown that cultured standards have sub-populations

of deletions among major genes for which assays target. This would lead to a mis quantification of viral load and improper assay calibration. Utilizing the combination of sequencing and digital PCR, we are assessing the potential quantification differences of the CMV assays calibrated with different strains of CMV. **Methods:** CMV Merlin and AD169 genomic DNA was diluted to a level of  $10^4$  copies/mL. Both strains were normalized to using the UL132 gene on the Digital Droplet PCR (ddPCR). Both strains were then tested on the DiaSorin CMV Primer Pair, and the Altona RealStar CMV ASR. Both strains were sequenced on the Illumina MiSeq using a MiSeq Nextera XT Kit and compared against AY446894.2 Human herpesvirus 5 strain Merlin, complete genome obtained from GenBank. Analysis was performed on Geneious v 11.1.4. **Results:** After normalization of the CMV Merlin and AD169 to  $10^4$  copies/mL, testing was performed on the Altona RealStar CMV ASR on the ABI 7500 FAST and the DiaSorin CMV Primer Pair on the 3M Integrated Cyclor. Results on both the DiaSorin CMV Primer Pair and the Altona RealStar CMV ASR for the AD169 was  $1.90 \times 10^4$  E4 copies/mL and  $2.58 \times 10^4$  copies/mL respectively. For the Merlin strain, DiaSorin CMV Primer Pair was  $2.29 \times 10^4$  copies/mL and Altona RealStar CMV ASR was  $4.39 \times 10^4$  copies/mL. Calculating the fold difference of AD169 to the Merlin Strain, the DiaSorin CMV Primer Pair is 0.83-fold difference while the Altona RealStar CMV ASR is 0.59-fold difference. Sequencing results at the UL83 gene target for the DiaSorin CMV Primer Pair indicates that the AD169 has lower coverage than CMV Merlin. **Conclusion:** Calibrating CMV assays using standards manufactured from different strains of CMV can lead to quantification differences. Due to the nature of the cultivation of these viral strains, sequencing provides information if sub-population with deletions exist within the culture and the strain. Digital PCR can confirm the quantification differences and provide correction factors. Assay calibration can be improved with a better understanding of the different CMV standards available.

**ID070. Detection of Microorganisms and Antibiotic Resistance Genes in Skin and Soft Tissue Infections by a PCR-based Diagnostic Test**  
G. Zhu, E. Dawson, K. Balasubramanian, F. Espin, A.L. Del Tredici  
Millennium Health, LLC, San Diego, CA.

**Introduction:** Identification of pathogens in chronic wounds and skin and soft tissue infections (SSTIs) is recommended for targeted antibiotic therapy (Stevens, *et al.*, Clin Infect Dis 2014). Delayed initiation of appropriate antibiotic treatment targeted to the causative pathogen has been associated with treatment failure (Amin, *et al.*, Mayo Clin Proc 2014). Molecular diagnostics offers an accurate, fast and culture-independent alternative that is especially well suited for identifying microorganisms requiring extended time for growth in culture. **Methods:** Retrospective analysis was performed on more than 800 de-identified outpatient wound samples subjected to PCR-based clinical testing. The test targets 28 organisms (aerobic bacteria, anaerobic bacteria, and fungi) that were selected based on their frequency and pathogenicity in various types of wounds and SSTIs. The test also includes 13 antibiotic resistance genes and the staphylococcal virulence factor Pantone-Valentine leukocidin. **Results:** The majority of samples (90%) had  $\leq 3$  detected organisms and  $\leq 2$  detected antibiotic resistance genes. The overall frequencies of detected organisms are consistent with previously published culture-based studies of SSTIs (Moet, *et al.*, Diagn Microbiol Infect Dis 2007). For example, the top three most frequently detected organisms included *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*. In addition, *mecA* was the most commonly found antibiotic resistance gene. Interestingly, there were significant differences in the frequency of certain bacterial and fungal species among samples from different types of wounds. For example, the frequency of *P. aeruginosa* was the highest in venous leg ulcers (VLUs) compared to other types of wounds. This is consistent with previous reports (Andrejcek, *et al.*, Pol Przegl Chir 2016; Renner, *et al.*, Eur J Dermatol 2012; Thomsen, *et al.*, Wound Repair Regen 2010) that showed high frequency of *P. aeruginosa* in VLU samples. Notably, results for all organisms and antibiotic resistance genes tested were reported to clinicians the day after specimens were received. **Conclusions:** PCR-based testing of organisms and antibiotic resistance genes offers a more rapid alternative to traditional culture and susceptibility testing for wounds and SSTIs.

**ID071. Comparison of Three Nucleic Acid Amplification Tests (NAATs) to Culture for Detection of Group B Streptococcus (GBS)**  
J. Shin<sup>1</sup>, C. Hentzen<sup>2</sup>, D.T. Pride<sup>1</sup>

<sup>1</sup>University of California, San Diego, CA; <sup>2</sup>Hologic, Inc., San Diego, CA.

**Introduction:** Group B *Streptococcus* (GBS) is a commensal bacterium found in the normal microbiota of the gastrointestinal and genitourinary tract of healthy women. While GBS colonization is usually asymptomatic, GBS can infect neonates through vertical transmission during labor and delivery, resulting in early-onset disease (EOD). Current recommendations for the prevention of EOD include a universal culture-based screening method for pregnant women 5 weeks before delivery to administer intrapartum antibiotics. It is important to accurately determine GBS colonization status in order to limit antibiotic use to the appropriate risk group. This study compares three NAATs, Hologic Panther Fusion GBS (pre-market), Luminex Aries GBS (FDA-approved), and GeneXpert GeneXpert GBS (FDA-approved), to the culture-based method recommended for GBS screening. **Methods:** Five hundred vaginal-rectal swabs from women pregnant at 35-37 weeks were incubated for 24 hours in Lim broth. Culture results were determined by subculture on a blood agar plate and positive identification of GBS by MALDI-TOF mass spectrometry (Bruker Daltonics, Billerica, MA). 1 mL and 200  $\mu$ L of enriched Lim broth were used for Panther Fusion and Aries assays, respectively. A sterile swab dipped into the enriched Lim broth was used for GeneXpert. All tests were performed within 72 hours of enrichment. A composite standard was determined for each sample using a majority result for the 3 NAATs. To aid interpretation of discordant results, threshold cycle (Ct) data were retrieved. **Results:** Culture was less sensitive than NAATs, with a relative sensitivity of 70.7% compared to the composite standard. Compared to culture, GeneXpert and Aries each had one false negative while Panther Fusion had none. Sensitivity/specificity (%) of NAATs compared to the composite standard was 96.0/99.4 for Panther Fusion, 96.0/98.3 for GeneXpert and 96.6/96.3 for Aries. The average positive Ct value was 22.2 for Panther Fusion, 25.95 for Aries, and 27.09 for GeneXpert. The average false positive Ct value was 38.3 for Panther Fusion, 36.5 for Aries, and 39.4 for GeneXpert. **Conclusions:** Culture-based identification of GBS was less sensitive than identification by all three FDA-cleared NAATs after 18 to 24 h of Lim broth enrichment culture. The high Ct values for false positives suggests the discordant specimens contained a small amount of target organism. While culture-based screening is the most widely used method to confirm the presence of GBS, the development of molecular-based screening methods may lead to rapid detection, faster treatment, and improved clinical outcomes. Sensitivity, accuracy, cost, and turnaround time are important factors to consider in determining the primary method of GBS detection.

**Informatics**

**I001. Evaluation of a Guideline-adherent Microsatellite Instability (MSI) Module for Calculation of MSI Using a Comprehensive 170 Assay on an NGS Platform.**

R. Kolhe<sup>1</sup>, T. Boyle<sup>2</sup>, T. Edmonston<sup>3</sup>, A.K. Mondal<sup>1</sup>, C. Pundkar<sup>1</sup>, P.K. Ahluwalia<sup>1</sup>, A. Chaubey<sup>1</sup>

<sup>1</sup>Medical College of Georgia, Augusta, GA; <sup>2</sup>Moffitt Cancer Center, Tampa, FL; <sup>3</sup>Cooper University Hospital, Camden, NJ.

**Introduction:** Microsatellite instability (MSI) is caused by DNA mismatch repair (MMR) deficiency and results in the inability to repair errors during normal DNA replication of repetitive DNA sequences. MSI has traditionally been tested via PCR fragment analysis using a either Bethesda panel or MSI analysis system (Promega). However there are hundreds of thousands of microsatellite loci across the genome that can be used for MSI testing depending on the coverage profile of a particular panel. Next generation sequencing (NGS) enables interrogation of these MSI loci in a more comprehensive manner than previously possible via PCR based testing. Tumors exhibiting high MSI are eligible for FDA approved immunotherapy by checkpoint inhibitors, making this type of testing an important component in determining a patient's likely response to immunotherapy. The TST170 panel is a comprehensive gene panel covering the coding regions of 170 genes with clinical relevance in solid



tumors and can be used to estimate MSI with results comparable PCR. **Methods:** This validation is a joint effort by multiple molecular academic diagnostic laboratories in United States. The group termed as Sequoia Team (Sequencing and Oncology Informatics Academic Team) includes laboratories from multiple academic hospitals including Augusta University, Moffitt Cancer Center, Copper University hospital *et al*. The validation was guided by the joint consensus recommendation for validation of NGS assays by the AMP & CAP and NYSDOH. The validation included evaluations of precision, analytic sensitivity, analytic specificity, accuracy, reportable range, and reference range for MSI. DNA from 50 samples which were characterized by either Promega PCR or dMMR IHC were used. Libraries were prepared using the Illumina TruSight Tumor 170 (TST170) kit and sequenced on NextSeq 550. The MSI module used for calculation of TMB in this validation was designed and developed by PerianDx. **Results:** *In silico* analysis using the TCGA data both by PerianDx and Illumina (poster) showed that the results from whole exome NGS using the MSI module were highly concordant with PCR based methods using both the full list of MSI loci and the list limited to mono and dinucleotide loci. In the same dataset TST170 target space shows high sensitivity and specificity for MSI detection using the MSI module. In our clinical validation there was >99% concordance with MSI detection using MSI module on TST170 panel in comparison to MSI by PCR (Promega). **Conclusions:** In this validation we demonstrate that the TST 170 assay can be used to reliably predict MSI across a wide range of cancer types using the PerianDx TMB module. Our lab's experience provides an example for others that may wish to implement MSI testing by NGS on a comprehensive panel.

#### 1002. Clinical Implications of the Reference Sequence Used for Diagnostic Interpretation

J.A. SoRelle, M.A. Wachsmann, B. Cantarel, E. Villa, A. Quinn, D.A. Oliver

University of Texas Southwestern Medical Center, Dallas, TX.

**Introduction:** More than one reference gene annotation is available for next generation sequencing (NGS) labs but the influence of the geneset used has not been extensively analyzed. The GENCODE reference gene annotation arose out of the ENCODE Project and uses a combination of manual (HAVANA) and automated annotations along with experimental validation. This extensively manually curated database strives to capture all transcript variants (alternative splicing) of a gene. The NCBI RefSeq geneset contains a significant number of computationally-predicted [modeled, designated XM\_, XP\_] transcripts in addition to manual and automatic annotation, and works from the single canonical transcript. These two annotations can differ when comparing the boundaries of exons. This study was designed to determine the frequency of annotation discrepancies between GENCODE and RefSeq in a large NGS cancer panel and their clinical impact. **Methods:** Sequencing data generated by our institution's 1,385-gene oncopanel was analyzed by an in-house bioinformatics pipeline that uses multiple callers to generate a file that is subsequently sent to a commercial annotation application. This system (Philips IntelliSpace Genomics) provides GENCODE/Ensemble as well as RefSeq annotations on each mutation. Discordant annotations were recorded, manually reviewed using Integrative Genomics Viewer on GRCh38, and researched using literature and online oncology databases. **Results:** We found numerous genes in our panel had RefSeq annotations based only on modeled, predicted transcripts (XM, XP) including ATM, BCOR, DNMT3A, FANCA, MTOR, NF1, POLD1, and SMARCB1. We noted that the most recent RefSeq release (108) contains 113,620 transcripts [of which 63,555 are model transcripts (XM\_)] for 20,203 protein-coding genes while GENCODE Version 28, released November 2017 has information on 82,335 protein-coding transcripts (none modeled) for 19,001 genes. Discrepancies (intronic vs exonic, frameshift vs intronic) between RefSeq and GENCODE annotations were found in nearly all our clinical tumor samples, within clinically relevant genes including *ERBB3*, *FANCA*, *POT1*, *RPTOR*, and *TCF3*.

**Conclusions:** Many issues with variant annotation still exist and may explain in part the differences found in two reports from two labs on the same sample. Here we highlight the differential impact of choosing one transcript annotation versus another to make diagnostic interpretations. Awareness of the annotation geneset used by an NGS lab's in-house,

commercial, or reference bioinformatics software is important, especially for the future as more genes are discovered to have clinical relevance. A careful choice should be made to determine which transcript to select and which reference genome to use.

#### 1003. CCKB: A High-Performance and Genome-Scale Informatics Portal for Analysis and Multi-Institutional Sharing of Pediatric Cancer Variants

X. Gai<sup>1,2</sup>, A. Govindarajan<sup>1</sup>, R. Schmidt<sup>1</sup>, D.T. Maglinte<sup>1</sup>, T.J. Triche<sup>1</sup>, M.C. Hiemenz<sup>1</sup>, J.A. Biegel<sup>1</sup>

<sup>1</sup>Children's Hospital Los Angeles, Los Angeles, CA; <sup>2</sup>Keck School of Medicine of USC, Los Angeles, CA.

**Introduction:** Unique challenges exist in interpreting genetic variants in pediatric cancers, which are rare, and more often initiated by germline alterations in cancer susceptibility genes, copy number changes, and gene fusions compared to somatic mutations in adult tumors. In contrast, mutations like TP53 and other cancer gene mutations common in adult cancer are infrequent in young cancer patients. To address the unmet need for a pediatric cancer focused knowledgebase, we have implemented an informatics portal, CCKB (Childhood Cancer KnowledgeBase), to share knowledge regarding DNA and RNA variants across the spectrum of benign and malignant tumors in children. CCKB is cloud-based and web-accessible, and is sequencing-platform and test-agnostic. CCKB takes advantage of the latest computational and bioinformatics advancements to enable genome-scale cancer variant analysis and sharing of data from thousands of patients across multiple institutions. **Methods:** CCKB is implemented with open-source packages, including OpenCGA; a big-data analytic framework supporting the Genomics England Project, and OncoTree disease ontology. Genomic variants are managed using OpenCGA. CCKB is hosted in the AWS cloud using MongoDB, and web-accessible via a customized Interactive Variant Browser (IVA). Generic annotations are provided via OpenCB. RESTful APIs are leveraged to provide cancer-specific annotations using a variety of genomic resources including Ensembl, ICGC, CIViC, COSMIC, and Oncotator. **Results:** CCKB currently hosts genetic data for 400 pediatric cancer patients that we have tested at CHLA with our OncoKids<sup>SM</sup> panel, which is based on the OncoPrint Childhood Cancer Research Assay (OCCRA) that we developed in collaboration with Thermo Fisher Scientific. Over the web, genomic variants in VCF format, as well as OCCRA data in BAM format, can be directly uploaded into CCKB. With a variant-centric way that is capable of storing and managing hundreds of millions of variants and metadata of thousands of patients, CCKB allows the user to analyze cancer variants of individual patients or cohorts of patients over the web. De-identified data can be securely shared among collaborating institutions via the sophisticated authentication system at multiple levels or anonymously via the Beacon mechanism. **Conclusions:** The CCKB platform will enable data sharing to support the International Childhood Oncology Network (ICON), initiated by Thermo Fisher Scientific, the Sanford Consortium, AACR GENIE, as well as the larger community of pediatric oncology investigators who participate in the project.

#### 1004. A Machine-Learning Framework for Accurate Classification and Quantification of Oncogenic Variants Using the QuantideX NGS DNA Hotspot 21 Kit

L. Ringel, B. Printy, K. Kaplan, B.C. Haynes, J.L. Larson  
Asuragen, Inc., Austin TX.

**Introduction:** Targeted amplicon-based next-generation sequencing (NGS) is a common and critical tool for profiling somatic mutations within tumor genomes in both clinical and research settings. Accurately classifying variants remains an ongoing challenge due to the limited DNA quantities and compromised integrity of many formalin-fixed paraffin-embedded (FFPE) tumor specimens. A comprehensive machine-learning approach was developed to accurately differentiate true biological variants from process-related artifacts as part of companion automated analysis for the QuantideX NGS DNA Hotspot 21 Kit. **Methods:** DNA was extracted from tumor FFPE specimens and cell-line admixtures with independently-verified variants. Libraries for each sample were prepared and quantified using the QuantideX NGS DNA Hotspot 21 Kit\* (Asuragen), a targeted amplicon panel, and sequenced on the Illumina MiSeq. This dataset was

augmented by an *in silico* cohort that added the most frequent COSMIC single nucleotide variants (SNVs) and insertion/deletions (INDELs) at distinct allele frequencies to the sequencing output of mutation-negative samples. Sample DNA quantity and read sequence quality, frequency, and variability were characterized to capture the complex sample and site-specific noise profiles for all non-reference events in each library. After splitting the data into training and testing cohorts, a machine-learning model was trained to accurately identify both SNVs and INDELs. This model, incorporated into QuantideX NGS Reporter software, was then evaluated with an independent testing cohort of 36 FFPE libraries.

**Results:** Under cross-fold validation, the machine-learning strategy recognized >600 true SNVs at  $\geq 5\%$  variant allele frequency. These variants were detected with  $\geq 99\%$  sensitivity and PPV in the training cohort of >150 libraries (including 8 *in silico*) containing >300,000 non-reference observations. More than 20 INDELs were also identified with 100% sensitivity and PPV in the same cohort, including 6 from *in silico* libraries. The trained variant caller correctly identified >100 SNVs and >50 INDELs (up to 20 nucleotides long) in the independent testing cohort of 31 FFPE and 5 *in silico* specimens. Variants were detected with  $\geq 99\%$  sensitivity and PPV in this sample set. **Conclusions:** The presented variant caller addresses the challenges of FFPE quantity and quality by directly accounting for the quantity of amplifiable DNA input and both the FFPE specimen and site of interest error profiles. This machine-learning approach offers a robust and sensitive framework for oncology diagnostics and clinical trials research. \*Product under development.

#### 1005. Improving Variant Call Accuracy by Combining Torrent Variant Caller and PLATYPUS

Z. Siddiqui<sup>1</sup>, T. Ahn<sup>2</sup>, D.Y. Zhang<sup>3</sup>, J.A. Longtine<sup>4</sup>, F. Ye<sup>5</sup>, H. Guo<sup>2</sup>

<sup>1</sup>Saint Louis University, Town and Country, MO; <sup>2</sup>Saint Louis University, St. Louis, MO; <sup>3</sup>Mount Sinai Hospital, New York, NY; <sup>4</sup>Yale University, New Haven, CT; <sup>5</sup>New York Medical College, Valhalla, NY.

**Introduction:** Torrent Variant Caller (TVC) is a genetic variant caller for Ion Torrent sequencing platforms designed to call single-nucleotide variants (SNVs), multi-nucleotide variants (MNVs), insertions and deletions (INDELs), and block substitutions. TVC has optimized pre-set parameters to accurately call SNVs, but we found that it is sometimes inaccurate when calling INDELs and MNVs. For example, on the Ion AmpliSeq Cancer Hotspot panel, a 15-bp deletion in the *EGFR* gene was called a 12-bp deletion, and a two-nucleotide MNV in the *MPL* gene was called as two separate SNVs. The inaccurate calls on INDELs and MNVs using TVC put a burden on pathologists, as they spend a significant amount of time and effort manually correcting them in addition to categorizing mutations into tiers to generate a pathology report. **Methods:** We developed a novel tool, HOTPIPE, which analyzes Ion Torrent next generation sequencing (NGS) data (Ion AmpliSeq Cancer Hotspot panel) using TVC and PLATYPUS. PLATYPUS is another variant caller tool designed for efficient and accurate variant-detection in high-throughput sequencing data, and it performs well on INDELs and MNVs. For every INDEL and consecutive SNV called from TVC, PLATYPUS is called by HOTPIPE to generate a more accurate variant calling file. In addition, a NGS pathology report is automatically generated based on the variant calls and predefined BED files containing tiered mutations. **Results:** Thirty NGS datasets from lung/colon/melanoma cancer specimen were processed by HOTPIPE. Verified by manual review of sequence alignment, all INDELs and MNVs were called correctly, including the 15-bp deletion in the *EGFR* gene and the two-nucleotide MNV in the *MPL* gene. **Conclusions:** HOTPIPE significantly improves the accuracy of variant calls on the Ion AmpliSeq Cancer Hotspot Panel by harnessing the power of two variant callers, TVC and PLATYPUS.

#### 1006. Improving the Molecular Pathology Workflow with Machine Learning: Automated Calculation of Tumor Percentages on H&E Digital Whole Slide Images

C.M. Cirelli, P.D. Velu, M.D. Feldman, J.N. Rosenbaum  
University of Pennsylvania Health System, Philadelphia, PA.

**Introduction:** Molecular oncology testing, particularly massively parallel sequencing, or next-generation sequencing (NGS), makes use of estimates of tumor burden for a number of purposes. Assessment of sample acceptability, accurate quantitation of variants, and assessment of

copy number changes (among other applications) may all be improved with more accurate and reproducible estimates of tumor content. Currently, tumor percentages of samples submitted for molecular testing are estimated visually by pathologists on H&E stained tissue slides. These estimations can be automated, expedited, and rendered more accurate by applying machine learning methods to digital whole slide images (WSI). Here we detail how QuPath, a free and open-source digital slide analysis software, can be used to estimate tumor content from non-small cell lung cancer (NSCLC) and melanoma digital WSI. **Methods:** H&E guide slides submitted with NSCLC and melanoma samples sent for testing on our institutional solid tumor-targeted next-generation sequencing panel were scanned at 40x and split into 2000 x 2000 pixel tiles. Representative tiles were chosen for analysis in QuPath. Automated cell segmentation was performed using a watershed algorithm with customized parameters for melanoma and lung. A random forest classifier was trained on 42 NSCLC tiles (n=21 patients) and 46 melanoma tiles (n=23 patients) with cells labeled as tumor or non-tumor by a pathologist. This trained algorithm was then used to classify tumor cells versus non-tumor cells on 16 NSCLC test tiles (n=16 patients) and 10 melanoma test tiles (n=10 patients) that were not used during the training phase. The tumor percentages determined from the test classifications were then compared with tumor percentages determined from test tiles with manually annotated tumor and non-tumor cells. **Results:** The lung tumor estimation error on test tiles was 17.6% (range 0.3-56%) and the melanoma tumor estimation error on test tiles was 11.3% (range 2-30%). Separate watershed cell segmentation parameters were used on NSCLC and melanoma for optimal tumor and non-tumor cell classification. Proper cell segmentation was a crucial determinant of classification performance, and the ideal watershed parameters within the training dataset for NSCLC were Gaussian filter (sigma) =4.5, minimum area=10, maximum area=1000, and threshold=0.01 and for melanoma were Gaussian filter (sigma) =2.5, minimum area=10, maximum area=800, and threshold=0.01. **Conclusions:** Programs like QuPath can be used to provide automated estimates of tumor percentage from digital WSI. Further optimization of cell segmentation and further training of classifiers on larger and more diverse sets of cases is necessary for the improvement of estimation accuracies.

#### 1007. Assessment of a Somatic Mutation Detection Pipeline Using a Simulated Tumor Genome

Z. Li, R. Zhang, J. Li

Beijing Hospital, Beijing, China.

**Introduction:** The molecular diagnostics of cancer by the adoption of next-generation sequencing (NGS) is transforming to match identified genetic alterations with clinical actionable strategies. However, bioinformatics analysis pipelines used for extracting information from raw sequence data remains in their infancy and their outputs are highly divergent, especially for tumor-specific (somatic) mutations calling of cancer. Thus, a full assessment of the variables affecting bioinformatics analysis output is required. Benchmark data is very important for clinical laboratories to benchmark and calibrate their analysis pipelines. In this study, we developed a cancer genome simulator, which generate somatic mutation benchmark data, to investigate the factors that need to be considered to generate high-confidence variant calls from tumor-normal pairs, including sources of bias and pitfalls encountered in targeted sequencing data analysis. **Methods:** We developed a simulator for mutation simulation by editing the existing sequence reads. Our developed simulator can add comprehensive mutations, including single-nucleotide variants (SNVs), small insertions and deletions (Indels), large structural rearrangements and copy number variations to any alignment stored in BAM format, such as whole genome, whole exome and targeted sequence data. To investigate the factors of somatic mutation calling, we invited 92 clinical laboratories to process our synthetic tumor-normal pairs by applying our simulator to an already sequenced data that generated from Illumina HiSeq2500 platforms with a custom designed hybridization capture-based panel. **Results:** In total, 95 randomly selected variants, including SNVs and Indels, were introduced to the sequenced data. The variant allele fractions (VAFs) ranged from 2% to 35%. All simulated mutations were manually validated using the integrative genomics viewer (IGV). In total, 92 results along with the corresponding analysis pipelines

were received. On average, laboratories identified 92.9 of 95 variants (97.8%) overall. For SNVs, on average 73.3 of 74 variants (99%) were identified. However, on average only 14.1 of 21 Indels (67.1%) were identified. In addition, we observed that variant filtering criterion, VAFs, sequencing depth, as well as tumor-normal sequencing depth ratio are also important factors for variant calling accuracy. **Conclusions:** In summary, our survey provides a comprehensive assessment of bioinformatics analysis pipelines for somatic mutation detection of target sequencing which may propose useful guidelines for testing or developing new somatic mutation calling pipelines. In addition, our cancer genome simulator provides a comprehensive resource for somatic mutation detection benchmarking.

#### 1008. Interpretation of Mutational Signatures Associated With Smoking from an Amplicon-Based Clinical Oncology Sequencing Panel

J.E. Adler<sup>1,2</sup>, S. Shaffer<sup>1</sup>, J.J. Roth<sup>1</sup>, J.N. Rosenbaum<sup>1</sup>

<sup>1</sup>University of Pennsylvania, Philadelphia, PA; <sup>2</sup>Pennsylvania Hospital, Philadelphia, PA.

**Introduction:** Recent work has demonstrated that distinct oncogenic exposures, for example smoking, generate unique patterns of genomic alterations or mutational signatures (MS). Different types of cancers are characterized by the relative prevalence of these MS. Characterization of these signatures from clinical oncology data may permit correlation with other clinco-pathologic parameters for quality control purposes. Various informatics methods are utilized to identify MS and stratify patients into groups based on the prevalence of the signature. The mutational space is modeled as a single nucleotide base substitution matrix with each position representing a base substitution with respect to the preceding and succeeding bases. Each value generated represents the probability of such a mutation relative to the set of patient sequencing assays. Non-negative matrix factorization (NMF) is used to derive a set of mutational signatures which represent the centroids of the largest clusters of mutational matrices for each of the individual sequencing assays that comprise the dataset. **Methods:** This study analyzes MS generated from a 152 gene, amplicon-based next generation sequencing (NGS) panel to separate primary lung cancer patients into different groups based on smoking exposure history. The NGS panel encompasses full gene analysis +/- 10 intronic bp and covers approximately 0.5 Mb. Data are examined from 665 formalin-fixed primary lung tumor samples containing at least 10% tumor. Patients are stratified into cohorts based on their pack years and the correlation between pack year history and dosage of the various derived signatures. Derived signatures are then compared with the COSMIC database of previously published signatures. Data analysis is performed in R and MS are derived using the "MutationalPatterns" toolbox. **Results:** COSMIC signature #5 was the only signature that significantly separated non-smokers from heavy smokers (p value 0.0177). Combining COSMIC signatures implicated in lung cancer fared no better than just using signature 5 (via a Hotelling test). The signature most expressed in our dataset, COSMIC signature #1, did not significantly separate the two groups. **Conclusions:** Although the identification of MS typically relies on whole genome sequencing, this study demonstrates the possibility of performing signature-based analysis on a relatively small, amplicon based NGS panel. We've shown one COSMIC signature associated with lung cancer was able to separate two groups of patients, heavy smokers vs. non-smokers, in our patient cohort. Accurate and quantitative identification of these signatures may provide useful prognostic and therapeutic information.

#### 1009. Personalized Transcriptomic Drug Profiling in Non-small Cell Lung Cancer

Z. Abrams<sup>1</sup>, A. Joglekar<sup>2</sup>, G.R. Gershkovitz<sup>2</sup>, K.R. Coombes<sup>2</sup>

<sup>1</sup>The Ohio State University, Athens, OH; <sup>2</sup>The Ohio State University, Columbus, OH.

**Introduction:** Lung cancer is a particularly aggressive and deadly form of cancer, killing over 154,000 people per year in United States. Non-small-cell lung cancer is the most common form. It is well known that different genomic abnormalities have a significant impact on the therapeutic response of an individual's tumor. Notable examples include EGFR mutations and ALK fusions that identify tumors likely to respond to EGFR

inhibitors. We hypothesized that unusual transcriptomic (RNA) features in the profile of an individual patient's tumor would allow us to better understand and prioritize therapeutic treatment options tailored to that tumor. **Methods:** In order to test this hypothesis, we developed a novel statistic called the Newman paired test that allows the statistical analysis of RNA-Seq samples without the need for replicates. This test enables the statistical comparison of RNA-Seq data from paired tumor and normal samples collected from a single patient. This statistic enables us to analyze an individual patient without regard to a larger patient cohort. Using paired RNA-Seq data from The Cancer Genome Atlas (TCGA), we applied the Newman paired test to study 108 non-small-cell lung cancer patients (58 lung adenocarcinoma (LUAD) and 50 lung squamous cell carcinoma (LUSC)). **Results:** For each patient, we obtained a list of genes that were differentially expressed between normal and tumor samples from that patient. By comparing these lists from all LUAD and LUSC cases, we were able to find genes that were differentially expressed between these two subgroups. These results recapitulated the existing literature, which strengthened our belief that these individual transcriptomic profiles are representative of the underlying tumor biology. Individual patient gene lists then underwent gene enrichment analysis in ToppGene. Using the disease categories in ToppGene, we found that the majority of patients were "enriched" for the disease that they did in fact have. We then looked at the drug categories in ToppGene. The drug enrichment results showed that the majority of patients were significantly "enriched" for drugs currently used to treat non-small-cell lung cancer. We made personalized drug predictions using these transcriptomic profiles, which identified optimal treatment options on a per-patient basis. Based on our transcriptomic profile analysis with the Newman paired statistic, we were able to identify at least one FDA-approved cancer therapy for 94% of TCGA lung cancer patients. **Conclusion:** Our results suggest that the Newman paired test moves us closer to the goal of precision medicine by taking personalized measurements from an individual tumor, and using those measurements to tailor therapies to their specific disease.

#### 1010. NeGeSel-NIPT: A Web Based Tool for the Management of Non-Invasive Prenatal Screening Assays in the Clinical Laboratory

V. Williamson, B. Bullard, A. Kusmirek, I. Gonzalez, C. Yang, F. Sabato, A. Ferreira-Gonzalez  
VCU Health, Richmond, VA.

**Introduction:** Since its introduction, non-invasive prenatal screening (NIPT) has provided women a safe means of screening for chromosomal trisomies through cell free fetal DNA (cffDNA). Methods based on next generation sequencing (NGS) technology enable detection levels with high sensitivity (up to 99%) and low false positive rates (< 1%). The amount of data necessary for the successful performance of a NGS-based NIPT assay is immense and requires a specialized application for patient tracking in the clinical laboratory. We have created a clinical web based management tool, NeGeSel-NIPT, to manage all steps of the wet laboratory that interfaces with the cloud-based application Constellation (Natera) for data analysis and report generation. **Method:** We describe the design and use of NeGeSel-NIPT in the management of data generated by the Natera NIPT assay and how our application collects and monitors data necessary for test tracking and quality management. NeGeSel-NIPT acts as an interactive interface between our EMR, wet laboratory processes and Constellation (Natera's application) all the way to report generation. It leverages a user-friendly design against the stability of the Docker containment system for data uploading and storage. NeGeSel-NIPT works directly with Constellation through a series of API calls to identify and upload genomic data to Constellation and to monitor the analytical process as quality metrics are reviewed and the level of aneuploidy is assessed. Unique features of the NeGeSel-NIPT application include options that track and ensure consistent barcode/tracer usage, a fully dedicated audit system for tracking file transmission, role-based user system to authenticate access and screen-based visualization of allele frequency plots and quality metrics. In addition, NeGeSel-NIPT affords greater efficiency of the NIPT review process by providing real-time reagent control and automated methods of data de-multiplexing and file submission. **Results:** A total of 133 clinical and analytical samples were reviewed using the NeGeSel-NIPT tool as a part of its validation with 100% precision and accuracy. Samples included trisomies 13, 18, 21 and



monosomy X as well as a number of samples containing suspected cases of XXY and XYY. Fetal fraction levels ranged from 1.9 to 41.9%. Mean maternal and gestational age was  $34 \pm 6$  (years) and 14.37 (weeks) respectively. End-to-end testing analyses of the NeGeSel application against manual methodology show it to be far superior in terms on consistency, accuracy, and speed. **Conclusion:** An exceptional tool for the coordination of NIPT assays with outside cloud-based applications has been developed and implemented in the clinical laboratory that provides efficient and reliable NIPT testing management.

#### **1011. NeGeSel-Inheriseq: a scalable informatics solution for the management of assays for hereditary cancer**

V. Williamson, A. Kusmirek, B. Bullard, I. Gonzalez, C. Yang, C.N. Vlangos, F. Sábato, A. Ferreira-Gonzalez  
VCU Health, Richmond, VA.

**Introduction:** The advent of next-generation sequencing (NGS) in the clinical laboratory has revolutionized the molecular approach to testing for risk of hereditary cancer. Performance of this technology, however, sometimes necessitates that the results from accessory assays, e.g. Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA), be evaluated in conjunction with NGS findings to complete the clinical assessment. The pairing of these data types generates a need for an integrated end-to-end software solution that allows for complete data tracking and transparency. To satisfy this need, we have created the software tool, NeGeSel-Inheriseq. **Method:** We describe the performance of NeGeSel-Inheriseq tool for the management of the Inheriseq assay, a multi-dimensional assay based on NGS, MLPA, and Long Range PCR-Sequencing for the detection of hereditary risk of breast, ovarian and colon cancer. As a web-based application, NeGeSel-Inheriseq can be operated on any standard desktop with browser and network access. Its modular design allows the user to track the progress of patient samples through a dashboard that reports testing progress by assay type and analytical phase. The tool interfaces directly with the EMR without the need for specialized HL7-based language. Variants detected through NGS and MLPA are evaluated using current ACMG guidelines and a dedicated workflow is also employed by the software to address the confounding effects of pseudogenes for genes like PMS2 homolog2, mismatch repair system component (PMS2). **Results:** Twenty-four samples were processed with the NeGeSel-Inheriseq tool during the validation of the Inheriseq assay. Five assays (InheriseqBreast, InheriseqHBOC, Inheriseq Colon, Inheriseq Colon-Expanded and Inheriseq Comprehensive) were evaluated using the tool in a blinded sample set which included twenty-two clinical samples, one commercial reference sample (SeraCare) and DNA from NA12878 (Genome in a Bottle). The number of genes per test ranged from as low as nine to as many as forty and results were compared to Qiagen Clinical Insight with 100% reproducibility and repeatability (95% CI, 0.99-1) for identifying pathogenicity.

**Conclusion:** NeGeSel-Inheriseq is the first software tool of its kind that allows for the direct integration of NGS results with other assays performed in the clinical laboratory during the analytical process. This allows the user to directly access all pertinent data relevant to the case when assessing the level of hereditary risk.

#### **1012. A Local Population Allele Frequency Query Tool**

M. Leong<sup>1</sup>, B. Kosmo<sup>1</sup>, C. Lee<sup>1</sup>, B. Chor<sup>2</sup>, K. Tan<sup>1</sup>, Y. Teo<sup>1</sup>, B. Yan<sup>1</sup>, H. Lee<sup>1</sup>

<sup>1</sup>National University Hospital, Singapore; <sup>2</sup>Singapore General Hospital, Singapore.

**Introduction:** The advent of next-generation sequencing has made analyzing the human genome much cheaper and faster. Each individual has approximately 15,000 predicted protein-altering variants (Lek *et al.*, Nature 2016) that may be correlated with different traits, disease susceptibility/resistance, and efficacy of drug responses. The challenge in clinical genetics is to predict whether the variants are disease-causing or benign. The absence or low frequency of a variant in the ethnic-specific population is recognized as a criterion for categorizing whether a variant is disease-causing (Richards *et al.*, Genet Med 2015). There are 3 major ethnic groups (i.e. Chinese, Malay, and Indian) living in Indonesia, Malaysia, and Singapore. As such, knowing the population allele frequencies for each ethnic group would not only help with clinical variant

interpretation but may also identify ethnic-specific variants that have a pharmacogenetic influence on medical treatments, for instance, HLA-B\*1502 genotype causing Carbamazepine hypersensitivity that is mainly found in Han Chinese. We developed an ethnic-based population allele frequencies query tool using SQLite on standalone MacOSX machine (local filesystem, LFS) and Apache Spark framework running on Hadoop distributed filesystem (DFS) server-based environment. **Methods:** Briefly, 93 whole genomes of Chinese Dai available in the 1,000 Genomes Project, 100 whole genomes of Malays, and 38 whole genomes of Indians available from the Singapore Public Health Genomics were downloaded. The VCFtools and bcftools were used to generate database (DB) inputs from the vcfs. The query was performed using python/pandas DataFrame (DF) or PySpark DF. The entire framework was built on Unix/Linux-based operation system. Query input should consist of nucleotide position (with hg19 build GRCh37 as reference during variant calling) and Chromosomal number. The detailed installation and execution can be obtained from <https://github.com/hkailee/GenoSpark>. **Results:** A query performed on 4,106 variants obtained from breast cancer panel tests on 12 samples was completed within 4 seconds, using the SQLite DB and pandas DF built on the LFS. In comparison, similar DB query performed using the PySpark DF on the Hadoop DFS (5 replicas) was significantly slower, i.e. 20-30 seconds. **Conclusions:** Larger whole genome datasets obtained from each ethnic group should increase the statistical power to aid local clinical variant interpretation. The DFS is not suitable for current small DB query because of significant overhead process/latency involved. However, with the continuous whole genome sequencing effort for the population, the DFS is likely to provide significant efficiency and benefit in terms of horizontal scalability.

#### **1013. Classification of Variants from Myeloid NGS Panel Testing Using a Scalable Evidence Based Variant Classification Workbench (SEBVaC)**

W. Chen<sup>1</sup>, S.A. Gardner<sup>2</sup>, E. Bogdanova<sup>1</sup>, W.S. Kelly<sup>2</sup>, K.S. Weymouth<sup>1</sup>, J. Suh<sup>1</sup>, D. Lupu<sup>2</sup>, G. Fenyofalvi<sup>2</sup>, H. Dong<sup>3</sup>, L. Cai<sup>4</sup>, A. Chenn<sup>4</sup>, S. Letovsky<sup>1</sup>, N. Nagan<sup>1</sup>, U. Geigenmüller<sup>1</sup>

<sup>1</sup>Integrated Genetics, Laboratory Corporation of America Holdings, Westborough, MA; <sup>2</sup>Integrated Genetics, Laboratory Corporation of America Holdings, Research Triangle Park, NC; <sup>3</sup>Integrated Oncology, Laboratory Corporation of America Holdings, New York, NY; <sup>4</sup>Center for Molecular Biology and Pathology, Laboratory Corporation of America Holdings, Research Triangle Park, NC.

**Introduction:** Classification of germline variants in Mendelian diseases is based on evidence tying the specific variant to a phenotype and/or to a surrogate measure in controlled experimental systems. In contrast, assigning clinical significance to a somatic variant is often based on studies measuring outcomes in groups of patients with or without potentially relevant variants in the gene, domain or exon in question. Classification of a somatic variant thus involves 1) ascertaining available evidence that applies to the levels of gene/domain/exon and 2) deciding on the relevance of this evidence to the specific variant of interest. A workbench that supports scalable and traceable classification of somatic variants by incorporating the concept of multi-level evidence into the Tier classification system proposed by AMP guidelines was developed. Our experience in developing and implementing SEBVaC for next generation sequencing (NGS) testing in myeloid neoplasms is described herein. **Methods:** The assay (IntelliGEN Myeloid) uses amplicon-based NGS to identify alterations in 50 genes of significance to myeloid neoplasms. For each gene, the predominant molecular mechanism of disease was pre-curated, namely loss of function (LOF) or gain of function (GOF), and supporting evidence at gene/domain/exon levels. During variant classification, all relevant evidence is automatically loaded into the SEBVaC workbench together with other programmatically available data from ClinVar/COSMIC/ExAC databases and *in-silico* predictions. Reviewers supplement this information with variant-specific evidence and decide whether to apply the available gene/domain/exon-level evidence to the variant of interest. A hierarchical rules-based algorithm then generates tiered classifications across all myeloid test indications. **Results:** The predominant molecular mechanism of disease for the 50 panel genes is LOF in 29 genes and GOF in 21 genes. Thirty-four genes had reported prognostic evidence, 16 had reported diagnostic evidence and 22 had

reported therapeutic evidence. Nine had evidence across all three categories. Among 404 variants in 43 genes that were classified in an initial series of 126 consecutive patients referred for testing, 187 were unique Tier I or Tier II variants. One hundred and thirty-eight of these occurred in genes with LOF mechanism, and almost all (137) were classified using gene/domain/exon level evidence. Out of 49 variants in genes with GOF mechanism, 15 were classified using gene/domain/exon level evidence, while 34 variants were classified using codon/variant level evidence. **Conclusions:** Using a novel strategy to integrate multi-level evidence, SEBVaC supports scalable and efficient classification of somatic variants in a high-throughput laboratory setting.

#### I014. A Molecule-Centric Approach to Phasing

M. Debeljak<sup>1</sup>, E. Mocci<sup>1</sup>, W.W. Grody<sup>2</sup>, A.P. Klein<sup>1</sup>, J.R. Eshleman<sup>1</sup>

<sup>1</sup>Johns Hopkins University, Baltimore, MD; <sup>2</sup>UCLA School of Medicine, Los Angeles, CA.

**Introduction:** Correctly phasing genetic variants is important in fields such as genetics and transplantation. We developed a molecule-centric approach to phasing short reads that does not rely on probability or inference. **Methods:** Using 1,000 genomes data at a SNP dense locus, we converted all genotypes to base-10 numerical codes. We identified homozygotes, termed "Hom-alleles" and sorted remaining genotypes by frequency. From the identified Hom-alleles we created all pairwise combinations, *in silico* heterozygotes (IS-hets), and excluded these genotypes from further consideration. Starting with the most frequent unanticipated genotype, we considered if any of the previous hom-alleles could be a contributor to the genotype. If one and only one of the existing hom-alleles could contribute, then that allele was subtracted from the genotype to reveal a new putative allele. The new putative allele was confirmed by producing all pairwise combinations of that allele with the hom-alleles and identifying the resulting genotype in other heterozygotes. This process was iteratively repeated until all alleles were identified. **Results:** We validated this novel approach using single molecule sequencing of 8 loci and were able to identify 145 (8-35/locus) alleles and explain an average of 98.2% (95.0-99.9%) of 1,000 genomes individuals at these loci. **Conclusion:** We compared the accuracy of our new method to PHASE and Shape-IT and conclude that our method is slightly better.

#### I015. Using Autolt to Automatically Enter Molecular LDT Results into the Laboratory Information System

J. Grojean, M. Hoyer, H. Guo

Saint Louis University, St. Louis, MO.

**Introduction:** Routinely, the results of molecular LDTs (laboratory developed tests) are manually entered into the laboratory information system (LIS) by lab technologists. This manual process takes time and sometimes introduces errors. To save entry time and improve accuracy, we automated the manual entry process using a free computer software tool, Autolt. **Methods:** Autolt is a free-ware BASIC-like scripting computer language designed for automating the Microsoft Windows graphic user interface. It can read electronic test result files and do keystrokes and mouse clicks to automate the tasks done by laboratory staff. First, the details of the staff work flow were analyzed and recorded, including: 1) how to read and extract information from the raw test result file; 2) the sequence of keystrokes and mouse clicks to open and enter data into the LIS. Second, an Autolt computer program was developed to automatically extract information from the raw test result file and enter it into the LIS. Third, after we validated the Autolt program in the LIS testing environment using dummy cases, we moved to the production system and started automatic resulting. **Results:** We have switched from manual to automatic resulting for hemochromatosis (C282Y and H63D), Factor V Leiden, Prothrombin (G20210A), and EBV quantitative tests. The accuracy of the automatic resulting is 100% and the time spent on reporting was shortened to one third of manual resulting. For example, manual resulting 30 EBV quantitative test results took about 30 minutes. In contrast, automatic resulting takes less than 10 minutes. **Conclusions:** Using Autolt to automate the molecular LDT result entry process saves time and improves accuracy. It has general application for most molecular LDT tests.

#### I016. Fragment Size Characterization of Cell-Free DNA Mutations from Clonal Hematopoiesis

T. Jiang, A. Jager, S. Katz, J. LoCoco, P. Le, D. Baker, T. Pawlowski, S. Bilke, C. Zhao

Illumina, Inc., San Diego, CA.

**Introduction:** Circulating tumor DNA (ctDNA) is a noninvasive, real-time biomarker that can provide diagnostic and prognostic information for cancer patients before and after treatment. However, only a small fraction of cell-free DNA (cfDNA) originates from tumor cells, and the majority of fragments come from hematopoietic cells. Somatic mutations harbored by hematopoietic cells (clonal hematopoiesis, CH) can be a major source of false positive mutations in cfDNA affecting clinical decisions. Therefore, it is critical to characterize and identify somatic mutations by clonal hematopoiesis. **Methods:** Cell-free DNA (cfDNA) and matched tumor samples were collected across four original tissue types by different tumor stages, including solid tumors and leukemia. Using a cfDNA assay targeting 500+ genes, molecular profiling was performed with unique molecular identifiers (UMIs), sequenced on Illumina platforms, and analyzed using an internal pipeline for variant frequencies down to 0.4%. Fragment size was inferred by consensus sequence after read collapsing using genomic coordinates. Matched tumor samples were also analyzed using the same assay. By matching tumor and cfDNA variant results, we constructed a fragment size profile that originates from tumor, healthy and abnormal hematopoietic cells. To identify somatic mutations from CH, we applied a likelihood ratio test of fitting observed fragment sizes of different cell origins. **Results:** Our assay and pipeline yielded a variant concordance of 70% between cfDNA and tissue (23/33) in recurrent COSMIC variants. A majority of driver mutations only found in plasma (7/10) were enriched in *TET2*, *DNMT3A* and *TP53*, which suggests clonal hematopoiesis. The size distribution of fragments carrying somatic mutations from solid tumors (peak at 147 bp) was shifted relative to fragments carrying somatic mutations from CH or leukemias (peak at 166 bp). There was no significant difference in size distributions between fragments carrying somatic mutations and healthy hematopoietic cells (*p*-value=0.86). In addition, we simulated different levels of CH mutations *in silico* with 2000x coverage. By fitting the fragment size distribution, we achieved sensitivities of 81.5%, 92.5%, 98.3% and 100% and specificities of 82%, 92.5%, 97.5% and 100% for 1%, 2.5%, 5% and 10% CH mutations, respectively. **Conclusions:** Fragment size distribution of cfDNA released by malignant or healthy hematopoietic cells is different from that of cfDNA released by solid tumors. Accordingly, we leveraged the fragment size distribution to differentiate somatic mutations from different cell origins.

#### I017. NGS Panel Analyzer: A Software Tool to Assess NGS Panel Design

S.B. Patel, M.B. Raffeld, S. Davis

National Institutes of Health, Bethesda, MD.

**Introduction:** Next generation sequencing (NGS) is routinely performed on tumors to aid diagnosis or identify therapeutic targets. However, parameters of practical importance in assessing the suitability of an NGS panel for a particular tumor type are not readily ascertained. To address this limitation, we developed a software tool using the R Project for Statistical Computing that results the following data for each gene on any targeted NGS panel: Coding sequence coverage, percent of unique COSMIC mutations detectable, and percent of COSMIC mutation counts detectable. **Methods:** The code for NGS Panel Analyzer was written in the R Project for Statistical Computing v3.4.4. The Analyzer requires the user to input a BED file of his targeted NGS Panel. Ensembl GRCh37, release 75 gene annotations, including gene symbols and genomic coordinates of coding regions of genes, were derived via the Bioconductor package AnnotationHub. A well-curated cancer gene mutation dataset, CosmicMutantExport.tsv, v83, GRCh37, was obtained from the COSMIC database ftp site. **Results:** The OncoPrint Comprehensive Assay v3 (Thermo Fisher Scientific) was used as an example targeted NGS panel to demonstrate the functionality of the Analyzer. The outputs of the Analyzer were an R markdown document and plots of the coding sequence coverage and mutation coverage. **Conclusions:** The NGS Panel Analyzer allows users to evaluate the design of an NGS assay, which may become important when deciding whether an NGS panel is

suitable to evaluate mutations in a particular tumor type or identifying areas where an NGS panel could be improved in its next version. The code has been made freely available on GitHub (<https://github.com/sbpatel2009/NGS-Panel-Analyzer>).

#### 1018. WITHDRAWN

#### 1019. Database of High-Resolution Melting Publications with Data Mining and Statistical Reporting

Z.L. Dwight, K.L. Brown, C.T. Wittwer  
University of Utah, Salt Lake City, UT.

**Introduction:** Querying public literature resources for specific research aims can be cumbersome. Though comprehensive, large-scale publication stores lack the granularity and/or specific details needed. To aid researchers, we developed a searchable and curated database consolidating pertinent information for the area of melting curve analysis. The database and web interface is accompanied by real-time reporting of statistical trends such as instrument use, PCR techniques, and genes. By mining usage data of these manuscripts, opportunities for future research can be identified, research aims can be validated, and better perspective of the evolution of DNA melting analysis can be obtained. A data-driven view of melting research can educate new scientists and reveal missed opportunities for future innovation. **Methods:** The publications database was designed around the common data all applications of melting curve analysis share. This data is acquired by a human reader due to protected publication access and dense material. Many categories, such as sensitivity and specificity of results, were calculated if necessary. The primary applications were identified as gene scanning, human tumor scanning, human genotyping, analysis of monoploid organisms such as bacteria or viruses, methylation studies, and non-human polyploid organisms. Additional tables include PCR speed optimization and comprehensive literature reviews. Tables are designed and built in a relational database (MySQL). The web interface is built with PHP and jQuery. Real-time database statistics and trends are presented via Google Charts. **Results:** The database, presentation layer, and accompanying statistics are freely available at [dna.utah.edu/db](http://dna.utah.edu/db). A database query of a topic such as BRAF using the web interface provides the user visualization and statistics of the query which identifies the areas found as total counts and percentages and includes a listing of related melting publications. If topic is known, users can view the entire manuscript listing quickly as well as sort, search and filter rows for columns of interest. All results provide links to related PubMed manuscript listings and author contact information. By mining our own database (> 1,700 manuscripts), we found that genotyping is the most popular use (24.4%) of melting curve analysis followed by tumor scanning (19.7%). The most widely used instruments include the Roche LC480 (33.5%) and the Qiagen RotorGene (31.7%). **Conclusions:** This database, found at <https://dna.utah.edu/db>, provides detailed and curated high-resolution melting publications. Accompanied by statistical reporting and data-driven charts, researchers can quickly obtain relevant information and references to help discover research opportunities and focus research aims.

#### 1020. A Clinical Decision Support Tool to Integrate Next-Generation Sequencing and Cytogenetics Assays for Myeloid Cancers

S. Bandla<sup>1</sup>, D. Galimberti<sup>1</sup>, K. Kopp<sup>1</sup>, N. Khazanov<sup>1</sup>, S. Anstead<sup>1</sup>, C. Zurenko<sup>1</sup>, N. Bonevich<sup>1</sup>, C. Gates<sup>2</sup>, D. Cyanam<sup>1</sup>, J. Faershtein<sup>3</sup>, J. McIntyre<sup>1</sup>, J. Sherlock<sup>4</sup>, C. Nikoloff<sup>2</sup>, M. Hogan<sup>1</sup>, S. Sadi<sup>1</sup>  
<sup>1</sup>Thermo Fisher Scientific, Ann Arbor, MI; <sup>2</sup>Thermo Fisher Scientific, Santa Clara, CA; <sup>3</sup>Thermo Fisher Scientific, Pleasanton, CA; <sup>4</sup>Thermo Fisher Scientific, Paisley, United Kingdom.

**Introduction:** Sanger sequencing and karyotyping are traditionally used to detect molecular alterations in myeloid cancer that inform diagnosis and treatment. Recently, new myeloid biomarkers have expanded the scope of molecular testing. To address the need for broader profiling, we developed a next-generation sequencing (NGS) assay to research alterations in 58 myeloid cancer genes and a cytogenetics assay to detect chromosomal alterations. Furthermore, we developed a clinical decision support software that integrates results from both platforms into a single report. **Methods:** The NGS assay was developed on Ion Ampliseq targeted sequencing technology to detect DNA and RNA variants from

10ng of input. The cytogenetics assay was developed on an Affymetrix platform with enriched coverage for cancer genes. Evidence from drug labels (FDA, EMA), guidelines (NCCN, ESMO), and global clinical trials were identified using automated text searches and manual curation. In addition, all curated evidence was associated with appropriate AMP/ASCO/CAP tiers. A web application was developed to generate biomarker specific custom reports in 11 languages. **Results:** The NGS assay detects single-nucleotide variants, insertion/deletions, and fusions in 58 genes with an average read depth of >2,000 reads per amplicon and average uniformity of >95%. The cytogenetics assay contains 2.6 million markers for the detection of amplifications/deletions, LOH, and chromotripsis. The clinical decision support software integrates outputs from the two platforms to report relevant clinical evidence including the AMP/ASCO/CAP tiers for the biomarkers. **Conclusion:** We developed two complementary molecular profiling platforms to detect relevant mutations and chromosomal alterations in myeloid cancer research. Combined with an integrative clinical decision support software, we demonstrate a streamlined and robust sample-to-report workflow.

#### 1021. Development and Analysis of a Machine Learning Variant Caller

D.E. Wood<sup>1</sup>, A. Georgiadis<sup>1</sup>, J.R. White<sup>2</sup>, B. Van Emburgh<sup>1</sup>, S. Parpart-Li<sup>1</sup>, J. Mitchell<sup>1</sup>, E. Papp<sup>1</sup>, C. McCord<sup>1</sup>, P. Lovero<sup>1</sup>, D. Riley<sup>1</sup>, S. Jones<sup>1</sup>, M. Sausen<sup>1</sup>, J. Simmons<sup>1</sup>, S.V. Anguoli<sup>1</sup>  
<sup>1</sup>Personal Genome Diagnostics, Baltimore, MD; <sup>2</sup>Resphera Bio, Baltimore, MD.

**Introduction:** Variability in the accuracy of somatic mutation calls may have important implications for the therapeutic management of cancer patients. To identify true somatic variants with high confidence, we developed a novel machine learning based-approach and integrated it into our clinical gene panels. **Methods:** We developed a random forest classification model, named Cerebro, which evaluates a large set of decision trees to generate a confidence score for each variant. The algorithm was trained using matched tumor and normal pairs. Validation was carried out using both *in silico* and experimental data sets. For experimental validation, Cerebro was evaluated against all leading publicly available variant callers. **Results:** Analysis of matched tumor-normal exome data from 1,368 TCGA samples using this method revealed concordance for 74% of mutation calls but also identified likely false positive and negative changes in TCGA data, including in clinically actionable genes. Integrating high-quality mutation detection by Cerebro into our clinical next generation sequencing (NGS) panels analyses improved the accuracy of test results compared to other clinical sequencing analyses (sensitivity of 100% vs. 50-98%; positive predictive value of 100% vs. 9-100%). In addition, determination of high-quality somatic mutation calls improved tumor mutation load-based predictions of clinical outcome for melanoma and lung cancer patients treated with immune checkpoint inhibitors. **Conclusions:** These analyses provide a novel method for improved identification of tumor-specific mutations. Our machine learning strategies to optimize sensitivity and specificity for detection of true alterations have important implications for treatment options for patients with cancer.

#### 1022. Evaluation of SOPHiA DDM v4 for NGS Analysis of Ampliseq Cancer Hotspot Panel

D.C. Green, J.D. Peterson, L.J. Tafe, F.B. deAbreu, G.J. Tsongalis, J.A. Lefferts  
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

**Introduction:** Advances in next-generation sequencing (NGS) technology has made high throughput sequencing more accessible to many clinical molecular laboratories but data analysis continues to be a major bottleneck to clinical NGS. Third-party software that can be easily implemented to provide bioinformatics pipelines for various types of NGS assays may be an important component of NGS testing in many laboratory settings. These products must result in properly aligned sequencing reads with accurate and reproducible variant calling, filtering and annotation to add in clinical reporting. In this study we evaluated the ability of SOPHiA DDM v4 (SOPHiA GENETICS), a comprehensive bioinformatics platform, to serve as an alternative to our Ampliseq CHPv2 pipeline. **Methods:** Performance of an out-of-the-box SOPHiA DDM



pipeline for CHPv2 NGS analysis was compared to our current clinical pipeline. A total of 32 formalin-fixed, paraffin-embedded (FFPE) DNA samples from solid tumor patients and control replicates were used in this evaluation. Libraries were prepared using the CHPv2 chemistry and sequenced on four 318v2 chips with the Ion Torrent PGM HighQ enzyme. Signal processing, alignment, and variant calling was performed on the Torrent Suite version 5.6.0 and FASTQ files were annotated using Golden Helix according to our validated workflow. The analysis hands-on time, total processing times and final variant calls and annotations were compared. **Results:** The total analysis hands-on time per chip with 10 samples was approximately five minutes using SOPHiA DDM with an additional hour needed for automated processing. Using our current pipeline, the total hands-on time was on average 170 minutes for a chip with 10 samples. Using our current pipeline, a total of 17 different variant calls deemed "clinically actionable" were made among the 32 patient samples. All of these variants were called and categorized as level A or B variants (highly pathogenic and potentially pathogenic) by SOPHiA DDM. There was a high level of concordance between reportable, non-actionable variants as well. An *FGFR1* deletion, c.379delG, was called as a level A variant by SOPHiA in six samples, which is likely a PGM-specific artifact and is filtered out by our pipeline based on strand bias. Additionally, SOPHiA DDM also identified a complex *PDGFRA* variant (c.2525\_2538delACATCATGCATGATinsCA) that was missed by our current pipeline but present upon manual review of this region in IGV. **Conclusion:** SOPHiA DDM is a powerful NGS bioinformatics platform which can support a clinical workflow from variant alignment to report generation. In this initial evaluation, we found that the total hands-on analysis time required for our bioinformatician to produce a list of annotated variants was reduced by approximately 97%.

#### 1023. Precision Medicine Requires Molecular Pathologists Have Clinical Decision Support and Automation Found in Agilent Alissa to Analyze and Interpret Large Numbers of Variants from NGS Assays

S. Van Vooren<sup>1</sup>, E. Hage<sup>2</sup>, K. Bungartz<sup>3</sup>, J. Taminiau<sup>2</sup>, N. Van-der-aa<sup>2</sup>, S. Van-Vooren<sup>4</sup>

<sup>1</sup>Agilent Technologies, Santa Clara, CA; <sup>2</sup>Agilent Technologies, Diegem, Belgium; <sup>3</sup>N-of-One, Concord, MA; <sup>4</sup>Agilent Technologies, Boston, MA.

**Introduction:** Tumor profiling using next generation sequencing (NGS) is becoming increasingly important in the molecular pathology setting. At the Association of Molecular Pathology's AMP Europe 2018 meeting, Agilent Alissa Interpret teamed up with N-of-One to participate in a 'Battle of the Bioinformatics Pipelines' to see how our NGS variant analysis and interpretation pipelines with sequencing data from real patient samples, generated by a routine molecular diagnostics laboratory, would compare with 3 other vendors. In parallel, the Erasmus MC lab performed its own data analysis of the sequence data but did not share those results.

**Methods:** Erasmus University Medical Center (Erasmus MC)'s pathology department selected 5 patient samples — one non-small cell lung cancer (NSCLC) formalin-fixed, paraffin-embedded (FFPE) tissue sample, one pancreatic cancer FFPE tissue sample, and 3 non-small cell lung cancer pleural fluid cytology samples — that differed in tumor content from 50 percent to 80 percent. They sequenced these samples on their in-house Ion S5 XL, using a custom panel and provided FASTQ files, where the reads are not aligned, and as BAM files, which has aligned reads to the vendors. We were asked to identify and annotate the variants, state their allele frequency, and classify them according to their clinical relevance.

**Results:** Alissa Interpret's report overlapped Erasmus MC laboratory's results identifying clinically significant variants in the different tumor types. In the pancreatic cancer sample, variants within *CDKN2A* and *KRAS* genes were flagged as alterations with potential clinical significance. On the other hand, several variants with strong or potential clinical significance were called in the NSCLC samples, including a variant in the promoter region of the *TERT* gene, a 19bp deletion in the *MET* gene affecting an exon 13/14 splice site, and missense variants within the *TP53* and *EGFR* genes. For example, an activating mutation in the *EGFR* gene (G719E) was identified in a NSCLC sample. *EGFR* activating mutations may predict sensitivity to EGFR-targeted therapies, of which several have received FDA approval for certain indications. To this extent, and with the integration of N-of-One's knowledgebase, we identified Afatinib as an FDA approved therapy while

taking into consideration both the alteration and the tumor type.

**Conclusions:** There is no gold standard for data interpretation, both organizers and participants acknowledged the importance of the expertise of the clinical professional in making the right decision and to decide on reporting criteria. To do this, it becomes more and more clear that molecular pathologists require an advanced and robust clinical decision support system to analyze and interpret the large number of variants originating from NGS assays.

#### 1024. Identification of Germline Mutations in Tumor DNA Samples Absent a Matched-normal

A. Bigdeli, P. Velu, R. Sussman, J. Rosenbaum, J.J. Morrisette, R.B. Faryabi

University of Pennsylvania Health System, Philadelphia, PA.

**Introduction:** Somatic mutation detection using next-generation sequencing (NGS) can utilize companion healthy tissue to allow for the discrimination of germline variants during analyses. In clinical cancer diagnostics when matched-normal tissue is unavailable, population allele frequencies in genetic variation databases are often used as a surrogate for germline variant identification. This approach introduces limitations in both accuracy and reproducibility as conventions and resources change over time. Here we evaluate three methods of germline filtration and develop a novel method for germline identification comparable to matched-normal informatics analysis. **Methods:** Three methodologies for germline identification are evaluated: presence in normal genetic variation databases at population frequencies greater than 0.1%, an in-house algorithm designed to mimic manual genomic analyst review, and the variant caller MuTect2 which provides germline probability estimates further refined using aggregate normal samples. Filtration results are compared to germline variants identified in 8 tumor-normal analyses run at an outside clinical institution. Additional comparisons are performed on germline HapMap samples NA12878 and NA19240. Variant identification is performed by clinically validated informatics pipelines and all post processing and visualization is performed using the R programming language. **Results:** We observe a wide range of variability in filtration among the three methods and while all prove highly sensitive, none yielded adequate specificity. Through orthogonal validation using known germline variants as determined through matched normal tissue, we are able to determine which features from each method perform optimally for the development of a combinatorial computational approach.

**Conclusions:** By using a combinatorial algorithmic approach of probability, variant databases (both public and internal), and normal samples evaluated using identical technologies we increase accuracy and reliability while limiting burden on analyst review. Additionally, by utilizing samples that have undergone previous manual interpretation, independent of technology, we hope to train a classifier to enhance performance of the developed method.

#### 1025. Identification of Viral Integration Sites in Cancer Genomes Using Unmapped Reads in Targeted Next-generation Sequencing Data

A.S. Bowman, S. Middha, C. Vanderbilt, M. Ladanyi, M. Berger, A. Zehir

Memorial Sloan-Kettering Cancer Center, New York, NY.

**Introduction:** Integration of viral DNA into the human genome has been shown to drive tumorigenesis in certain cancer types. Nearly 80% of human papillomavirus (HPV)-related tumors such as cervical and head and neck cancers are associated with virus integration. Additional malignancies with a viral etiologic association include neuroendocrine carcinoma of the skin associated with Merkel cell polyomavirus (MCPyV), hepatocellular carcinoma associated with Hepatitis B virus (HBV), and nasopharyngeal cancer and Burkitt lymphoma associated with Epstein-Barr virus (EBV). Here, we show how unmapped DNA reads in targeted next-generation sequencing (NGS) can be used to detect the presence of viral genomes and to identify integration sites across diverse tumor types.

**Methods:** Data from our institutional prospective sequencing program, MSK-IMPACT, were screened for evidence of viral reads using KRONA, which outputs all ntBLAST hits of unmapped reads in a given sample. Samples with more than 10 viral reads were further interrogated to identify an integration site by re-aligning the fastq files using a combination of the human genome (hg19) and a "SuperViral" reference. This reference

contains a total of 197 viral genomes comprised of 185 HPV subtypes, 10 human herpes virus (HHV) subtypes including the two major EBV strains, HBV, and MYCPyV. Integration sites were detected by analyzing the output of DELLY, a structural variant caller. We compared our results to GENE-IS, a vector gene therapy detection pipeline, in targeted-sequencing mode to demonstrate concordance. At least three supporting reads of an integration site were required for both tools. **Results:** Fifty-five samples from 16 cancer types were chosen with evidence of at least one of the aforementioned viruses. Both DELLY and GENE-IS reported equivalent integration sites in 14 samples, including 5 HBV cases, 4 HPV type 16 cases, and 5 HPV type 18 cases while returning negative results for 36 samples. HBV integration sites were found in or near *TERT* promoter in 5 of the 7 hepatocellular carcinoma samples, with mutual exclusivity with *TERT* promoter mutations. HPV16 integration sites in or near *ERBB2* were found in 2 HPV samples, one of which was a head and neck cancer with concurrent *ERBB2* amplification. PVT1, a long ncRNA known to be a site of HPV18 integration in cervical cancers, was also observed in the results from this small cohort. **Conclusion:** We identify the presence of viruses in a large number of cases using unmapped reads from MSK-IMPACT data. We further show evidence that virus integration sites can be identified using a simple bioinformatics pipeline.

#### **1026. Variant Inspector: A Computational Approach for Somatic Variant Prioritization in Routine Clinical Practice**

R.J. Maglantay, W. Ernst, M. Barbi De Moura, Y.E. Nikiforov, M.N. Nikiforova, S. Roy

University of Pittsburgh Medical Center, Pittsburgh, PA.

**Introduction:** Next-generation sequencing (NGS) has enabled comprehensive and sensitive detection of somatic variants in cancer. However, interpretation and clinical reporting of somatic variants pose several challenges. The AMP published the joint consensus guidelines with CAP and ASCO for standardizing the annotation, interpretation, and reporting of somatic sequence variants. We have developed a computational approach, Variant Inspector (VI), for prioritizing somatic variants to assist with clinical interpretation using the AMP guidelines as a framework and present the preliminary results. **Methods:** The AMP 2017 guidelines were reviewed to identify variant classification criteria that could be incorporated in a computational algorithm. Criteria requiring manual input (e.g. review of literature/clinical trials results) could not be incorporated. For algorithm development, we used 66 known cancer driver genes that are sequenced for clinical testing in our laboratory. Variants classified either as Tier I/II or Tier IV in our clinical variant knowledgebase were used for validating VI's output. Fifty-six variants, comprising of 40 Tier I/II (strong/potential clinical significance) and 16 Tier IV (benign), were included. The variants consist of 27 single-nucleotide variants (SNVs; 48.2%), 11 deletions (19.6%), 5 duplications (8.9%), 10 horizontally complex insertion/deletions (indels) (17.9%) and 3 insertions (5.4%). VI is implemented using Python v2.7 and uses the following python libraries and databases; hgvs python package, 1000genomes, ExAC, ESP6500, COSMIC, ClinVar, dbNSFP, dbcsnsv, and cancerhotspots. The application is deployed as a RESTful web service using Docker containers in a Docker swarm cluster. VI is currently under alpha testing with ongoing validation. **Results:** The overall concordance rate was 89.3% (50/56). Of the discordant variants (Tier I/II miscalled as VUS), there were 3 SNVs, 2 insertions and 1 duplication. All of the discordant SNVs were missense changes in tumor suppressors (CIC, p.R215W; CTNNB1, p.G34R; TP53, p.H179R) whereas the 3 discordant insertion and duplication variants were inframe changes in oncogenes (BRAF, p.A598\_T599insV; BRAF p.T599dup; EGFR; p.V769\_D770insGSV). All Tier IV (benign, 16/16) and splice site variants (8/8) showed 100% concordance. **Conclusions:** VI showed good concordance with manual variant curation for most types of variants, with the exception of 6 missense and inframe alterations. Accurate interpretation of these 6 variants requires manual review of published functional studies by a molecular pathology professional. Overall, the preliminary results indicate that VI can potentially improve the efficiency of variant interpretation workflow by limiting the need of manual variant review to less common and novel missense and inframe variants.

#### **1027. Detection of Microsatellite Instability Using a Large Next-generation Sequencing Panel across Diverse Tumor Types**

J. Pang, M.M. Mansukhani, S.J. Hsiao

Columbia University Medical Center, New York, NY.

**Introduction:** Microsatellite instability (MSI), a hallmark of DNA mismatch repair deficiency, is a key molecular biomarker with multiple clinical implications including selection of patients for immunotherapy, identifying patients who may have Lynch syndrome, and predicting prognosis in patients with colorectal tumors. Defective mismatch repair can be demonstrated clinically by PCR for MSI, or by immunohistochemical (IHC) staining for mismatch repair proteins. There are reports that these approaches may have differing performance characteristics in tumor types other than colorectal carcinoma and may, at a low but significant frequency, be discordant. Next-generation sequencing (NGS) provides the opportunity to interrogate large numbers of microsatellite loci simultaneously, potentially improving performance in different tumor types. We sought to develop a MSI classifier that would not require paired normal tissue and would leverage the sequence data obtained from a broad range of tumors tested using our 467 gene NGS targeted cancer test (Columbia Combined Cancer Panel, CCCP). **Methods:** Mononucleotide and dinucleotide regions across the genome were identified using the RepeatFinder tool (part of the Mantis software package) and intersected with the regions covered by the CCCP panel. Insertion/deletion (indel) variants in these regions, following removal of commonly seen variants in our internal dataset, were evaluated in clinical samples encompassing a diverse range of tumor types (n=18). MSI or defective mismatch repair status was assessed by fluorescent multiplex PCR (Promega) or IHC staining for MLH1 (clone G168-15), MSH2 (clone FE11), MSH6 (clone 44) and PMS2 (clone MRQ-28). **Results:** 8,732 mononucleotide and dinucleotide repeat regions covered by the CCCP assay were identified. Following removal of commonly seen variants in our dataset, 8,682 mononucleotide or dinucleotide loci remained. Indel variants in these regions were evaluated in a training set (n=107) of clinical tumor cases sequenced by the CCCP assay and were used to develop a classification rule. This algorithm was then used to analyze a test set of clinical cases (n=64) and was able to correctly classify 100% of cases as microsatellite stable or microsatellite unstable. **Conclusion:** We show that we were able to accurately classify a diverse range of tumors as microsatellite stable or unstable by interrogation of mononucleotide and dinucleotide loci for indel variants in the CCCP assay. This study highlights the utility of this approach which should be applicable to other laboratories performing similar testing.

#### **1028. A Rigorous Interlaboratory Examination of the Need to Confirm NGS-Detected Variants by an Orthogonal Method in Clinical Genetic Testing**

S.E. Lincoln<sup>1</sup>, J. Zook<sup>2</sup>, C. Lin<sup>3</sup>, R. Truty<sup>1</sup>, M. Salit<sup>4</sup>, H. Rehm<sup>5</sup>, R. Nussbaum<sup>1</sup>, M. Lebo<sup>3</sup>

<sup>1</sup>Invitae, San Francisco, CA; <sup>2</sup>National Institute of Standards and Technology, Gaithersburg, MD; <sup>3</sup>Brigham and Women's Hospital, Cambridge, MA; <sup>4</sup>Stanford University, Palo Alto, CA; <sup>5</sup>Harvard Medical School, Cambridge, MA.

**Introduction:** The confirmation of variants identified by next-generation sequencing (NGS) using orthogonal assays (e.g., Sanger sequencing) is standard practice in many laboratories. Published studies have examined this issue, concluding that confirmation of the highest-quality NGS calls may not always be necessary. However, these studies are often small, lack statistical justification, and explore limited aspects of the underlying data. Defining criteria that separate high accuracy NGS calls that do not benefit from confirmation from those that do remains a critical and pressing issue. **Methods:** We conducted a rigorous examination of NGS data from two clinical laboratories. Five Genome in a Bottle reference samples and over 80,000 clinical patient specimens were analyzed, with the combined data providing insights that neither data type alone could provide. In total, almost 200,000 variant calls with orthogonal data were examined including 1,684 diverse false-positives detected by confirmation. **Results:** We used these data to identify laboratory-specific criteria that flag 100% of false positives as requiring confirmation (CI lower bound: 98.5 to 99.8% depending on variant type and laboratory) while minimizing the number of flagged true positives, i.e. variants that would

unnecessarily undergo confirmation. Rather than relying on one or two quality metrics, as current publications do, a battery of criteria proved superior, consistent with the metrics recommended by recent AMP/CAP practice guidelines. Indeed, our expanded criteria identify some false positives as requiring confirmation that the currently published criteria miss. We also find that historical performance (observing a variant as a true positive some number of times) without applying additional stringent criteria can also lead to false positives escaping confirmation. This particular observation has implications for certain current practice guidelines. **Conclusions:** Although we found limitations with some currently published criteria and guidelines, our large, inter-laboratory study reaffirms prior findings that high accuracy variant calls can be separated from those of lower confidence. Our methodology for determining test and laboratory-specific criteria can be generalized into a practical approach, usable by many laboratories, which can help reduce the cost and time burden of confirmation without impacting clinical accuracy.

#### 1029. Dual-Assay Demultiplexing with Preferential Read Allocation and Unequal Index Size Presents Bioinformatics Challenges

A. Chitturi, A. Bigdeli, A. Oran, R. Sussman, J. Rosenbaum, J. Morrisette, R.B. Faryabi  
Hospital of the University of Pennsylvania, Philadelphia, PA.

**Introduction:** In high-throughput clinical next-generation sequencing, combining samples from different assays on a single sequencing run can improve turn-around times while reducing expenses. However, demultiplexing pipelines are often assay-specific, as they utilize adapter length, which can change with assay chemistry. Here, we present a pipeline to demultiplex mixed sequencing runs containing samples prepared on both a HaloPlex-based assay (Halo) employing 10-basepair (bp) unique molecular identifiers (UMIs) and an Archer Fusionplex-based assay (Archer) that includes dual 8-bp indices. We establish the advantage Halo samples enjoy in preferential read allocation, and the effect this has on the number of reads allocated to Archer FASTQs at different sample ratios. We also examine the effects of downstream analysis, including downsampling and read depth normalization, on the reported variant frequencies. **Methods:** Samples from two different assays were run on the same HiSeq sequencer: A Halo custom assay with a 10-bp UMI, and an Archer translocation assay with dual 8 bp-indices on each adapter plate. Two separate sample sheets for each assay are input to bcl2fastq 2.17 independently. The read boundaries are modified and assay-specific cycle information is passed into bcl2fastq to separate reads and produce distinct FASTQ files for each sample in both assays. For the second indexed read the program is told to read 10 bp in the RunInfo file, with a 10-bp UMI read for Halo (i\*) and the first eight bases read for Archer (i8nn). **Results:** Read allocation heavily favors Halo FASTQs; in a given combined run, the predicted number of reads per sample for Halo outnumbers those for Archer, and the degree to which Halo samples preferentially use reads depends on the total number of samples sequenced together. The discrepancy results in fewer than expected reads for Archer FASTQs, and causes inconsistent detection of fusion transcripts. The combined Halo-Archer sequencing run results in increased false-positive fusion calls and index contamination in Archer libraries when compared with the same pools sequenced independently. This effect varies with the ratios of Halo and Archer samples present in the run. Read counts and variant frequencies for Halo samples are unchanged, likely due to the significantly larger size of Halo libraries. **Conclusions:** We observe a large decrease in read counts across Archer samples as the ratio of Halo to Archer samples is increased. The number of Halo samples on a combined run must be limited such that read allocation to Archer FASTQ files is not significantly inhibited. We look to establish a threshold ratio allowing for consistent variant frequencies with low deviation when compared to the same libraries sequenced independently.

#### 1030. Integrating Clinical Genomics into Electronic Health Records to Foster Precision Medicine

A. Sigaras<sup>1</sup>, K. Eng<sup>1</sup>, E. Sticca<sup>1</sup>, S. Ramazanoglu<sup>1</sup>, S. Parmar<sup>1</sup>, A. Aljaludi<sup>2</sup>, M. Israel<sup>1</sup>, D. Artz<sup>1</sup>, C. Leslin<sup>3</sup>, J. D'Agostine<sup>3</sup>, G. Cheang<sup>1</sup>, D. Pancirer<sup>1</sup>, M. Rubin<sup>1</sup>, W. Tam<sup>1</sup>, M. Kluk<sup>1</sup>, J. Catalano<sup>1</sup>, H. Beltran<sup>1</sup>, W. Song<sup>1,2</sup>

<sup>1</sup>Weill Cornell Medicine, New York, NY; <sup>2</sup>New York Presbyterian, New York, NY; <sup>3</sup>Standard Molecular, Cambridge, MA.

**Introduction:** Precision Medicine can be defined as the delivery of the "right treatment to the right person at the right time". Providing clinicians with understandable genomics test results in a timely manner is thus a key component of any precision medicine effort. While next generation sequencing (NGS) technologies can generate a lot of data very rapidly, delivering those data with clear reports to clinicians into the Electronic Health Record (EHR) is still challenging. **Methods:** At the Englander Institute for Precision Medicine (EIPM), we designed a modular framework to provide clinicians with high quality clinical genomics reports in a timely fashion. In addition to PDFs, each variant is imported into the EHR as discrete unit of information, enabling the development of advance querying and alerting tools. The EIPM Laboratory Information Management System (LIMS) provides the connection between the legacy systems and the computational pipelines and the case review and sign-out system (NGSReporter). Variants are classified into tiers based on relevance to clinical management, tumor type and primary site and are linked to interpretations based on our precision medicine knowledge base (PMKB: <https://pmkb.weill.cornell.edu>, Huang *et al.* JAMIA 2017). This ensures that variants use the same interpretation regardless of the assay performed. **Results:** As an example, we here discuss EXACT-1: a Whole Exome Sequencing test for cancer patients that requires matching tumor and normal specimens (Rennert *et al.* Genome Med, 2016). Clinicians request EXACT-1 via the EHR triggering retrieval of archival specimens and blood drawing. Samples are processed and tracked via LIMS. Once on the sequencer, computational pipelines automatically generate the results while communicating their status to LIMS. Molecular pathologists (MPs) then review each case via NGSReporter, which provides easy access to the results: e.g. alignment files, variant allele frequency for point mutations, log2 fold-change for copy number alterations. MPs can add comments and electronically sign-out a case. Upon signing-out, both the PDF and the discrete variants are sent to the EHR via HL7 messages. This framework enabled EIPM to achieve an average turn-around-time of about 18 business days for EXACT-1 and has been extended to other NGS assays. **Conclusions:** NGS testing is rapidly becoming essential to the standard of care for cancer patients. Delivery of interpretable reports to clinicians in a timely matter is key component for any clinical genomics laboratory. A modular information infrastructure is thus crucial to fulfill the goal of precision medicine of "right treatment, right person, and at the right time".

#### 1031. Standardization of Molecular Diagnostic Testing for Non-small Cell Lung Cancer

A. Karimnezhad<sup>1</sup>, B. Lo<sup>2</sup>, P. Campbell<sup>1</sup>, D. Stewart<sup>2</sup>, T. Perkins<sup>1</sup>

<sup>1</sup>Ottawa Hospital Research Institute, Ottawa, Canada; <sup>2</sup>The Ottawa Hospital, Ottawa, Canada.

**Introduction:** Recent studies employing next generation sequencing (NGS) technologies have identified specific genetic mutations that predict therapeutic success with targeted treatment in many forms of cancer, including non-small cell lung cancer (NSCLC). However, mutation assays are generally developed using tissues from surgical samples, whereas many metastatic NSCLC patients only have fine needle aspirate (FNA) biopsies. Given the limited number and heterogeneity of cells in FNAs and the expanding number of clinically actionable mutations, the development of NGS testing strategies to detect driver mutations in NSCLC remains a challenge. **Methods:** Our study focuses on the identification of best methods (pre-analytical, analytical, and bioinformatic) to detect driver mutations in lung FNAs to standardize diagnostic targeted NGS testing for NSCLC. Here, we report performance comparisons of five variant callers (Mutect2, VarScan2, VarDict, Samtools, and Pisces) using TruSight 170 test data from replicates of a well-characterized Coriell cell line (GM12878) sequenced on an Illumina NextSeq, as well as replicates of an Acrometrix synthetic DNA panel sequenced on an Ion Torrent PGM. We



evaluated the variant callers by statistical measures including sensitivity, specificity, precision and reproducibility between technical and biological replicates, and we study the influence of coverage and allele frequency on variant detection. **Results:** We initially observed large discrepancies between the variant sets called, though many disagreements turned out to be due to different default values for different built-in parameters (such as minimum allele frequency, minimum map quality, etc.). Discrepancies are also partially based on the algorithms and model settings pre-defined in each mutation caller. Following the default parameters, VarDict was the best variant caller, detecting a significant percentage of the mutations present in GM12878 and Acrometrix data sets. VarScan2, Mutect2 and Pisces were our next preferences. Samtools had the weakest performance, failing to detect many of the mutations known to be present. When matching parameters between programs as far as possible, performance was much more similar, although VarDict remained the best performer. **Conclusions:** We recommend against using a mutation caller with its default parameters, as many clinical labs do, and instead suggest reviewing all parameters of any mutation caller, before applying it directly to any data. Differences in default parameters are a greater influence on algorithm disagreement than differences between algorithms. We are still assessing the callers in different settings, but so far, our analysis suggests that VarDict is the best caller.

### I032. Effects of Probe Regions on Somatic Variant Calling in TruSeq Amplicon Cancer Panel

P.B. Mayigowda<sup>1</sup>, P. Keswarpu<sup>1</sup>, M. Zhong<sup>2</sup>, J. Fallon<sup>2</sup>, N. Dimitrova<sup>1</sup>, F. Ye<sup>2</sup>

<sup>1</sup>Philips Healthcare, Valhalla, NY; <sup>2</sup>Westchester Medical Center, Valhalla, NY.

**Introduction:** The TruSeq Amplicon Cancer Panel is highly multiplexed and targeted assay which is used to detect somatic mutations in the hotspot region of 48 oncogenes and tumor suppressor genes. We were evaluating the use of this assay in the clinical setting. For this purpose, we developed our own bioinformatics pipeline to analyze the Fastq files and call variants. **Methods:** We sequenced 14 clinical samples using the TruSeq Amplicon Panel and ran through a custom pipeline which used BWA for alignment and VarScan2 for variant calling. We introduced additional steps to evaluate QC and also annotated the variants to filter out germline variants. Compared with commercial variant caller "Illumina VariantStudio 3.0", we found some of discrepancy variant calls, which were only called by our pipeline. The discrepancy variants were further verified by Sanger sequencing and confirmed that these were false calls. To eliminate the false calls, we used custom scripts to trim the Probe regions from Fastq reads, this approach is called Hard-clipping where the Probe regions are trimmed using Upstream and Downstream Locus Specific Oligo (ULSO and DLSO) sequences. We also used another approach where we Soft-clipped the probe regions from realigned bam files using a publicly available tool called *BAMClipper*. The bam file was then used to call variants. Then we compared the results of 2 approaches.

**Results:** During the initial pipeline validation stage, we observed a lot of variants in the flanking regions which looked like systemic artifacts. The Sanger sequencing results indicated that these calls were false positive. After a detailed investigation of bam files, we observed that these variants fall under Probe/primer regions. Hard-clipping successfully removed all the probe regions from the input Fastqs, however this approach seemed to affect the downstream steps. It resulted in poor Mapping qualities in all the 14 samples and the average read depth reduced by over ~33% when compared to the original pipeline where no clipping was performed. The Soft-clipping approach, however, worked very well and was able to mark 100% of the Probe regions in all the 14 samples. Since *BAMClipper* uses the probe information to mark (soft-clip) in aligned bam files rather than removing the probe regions, we were able to maintain same Mapping quality and average depth. **Conclusion:** In summary, Soft-clipping of probes worked better than Hard-clipping in terms of quality of the variants reported, so we decided to use just the Soft-clipping workflow and also recommend this to other users. This enabled us to successfully identify high-quality variants for clinical interpretation and reporting.

### I033. Modern Application Deployment Infrastructure for Supporting Clinical Next-generation Sequencing (NGS) Testing

L. Santana Dos Santos, L. Chen, C. Kaya, A. Wald, Y.E. Nikiforov, M.N. Nikiforova, S. Roy

University of Pittsburgh Medical Center, Pittsburgh, PA.

**Introduction:** Software development in a complex and regulated environment of the clinical laboratory is challenging. Typical bottlenecks in a traditional healthcare IT setup include slow development cycle, longer downtimes, system instability during upgrades, and limited scalability. Complexity of bioinformatics tool and integration with clinical systems further worsen the bottleneck. Modern deployment tools, coupled with software version control (VC) and virtualization provide a feasible long-term solution for the clinical laboratory. **Methods:** We have implemented a modern informatics (DevOps) infrastructure for the development and deployment of applications supporting next generation sequencing (NGS) in the past 2 years. This framework consists of three main components: Version control (VC), continuous integration and deployment (CI/CD) and virtualization. The code base contributed by multiple developers is managed using Git version control system. CI/CD pipeline is provisioned using Jenkins. Docker containers are used to create virtualized environment for various components of our custom web application. The containers are orchestrated using a Kubernetes cluster hosted within institutional firewall. In order to safeguard protected health information (PHI), electronic communications are encrypted using secure network protocols (SSH and HTTPS). **Results:** Our new infrastructure has provided several strategic advantages in terms of system stability, high availability, short release cycle, and scalability. Use of containers has completely eliminated manual server configurations. It has provided significant scalability by rapid allocation of CPU and memory resources on-demand and seamless server migrations. It has also facilitated a software-oriented architecture (SOA) for our application making it more stable and maintainable. CI/CD coupled with tight VC allows fast redeployment of the clinical system with an average time of only 2 minutes. Deployment rollbacks are also supported. Overall this has yielded a highly available system with uptimes of >99% since Go-Live (13 months). System redundancy and CI/CD pipeline permits different developers to work in parallel without affecting production environment. Newly modified code undergoes extensive testing and validation using simulated and archived clinical cases before deployment into production. This allows release cycles to be as short as 7 days. **Conclusions:** Our informatics infrastructure helps provide a highly available, scalable, secure, and reliable clinical system to support NGS operations in a clinical environment. This infrastructure also promotes and supports a multi-developer setup. Lastly, short release cycles and tight VC helps keep up with the rapid development NGS technology without compromising patient care.

### I034. Assessing Cancer Diagnosis from Clinical Genomics Data Using Machine Learning.

P.R. Hess, J.N. Rosenbaum

University of Pennsylvania, Philadelphia, PA.

**Introduction:** Cancer diagnoses are primarily rendered by histologic evaluation of involved tissues before genomic testing results become available. Data generated from clinical genomic testing performed for prognostic and therapeutic purposes can be used to generate mutational profiles corresponding to histopathologic diagnoses. Such tools provide an opportunity for internal quality control (confirming the correct specimen was sequenced, for example). These tools could offer further benefit to cases involving carcinomas of unknown primary, uncharacteristic genetic findings, or suspected coexisting malignancies. We investigate automated computational approaches based on machine learning to assess mutational profiling from genomic testing as a proof of concept.

**Methods:** Machine learning algorithms, including hierarchical clustering and decision tree approaches, were applied to a clinical data set of over 300 solid tumors, each with multiple disease associated variants reported from a 152 gene somatic cancer sequencing panel, and correlated with associated surgical pathology diagnoses. **Results:** Solid tumor genetic profiles were evaluated by multiple approaches, enabling automated genomics-based classification of cancer into diagnostic categories. Subgroup hierarchical clustering groups cancers into distinct diagnostic

categories, including lung cancers with 100% accuracy (49/49), GI cancers with 87% accuracy (41/47), and melanoma with 74% accuracy (14/19). Comparison to other approaches and expansion of diagnostic categories is ongoing. **Conclusions:** This proof of concept study demonstrates that cancer diagnoses can be evaluated by routine clinical genomic testing using machine learning. Future studies that integrate this approach into a clinical workflow will help to better define potential roles in challenging diagnostic cases and for quality control.

#### Other (e.g. Education)

##### OTH001. Virtual Case Sets for Genomics Education: Thinking Outside the Slide Box

J.N. Rosenbaum<sup>1</sup>, J.J. Roth<sup>2</sup>, D.B. Lieberman<sup>2</sup>, C. Orr<sup>2</sup>, S. Deihimi<sup>2</sup>, J.J. Morrisette<sup>2</sup>

<sup>1</sup>Hospital of the University of Pennsylvania, Center for Personalized Diagnostics, Philadelphia, PA; <sup>2</sup>University of Pennsylvania, Philadelphia, PA.

**Introduction:** A principal advantage of genomic testing is the massive amount of data extracted from specimens. With enormous data sets and diverse findings, adequate training in interpretation, classification, and reporting becomes a challenge. Traditional didactics and observation of sign-out do not provide the breadth of experience nor the practical skills necessary for proficiency. Allowing trainees to revisit previously signed-out clinical cases offers practical training, but such opportunities cannot be offered safely in active clinical databases. In anatomic pathology, curated slide review sets are commonly used training tools, allowing extensive exposure and opportunity to develop pattern recognition and interpretation skills necessary for independent sign-out. Here we describe a software solution for genomics education modeled on the pathology slide box.

**Methods:** Of approximately 10,000 specimens sequenced on 4 assays at an academic hospital offering clinical massively parallel sequencing (also referred to as next-generation sequencing or NGS), approximately 100 were selected for the initial case set. Samples were included for training value, including "normal" cases, cases with specific clinical relevance, bioinformatically challenging cases, and interpretive challenges. Cases are duplicated from the clinical database into a training environment, including de-identified clinical data and aligned sequence files. Cases are annotated for relevant clinical context and dissociated from the variant knowledge-base. Trainees are assigned a unique identifier, applied to their cases. Cases may be edited (variants removed) to highlight specific educational features. **Results:** The training case set permits independent review of cases in an environment cloistered from the clinical database. Key features include: 1) A "Data-to-report" training experience; 2) De-identified cases link back to the original clinical case for review; 3) Dissociation from the clinical knowledge-base enables independent research / review by trainees, without risk to clinical data; 4) Integrated links to external databases and tools for review; 5) Variant classifications and interpretations tied to specific trainees, thus preventing re-work without requiring "scrubbing" of the database for subsequent trainees; and 6) Mock reports and case logs are generated for faculty review and tracking. **Conclusions:** In diagnostic genomic medicine, the opportunity for practical "hands-on" training is essential, but difficult to provide. Here we describe a software solution enabling a "virtual case set" modeled on the anatomic pathology slide-box. This model enhances training with a curated experience enriched for cases with educational value, fostering skills necessary for independent sign-out, while protecting the clinical workspace.

##### OTH002. Standardized Protocol for Salvaging Quality or Quantity Not Sufficient (QNS) Samples in an Academic NGS Laboratory

S.F. Priore, D. Lieberman, J.R. Milano, R.T. Sussman, J.N. Rosenbaum, J.J. Roth

University of Pennsylvania, Philadelphia, PA.

**Introduction:** As the demand for genomic testing increases, the pressure to generate sequencing data from ever more limited specimens also increases. This dynamic risks increasing the number of specimens yielding insufficient quality or quantity of analyzable material

(quality/quantity not sufficient or QNS). By definition, QNS specimens do not permit identification of genetic aberrations which may be of diagnostic, prognostic, or therapeutic utility. A QNS result can result from factors including, but not limited to, pre-processing and specimen viability. We examined the incidence of QNS samples in our laboratory and generated a laboratory-driven workflow to optimize alternative testing strategies for inadequate specimens. **Methods:** A retrospective analysis of 16,500 oncology next-generation sequencing (NGS) samples was conducted in our laboratory. A subset of 36 QNS samples was manually examined to determine common specimen handling practices within our laboratory. Based on these data, a new standardized workflow was created and implemented. **Results:** Of 16,500 NGS samples, 483 were classified as QNS (3%). 36 manually examined cases revealed QNS samples fall into three broad categories: 1) Samples with an alternative specimen that could be submitted for testing (14%), 2) Samples without acceptable alternative material, but with sufficient extracted nucleic acid for an orthogonal molecular assay (72%), and 3) Samples insufficient for categories 1 or 2, or for which testing is no longer indicated (14%). With these categories, we generated a workflow including mandatory clinical chart review, examination of the specimen inventory for the patient, and communication with the relevant clinical and laboratory team members. Since pathology trainees have more exposure to anatomic pathology specimen processing than dedicated sequencing staff, they play an essential role in implanting the new workflow while also gaining exposure to evidence-based practice guidelines for genomic testing of malignancies. Applying this workflow retrospectively to 483 QNS cases, approximately 67 (14%) and 348 (72%) cases would have had an alternative specimen for testing or sufficient nucleic acid for a relevant alternative assay, respectively. **Conclusion:** We describe a logical, evidence-based algorithm to salvage QNS samples from an existing NGS workflow. This protocol enables reporting of results for patients with specimens initially determined to be QNS. Importantly, we emphasize the search for alternative specimens to maximize NGS testing, delivering the intended test and the most actionable results for the greatest number of patients.

#### Solid Tumors

##### ST001. Clinical Utility of Reflex Ordered Testing for Molecular Biomarkers in Stage IV Lung Cancer

T.L. Phung, K. Anand, P.L. Cagle, R.L. Olsen, E.L. Bernicker, J.L. Thomas

Houston Methodist Hospital, Houston, TX.

**Introduction:** Clinical testing of molecular biomarkers to guide therapy in non-small cell lung cancer has become routine and increasingly complex. To standardize and expedite molecular biomarker testing in these patients, our institution implemented reflex ordered testing for a group of targeted gene alterations in all newly diagnosed lung adenocarcinoma. The aim of our study is to evaluate the clinical utility of reflex ordered molecular testing in advanced lung cancer. **Methods:** Lung adenocarcinoma specimens received for molecular testing at our institution over a 6-month period in 2017 were identified with IRB approval. Testing for the entire group of molecular biomarkers is ordered at diagnosis (reflex ordered) or specific biomarkers are tested at request of oncologists (non-reflex ordered). Reflex ordered biomarkers include: *EGFR*, *KRAS*, *BRAF* and *ERBB2* mutations, *MET* exon 14 skipping, and *ALK*, *RET* and *ROS1* gene rearrangements, *MET* amplification, and *PDL1* gene expression.

**Results:** The cohort includes 98 patients with stage IV lung adenocarcinoma; 53% (n = 52) males, 47% (46) females, and median age of 75 years. 71% (n=70) of cases had reflex testing and 29% (28) had non-reflex testing. The mean number of days from the anatomic pathology report sign-out to the molecular report sign-out was 23 days for reflex ordered vs. 52 days for non-reflex testing. Reflex ordered cases had a higher variant detection rate than non-reflex (47% vs. 19%, p < 0.05). Specific variant detection rates were: *EGFR* = 11.7% (n = 11), *KRAS* = 29.4% (20), *BRAF* = 2.7% (2), *ERBB2* = 0% (0), *ALK* gene fusion = 5.4% (5), *RET* fusion = 5.8% (4), *ROS1* fusion =

2.4% (2), *MET* exon 14 skipping = 0% (0), and *MET* amplification = 2.1% (2). The majority (76.1%) of cases had <50% tumor PDL1 gene expression. **Conclusions:** The detection rates and types of pathogenic variants identified within our cohort are similar to those reported in literature. Mean turn-around-time was significantly reduced and the variant detection rate was 2.5 times higher for reflex-ordered testing compared to non-reflex. These findings show that a standardized comprehensive strategy for molecular testing increases timely implementation of personalized therapy in patients with advanced lung cancer.

#### ST002. Analysis of Urinary Cell-free DNA for Early Detection and Surveillance of Bladder Cancer

J. Dudley<sup>1</sup>, J. Schroers-Martin<sup>1</sup>, D. Lazzareschi<sup>1</sup>, W. Shi<sup>1</sup>, S. Chen<sup>1</sup>, J. Liao<sup>2</sup>, A. Alizadeh<sup>1</sup>, M. Diehn<sup>1</sup>

<sup>1</sup>Stanford University, Stanford, CA; <sup>2</sup>VA Palo Alto Health Care System, Palo Alto, CA.

**Introduction:** Current regimens for the diagnosis and surveillance of bladder cancer (BLCA) include urine cytology, which has low sensitivity, and cystoscopy, which is invasive and relatively expensive. Several ancillary tests have been developed to address these challenges, but none are widely used due to limited performance characteristics. By contrast, cell-free DNA (cfDNA) profiling has demonstrated high performance characteristics in previous studies for the noninvasive detection and surveillance of multiple cancer types. **Methods:** We developed a high throughput sequencing method for urinary cfDNA and applied it to the detection and surveillance of BLCA. Our approach combines an extraction protocol compatible with large fluid volumes, hybrid-capture based target enrichment with a 311 kilobase panel designed for BLCA, and an informatic pipeline that accommodates the wide distribution of fragment sizes found in urine. We used this approach to profile cfDNA in urine samples from 54 patients with early-stage BLCA (74% pTa, 6% pTis, 9% pT1, 11% pT2), 64 patients undergoing surveillance after treatment of localized BLCA, and 67 healthy adults. **Results:** We detected a median of 7 mutations per patient with 73% concordance between urine and tumor. Across both cohorts, the two most commonly mutated regions were the *TERT* and *PLEKHS1* promoters (74% and 46% of cases, respectively). In the cohort of patients with early-stage BLCA, the sensitivity of our method was 93% with an approach leveraging prior knowledge of mutations in each patient's tumor and 83% when blinded to tumor mutation status, compared to 14% for cytology performed on the same cases ( $p < 0.0001$ ). In a cohort of patients undergoing surveillance for recurrence, the sensitivities of tumor mutation informed and blinded approaches, respectively, were 91% and 84% while the sensitivity of cytology was 38% ( $p = 0.0001$ ). Concurrent cystoscopy and UroVysion results were available for a subset of patients in the surveillance cohort who developed recurrent disease, and cfDNA profiling outperformed the sensitivity of cystoscopy (34%,  $p < 0.0001$ ,  $n = 32$ ), the combination of cystoscopy and cytology (53%,  $p = 0.006$ ,  $n = 32$ ), and UroVysion (43%,  $p = 0.02$ ,  $n = 7$ ). Across both cohorts and methods, cfDNA profiling maintained high specificity (96-100%) and detected tumor DNA in 21/21 (100%) of cases that were positive by cytology and 54/66 (82%) of cases that cytology missed. **Conclusions:** We present a workflow optimized for urinary cfDNA profiling and apply it to samples from patients with BLCA. Our method significantly improves on the sensitivity of existing diagnostic modalities while maintaining high specificity. It could therefore facilitate the early detection and surveillance of BLCA through entirely noninvasive means.

#### ST003. Utility of a Comprehensive and Cost-effective DNA/RNA Panel (170 Genes) for Single Nucleotide Variants (SNV's), Small Insertions or Deletions (Indels), Copy Number Variations (CNV's) Splice Variations, and Gene Fusions on an NGS in Evaluation of Colon Cancer

R. Kolhe, A.K. Mondal, C. Pundkar, P.K. Ahluwalia, A. Chaubey  
Medical College of Georgia, Augusta, GA.

**Introduction:** Anti-EGFR monoclonal antibodies have been the main targeted therapies for CRC that require knowledge of the mutational status of genes in the pathway as predictive biomarkers of response to these therapies. Recently ASCP, CAP, AMP, and ASCO jointly approved guidelines for Molecular Biomarkers for the Evaluation of Colorectal

Cancer. Majority of labs use either DNA based small hotspots panels or two separate DNA and RNA panel. Unfortunately, this methodology can lead to an incomplete mutation profile because it lacks comprehensive screening of all known hotspots and tumor-suppressor genes. Many heterogeneous tumors carry multiple mutations and gene function can be altered by several types of variations including SNVs, insertion/deletions (indels), SV's and GF's. The assay is a targeted next-generation sequencing (NGS) assay designed to detect genetic alterations in 170 genes, including 148 genes for substitutions (single nucleotide and multinucleotide variants) and indel detection, 55 genes for fusion and splice variant detection, and 59 genes for CNV detection. Assessment of fusions, splice variants, indels and SNVs, and CNV's in one assay using DNA and RNA creates efficiencies in sample usage, time, and cost.

**Methods:** The validation was guided by the joint consensus recommendation for validation of NGS assays by the AMP and CAP. The validation included evaluations of precision, analytic sensitivity, analytic specificity, accuracy, reportable range, and reference range. RNA and DNA from 44 samples (mixture of known patient specimens with colonic adenocarcinoma, known CAP proficiency specimens as well as known synthetic reference standards (Acrometrix hotspot panel and Seracare fusion V2 panel)) was used. Libraries for were prepared using the Illumina TruSight Tumor 170 (TST170) kit and sequenced on NextSeq 550. Sequencing analysis and variant calling were performed using the BaseSpace TST170 App. **Results:** All the genes included in the guideline statement (*KRAS*, *NRAS*, *BRAF*) had great coverage in our panel. In addition, we were able to investigate newer and upcoming biomarkers by our panel. Overall we were able to validate manufactures analytical claims for all the variants groups in our validation e.g. for small variants,  $\geq 98\%$  sensitivity and 100% specificity at 5% allele frequency at positions with coverage  $\geq 250x$  was obtained. **Conclusions:** DNA and RNA libraries are prepared, sequenced, and analyzed simultaneously for efficient assessment of numerous types of somatic variants. We anticipate that this approach of obtaining high-resolution data from formalin-fixed, paraffin-embedded (FFPE) samples at reduced sequencing cost, will facilitate testing in different malignances which was not previously possible.

#### ST004. Validation of FFPE Tissue Punches for Detection of *KRAS* and *BRAF* Mutations with the Idylla PCR-based Molecular Diagnostics Assay

D. Morlote, A. Yemelyanova, R. Guo, G. DeFrank, D. Horton, Z. Grace, M.D. Musgrave, S. Harada

University of Alabama at Birmingham, Birmingham, AL.

**Introduction:** The Idylla *NRAS*-*BRAF*-*EGFR*-*S492R* Mutation Assay and the Idylla *KRAS* Mutation Test (Biocartis, Mechelen, Belgium) are fully-automated, real-time PCR-based molecular diagnostics assays with a 2-hour turnaround time that allow for the qualitative detection of the common hotspot mutations in *KRAS*, *NRAS*, and *BRAF* in formalin-fixed paraffin-embedded (FFPE) tissue samples. The Idylla system has been validated for FFPE tissue sections (5 to 10 microns), but it has not been validated for FFPE punches. The aim of this study is to compare the assay performance in both types of samples and to validate the use of a 1 mm tumor punch for the Idylla system. **Methods:** Ten colorectal cancer and two melanoma cases with known *BRAF* and *KRAS* mutations were selected based on availability of a FFPE block and a previous standard of care next generation sequencing (NGS)-based testing results. One FFPE tissue scroll (10 microns) and one 1 mm-diameter punch biopsy were obtained for each Idylla cartridge type. Deparaffinization, cell lysis, nucleic acid release, allele-specific PCR, and mutation detection occurred within the cartridges, per manufacturer's instructions. NGS testing had been previously performed using DNA extracted from three 1 mm tissue punches from the same FFPE block, and capture enriching for a custom cancer panel of 90 genes using the Agilent HaloPlex HS (Agilent Technologies) targeted sequencing method. Sequencing was performed on the MiSeq platform and analyzed by the PierianDX pipeline. Cohen's kappa statistics were used to calculate concordance amongst testing modalities. **Results:** The single 1 mm punch and the scroll tested by the Idylla *NRAS*-*BRAF*-*EGFR*-*S492R* and *KRAS* cartridges had a perfect agreement with each other and with the NGS *BRAF* and *KRAS* results ( $\kappa = 1$ ). Case 2, which was wild type for *KRAS*, yielded an "Error" report initially when testing the scroll with the *KRAS* cartridge, but was correctly



identified as wild type on repeat testing of another scroll obtained from the same block. **Conclusions:** A single 1 mm punch from FFPE tissue is a suitable alternative for use with the Idylla system for detection of *KRAS* and *BRAF* mutations. Advantages of using the punch versus the scroll include ease of handling and prevention of tissue loss due to fragmentation and flaking of the paraffin scrolls. In addition, the punch method can be used to select a tumor-rich area for the target enrichment. The Idylla system is a quick and reliable assay for initial hotspot mutational analysis that could be implemented into clinical workflow.

#### ST005. Evaluation of a Guideline-adherent Tumor Mutational Burden (TMB) Module for Calculation of TMB Using a Comprehensive 170 Gene Assay on a NGS Platform.

R. Kolhe<sup>1</sup>, T. Boyle<sup>2</sup>, T. Edmonston<sup>3</sup>, A.K. Mondal<sup>1</sup>, C. Pundkar<sup>1</sup>, P.K. Ahluwalia<sup>1</sup>, A. Chaubey<sup>1</sup>

<sup>1</sup>Medical College of Georgia, Augusta, GA; <sup>2</sup>Moffitt Cancer Center, Tampa, FL; <sup>3</sup>Cooper University Hospital, Camden, NJ.

**Introduction:** High TMB is an emerging biomarker for predicting response to immunotherapy treatment. TMB measured using whole exome sequencing has previously been shown to predict response to immunotherapy. Multiple studies have shown that measurement of TMB from large panels can be reflective of measurements made using whole exome sequencing and are more practical for implementation in clinical care. The TST170 panel is a next generation sequencing (NGS) assay designed to detect genetic alterations in 170 genes, including 148 genes for substitutions (single nucleotide and multinucleotide variants) and insertion/deletion (indel) detection, 55 genes for fusion and splice variant detection, and 59 genes for CNV detection and can be used to estimate TMB with results comparable to whole-exome sequencing. **Methods:** This validation is a joint effort by multiple molecular academic diagnostic laboratories in United States. The group termed as Sequoia Team (Sequencing and Oncology Informatics Academic Team) includes laboratories from multiple academic hospitals including Augusta University, Moffitt Cancer Center, Cooper University hospital *et al.* The validation was guided by the joint consensus recommendation for validation of NGS assays by the AMP and CAP and NYSDOH. The validation included evaluations of precision, analytic sensitivity, analytic specificity, accuracy, reportable range, and reference range for TMB. DNA from 50 samples which were characterized on Foundation CGP assay were used during the validation. Libraries for were prepared using the Illumina TruSight Tumor 170 (TST170) kit and sequenced on NextSeq 550. The TMB module used for calculation of TMB in this validation was designed and developed by PerianDx. TMB was calculated as the number of somatic mutations (single-nucleotide variants (SNVs) and insertion/deletions (Indels) only) per megabase (Mb) of the target region (0.524 Mb for TST170). **Results:** *In silico* analysis using the TCGA data both by PerianDx and Illumina (white paper) showed that the TST170 target space estimates TMB at a high level of concordance with whole exome sequencing (WES). The data show a high correlation ( $r^2 = 0.98$ ) between the TMB values determined using WES data and TMB using the TST170 limited data. Additionally the *in silico* analysis using the TCGA data both by PerianDx and Illumina (white paper) also showed that the TST170 estimates a higher TMB in tumors that respond to the checkpoint inhibitors pembrolizumab and ipilimumab, similar to the TMB from WES in non-small cell lung cancer and melanoma trials. **Conclusions:** In this validation we demonstrate that the TST 170 assay can be used to reliably predict TMB across a wide range of cancer types using the PerianDx TMB module. Our lab's experience provides an example for others that may wish to implement TMB testing by NGS on a comprehensive panel.

#### ST006. Comparison of Two DNA Polymerases in Detection of DNA Methylation via Pyrosequencing

C.M. Farrell<sup>1</sup>, G.K. Olson<sup>1</sup>, J.M. Gale<sup>1</sup>, P. Leach<sup>1</sup>, T.L. Ruetschilling<sup>1</sup>, M.A. Vase<sup>2</sup>, D. Chabot-Richards<sup>3</sup>

<sup>1</sup>Tricore Reference Laboratories, Albuquerque, NM; <sup>2</sup>University of New Mexico Health Sciences, Albuquerque, NM; <sup>3</sup>University of New Mexico, Albuquerque, NM.

**Introduction:** Epigenetic methylation is involved in transcriptional repression and is assessed clinically as a biomarker for diagnosis, prognosis and prediction of treatment response in many different diseases. There are several methods in clinical use for evaluation of methylation and pyrosequencing is regarded as one of the most sensitive and quantitative methods. However, a high rate of discordance in results makes interpretation and comparison between laboratories difficult. Given the importance of consistency in results of a clinical assay and the potential impact of inconsistent results on patient care, we compared the performance of two different DNA polymerases in pyrosequencing based methylation detection assays. **Methods:** Previously extracted DNA from formalin fixed paraffin embedded tissue (FFPE) was obtained from TriCore archives. These samples were previously assessed for methylation of MLH1 or MGMT promotor regions using bisulfite conversion and PCR amplification with TaqGold followed by pyrosequencing. For this study, the samples were subjected to the same process except using GoTaq for PCR amplification. The average methylation obtained using each polymerase was compared. Next, DNA from blood and FFPE was methylated by S.ssi Methyltransferase (or a highly methylated patient sample). This was diluted by a negative sample of equal or comparable quality to create dilution curves for each polymerase. When available, previous MMR IHC results were reviewed for assessment of expression of MMR genes. **Results:** Methylation levels for each sample differed by as much as 30% between the two polymerases. The difference was more pronounced as methylation increased and less pronounced as methylation values went <20%. GoTaq required fewer PCR cycles to reach comparable amplification to TaqGold. Amplification with TaqGold produced higher methylation percentages than expected based on the dilution curve. The dilution curves for GoTaq were more linear than those for TaqGold, however both had bias toward higher than expected methylation values when tested on DNA from FFPE. One case positive for methylation at a low percentage showed normal MMR IHC expression, indicating that MLH1 expression may not be affected at low methylation levels. **Conclusion:** The use of different DNA polymerases can lead to different methylation percentages even when the rest of the methodology is the same. This further emphasizes that there should be caution in interpretation of methylation testing alone and comparison of results from two different labs or studies should be done with attention to even small differences in methodology. Additionally, samples showing low percentage methylation may lead to misinterpretation of results with potential impact on patient care.

#### ST007. WITHDRAWN

#### ST008. Analyses of *BRAF* Mutations and MSI Status Frequencies in TKi Non-treatable Lung Adenocarcinoma Patients

G.N. Berardinelli<sup>1</sup>, R.d. Cavagna<sup>1</sup>, F.E. de Paula<sup>1</sup>, L. Viana<sup>1</sup>, C. Scapulatempo-Neto<sup>2</sup>, P. de Marchi<sup>1</sup>, G.D. Pinto<sup>1</sup>, L.F. Leal<sup>1</sup>, R.M. Reis<sup>1</sup>  
<sup>1</sup>Barretos Cancer Hospital, Barretos São Paulo, Brazil; <sup>2</sup>Diagnósticos da América, Barretos São Paulo, Brazil.

**Introduction:** Lung cancer is the most common cause of cancer death in both males and females in the world and the 5-years overall survival is lower than 20%. Mutations in *EGFR* and *KRAS* genes are mutually exclusive in lung adenocarcinoma patients and *EGFR*-mutated patients (around 20%) are benefited with tyrosine kinase inhibitors (TKi). *BRAF* mutations have been described in approximately 6% of lung adenocarcinoma and patients carrying activating and inactivating *BRAF* mutations may benefit from anti-BRAF and anti-MEK therapies, respectively. Recently, microsatellite instability (MSI) phenotype was reported as a predictive biomarker of response to immunotherapy, making it a crucial biomarker for clinical management for lung adenocarcinoma patients. However, there are no studies evaluating *BRAF* mutations and MSI frequencies in Brazilian lung

adenocarcinoma patients. This study aims to determine *BRAF* mutations and MSI status in Brazilian lung adenocarcinoma patients who are not eligible for TKI therapy. **Methods:** Formalin-fixed paraffin-embedded samples from 320 patients diagnosed with lung adenocarcinoma at Barretos Cancer Hospital, Brazil, were evaluated in the present study. Samples were analyzed for *EGFR* (exons 18, 19, 20 and 21), *KRAS* (codons 12/13) and *BRAF* (exon 15) mutations by Sanger sequencing. Molecular MSI evaluation was performed using a hexa-plex microsatellite-marker panel by PCR followed by fragment analysis in wild-type *EGFR*, *KRAS* and *BRAF* cases. **Results:** Mean age of patients was 61.4 ( $\pm$  11.0) years and 57.9% were male. *EGFR*, *KRAS* and *BRAF* wild-type status was identified in 39.7% (127/320) of cases. *EGFR* mutations and *KRAS* mutations were identified in 31.5% (101/320) and 28.1% (90/320) of cases, respectively. *BRAF* mutations were identified in 0.6% of cases (2/320), which are inactivating mutations located at codons 581 (p.Asn581Ser) and 594 (p.Asp594Gly). Both *BRAF*-mutated patients were male diagnosed at advanced stage of the disease and presented overall survival lower than one year. MSI-high status was identified in 0.8% of cases (1/125) with *EGFR*, *KRAS* and *BRAF* wild-type status. Patient harboring MSI-high was female diagnosed at advanced stage of the disease and presented overall survival lower than one year.

**Conclusions:** *BRAF* mutations and MSI-high status frequencies are low in Brazilian lung adenocarcinoma LA patients, yet these patients may benefit from anti-MEK therapies and immunotherapy, respectively. The identification of *BRAF* mutations and MSI status may provide an improved clinical management for lung adenocarcinoma patients who are not eligible for TKI treatment.

#### ST009. Evaluation of Microsatellite Instability Testing and Lynch Syndrome Screening Through Tumor Sequencing Using Illumina TruSight Oncology 500 panel

S. Zhang, S. Kaplan, J. Yao, A. So, P. Le, Y. Guo, T. Du, J.S. LoCoco, G. Berry, A. Kuraishy, A. Benjamin, A. Jager, T. Pawlowski, C. Zhao, S. Bilke, K. Kruglyak  
Illumina, San Diego, CA.

**Introduction:** Microsatellite instability (MSI) status has been approved by FDA to select for patients with metastatic tumors for cancer immunotherapy treatments. Additionally, MSI status assessment is the first step in the genetic diagnosis for Lynch syndrome (LS), which is most frequently linked to germline mutations in mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2*, or *EPCAM*. LS universal tumor screening, which involves up to 6 sequential tests, is recommended by NCCN guidelines with any newly diagnosed colorectal or endometrial cancers. Here we assess the performance of Illumina TruSight Oncology 500 panel for up-front tumor sequencing for MSI status, as well as LS tumor screening. **Methods:** We assessed DNA from 147 formalin-fixed, paraffin-embedded (FFPE) tumor samples from various cancer types to determine MSI status. With our 523-gene panel, we calculated the MSI score using 130 homopolymer microsatellite loci. The performance was compared to a commercially available MSI-PCR assay with matched tumor-normal DNA. Subsequently, we analyzed tumor sequencing results for *BRAF* p.V600E status in colorectal subjects (n=58), as well as potential germline or somatic mutations in MMR genes or *EPCAM* for both colorectal and endometrial tumors (n=92). Finally, we sequenced matched normal FFPE samples for colorectal and endometrial tumors with the 523-gene panel to confirm any germline mutations linked to LS. **Results:** For the 147 subjects assessed through the MSI-PCR assay, 37 were identified as MSI-high (MSI-H) and 110 as microsatellite stable (MSS). With the 523-gene panel, we calculated the MSI score based on tumor-only sequencing and achieved 98.0% (95% CI: 94.2% - 99.6%) overall percent agreement, 100% positive percent agreement (95% CI: 90.1% - 100.0%) and 97.3% negative percent agreement (95% CI: 92.2% - 99.4%) with MSI-PCR. Specifically, the 3 discordant subjects were endometrial tumors classified as MSS by MSI-PCR and MSI-H by tumor sequencing. In addition, we screened for LS in colorectal and endometrial subjects (n=92). Based on our data, we proposed a LS screening method with three filtering criteria: 1) MSI-H status (n=35), 2) without *BRAF* p.V600E mutations (for colorectal subjects only, n=13), and 3) at least 1 MMR gene variant or *EPCAM* deletion inferred as germline by variant allele frequency (n=6). Matched normal sequencing with the same 523-gene panel

confirmed all 6 cases of identified LS mutations as germline.

**Conclusions:** Collectively, our results demonstrated that MSI status can be determined accurately with Illumina's 523-gene panel. In addition, LS screening by tumor sequencing is proposed to identify *BRAF* p.V600E status, as well as potential pathogenic germline mutations linked to LS.

#### ST010. Identification of Different Levels and Spatial Patterns of Methylation of Promoter-Associated CpGs74-78 of the O6-Methylguanine Methyltransferase Gene (*MGMT*) in Gliomas

F. Khan, N. Tabish, J. Friedman, D. Gao, T. Stephen, A. Hormigo, N. Tsankova, J. Houldsworth  
Icahn School of Medicine at Mount Sinai, Mount Sinai Health System, New York, NY.

**Introduction:** In glioblastomas (GBM), hypermethylation of promoter-associated CpGs of the O6-Methylguanine Methyltransferase (*MGMT*) gene has been recognized as a favorable prognostic biomarker of response and outcome to treatment with temozolomide (TMZ). Given different methodologies and CpGs assessed by laboratories, we sought to determine if *MGMT* methylation analysis of 5 CpGs in a single institution, could identify different levels and spatial patterns of methylation that with ongoing clinical correlations could provide better patient risk stratification. **Methods:** The IRB-approved study of 153 gliomas (57 female, 96 male, average age 56y, 90% with >60% tumor cellularity) comprised 101 GBM (grade IV), 10 oligodendrogliomas (ODG, 4 grade II, 6 grade III), 34 astrocytomas (AC, 8 grade II, 26 grade III), and 8 others. *IDH1* R132H expression was assessed in 146 and 79 submitted to next generation sequencing (NGS) mutation analysis (50-gene hotspot panel). *MGMT* methylation analysis of CpG74-78 was assayed by pyrosequencing of genomic DNA after bisulfite treatment (standard procedures). To define spatial patterns across the 5 CpGs, methylation levels in each positive specimen were normalized to the highest and hierarchically clustered using average linkage. One-way ANOVA ( $p < 0.05$ ) and Chi-squared analysis were applied in another approach to identify groups with significantly different methylation levels across all cases.

**Results:** *MGMT* methylation was detected in 61 (5 CpG average >13%), indeterminate in 9 (8-13%), and not detected in 83 (<8%) cases, with 90% of ODG, 56% AC, and 32% GBM positive. *IDH1* R132H mutation was detected in 67%, 55%, and 7% respectively. Hierarchical clustering in methylated cases revealed 5 clusters with 5 (high CpG75 and 77), 2 (high CpG75 and 78), 11 (high CpG76), 9 (high CpG77), and 33 (high CpG74 and 75) cases respectively. In the first 4, GBM was the predominant histology (70%), while in the last cluster GBM comprised only 39%. Consistently, *IDH1* R132H mutation was evident in 28% cases in the first 4 clusters and 41% in the last (32% and 48% respectively including other *IDH* mutations identified by NGS). Within GBM alone, 4 clusters were identified (excludes the second cluster). In the second approach, two significant groups ( $p < 0.05$ ) were identified where the first comprised 28 cases (average methylation 37.8%) and the second 125 (average 1.8%). The level of methylation of CpG75 followed by CpG74, CpG77 had the most significant impact on grouping. **Conclusions:** In gliomas, different patterns of methylation were recognized, with some histologic enrichment. In GBMs, similar spatial clusters were found. Increasing the cohort size and testing of association of patterns of *MGMT* methylation with TMZ response and clinical outcome are currently in progress.

#### ST011. Evaluation of Molecular Spectrum of *BRCA* Gene Mutation in Indian Scenario using Next Generation Sequencing (NGS) Approach

B. Das<sup>1</sup>, F. Ahmad<sup>2</sup>

<sup>1</sup>SRL Ltd., Thane West, India; <sup>2</sup>SRL Ltd., Mumbai, India.

**Introduction:** Specific inherited mutations in *BRCA1* and *BRCA2* notably increase the risk of breast and ovarian cancers in females at a young age compared to people who do not have these mutations. Inheriting a harmful *BRCA1* mutation increases a female's chance of developing breast and ovarian cancer to about 87% and 68% respectively whereas inheriting *BRCA2* mutation increases those risks to about 84% and 30% respectively. Early screening of these mutations, combined with personal and family history of an individual will aid in better disease management measures by the clinicians. **Materials and Methods:** A total of 890 samples were screened using Next Generation Sequencing (NGS) for germline mutations in *BRCA1* and *BRCA2* genes.

**Results:** Overall, *BRCA* mutation was detected in 11.01% of the total cohort (98/890). Among the detected cases, *BRCA1* mutations were more commonly seen (77.55%, 76/98) in comparison to *BRCA2* mutations (22.44%, 22/98). Amongst *BRCA1* mutations, exon 10 was a major hotspot with 42.1% (32/76) mutations belonging to this exon alone. This was followed by exon 2 having 13.1% (10/67) mutations. In fact, all of the 10 mutations reported in exon 2 was c.68\_69delAG (p.Glu23Valfs), the most commonly reported *BRCA1* mutation. In *BRCA2* gene >50% mutations were that of exon 10 (8/22 or 36.36%) and exon 11 (6/22 or 27.27%). In *BRCA1* gene the most common type of mutation was "deletion" which made up 53.94% of all mutations (41/76) and mostly belonged to exon 2 and exon 10. Missense mutations leading to stop codon made up 30.26% (23/76) of all mutations whereas the remaining was made of duplications, insertions and other missense mutations. Similarly in *BRCA2* gene too, "deletions" made up most of the mutations with 54.54% (12/22) belonging to exons 10 (4/22 or 18.18%), 11 (3/22 or 13.63%) and 25 (3/22 or 13.63%). Missense mutation leading to stop codon was found in 22.72% (5/22) of all *BRCA2* mutations.

**Conclusion:** Next Generation Sequencing (NGS) based sequencing is an economical and less laborious approach for sequencing multiple *BRCA* exons. This is one of the largest reports on *BRCA* genes till date from Indian subcontinent. Mutations in exons 2 and 10 seem to be the mutational hotspot for *BRCA1* gene, while exon 10 and 11 were hotspots for *BRCA2* mutation in this cohort of cases. This further emphasizes the need for low cost mutation screening methods for identifying individuals who are at high risk of breast/ovarian cancer and to use the knowledge for better genetic counseling and preventive treatments.

#### ST012. Development of a Novel Pan-Cancer Biomarker Panel for Improved Detection of MSI in Tumor and Liquid Biopsies

J. Bacher<sup>1,2</sup>, R. Halberg<sup>2</sup>, P. Ward<sup>3</sup>, E. Udho<sup>1</sup>, K. Murphy<sup>4</sup>, M. Urh<sup>1</sup>, L. Dubeau<sup>3</sup>, J. Pettersson<sup>3</sup>, D. Storts<sup>1</sup>, S. Gallinger<sup>5</sup>, D. Buchanan<sup>6</sup>, M. Jenkins<sup>6</sup>, N. Lindor<sup>7</sup>, J. Eshleman<sup>8</sup>

<sup>1</sup>Promega Corporation, Madison, WI; <sup>2</sup>University of Wisconsin, Madison, WI; <sup>3</sup>University of Southern California, Los Angeles, CA; <sup>4</sup>ProPath, Dallas, TX; <sup>5</sup>University of Toronto, Toronto, Canada; <sup>6</sup>The University of Melbourne, Melbourne, Australia; <sup>7</sup>Mayo Clinic, Scottsdale, AZ; <sup>8</sup>Johns Hopkins University, Baltimore, MD.

**Introduction:** A new multiplexed biomarker panel is being developed for detection of microsatellite instability (MSI) that is more sensitive than currently available systems. Preliminary research data shows increased MSI sensitivity for colon polyps and endometrial, skin and prostate cancers. The sensitivity of this prototype Pan-Cancer MSI System is being verified on 15 different cancer types in both the primary tumor and circulating cell-free DNA (ccfDNA) obtained from blood.

**Methods:** Selection of the new microsatellite biomarkers was done by screening 160 patients ≤55 years with ≥1 polyp and 100 endometrial cancer patients ≤ 50 years for MSI. The expanded study uses samples from 100 Lynch syndrome MSI-High CRC, 100 sporadic MSI-High CRC, 100 sporadic MSI stable CRC and 219 extra-colonic cancers obtained from the Colon Cancer Family Registry. Extra-colonic cancer types include: colon, rectum, stomach, small intestine, duodenum, jejunum, skin, breast, endometrium, ovary, prostate, kidney, ureter, bladder and thyroid. DNA extracted from tumor, blood and plasma are being tested for MSI using two pan-cancer systems: Promega's MSI Analysis System version 1.2 and the improved prototype Pan-Cancer MSI System. Mutations in mismatch repair (MMR) and BRAF genes were tested, as well as MMR expression by IHC. **Results:** 2.3% of colon polyps were MSI-H for the MSI Analysis System compared to 5.4% with the new prototype Pan-Cancer MSI System. Sensitivity and specificity of the new biomarker panel for detection of MMR deficient lesions was 100% and 96%. Similarly, sensitivity of the new biomarker panel for endometrial cancer was about 2-fold higher. Allele size changes for MSI-H samples were significantly larger with the new biomarkers making MSI classification highly accurate and robust. The MSI and IHC results were highly correlated. Evaluation of the new biomarker panel is being performed on over 500 cancer samples from 15 different cancer types. Matching MSI profiles from primary colon tumors and ccfDNA has been observed. We are currently determining the frequency of detection of MSI in ccfDNA in cases of metastatic and non-metastatic colon and

extra-colonic cancers. **Conclusion:** Research results indicate that MSI sensitivity for colonic polyps and many extra-colonic cancers can be increased by at least 2-fold over current MSI systems using the new MSI biomarker panel. The improved sensitivity of the Pan-Cancer MSI System should improve detection of MSI in an expanded number of cancer types and facilitate identification of individuals with both sporadic and hereditary MSI-H cancers. Increased sensitivity for detection of MSI in liquid biopsies could further expand the utility of MSI testing by allowing for non-invasive testing.

#### ST013. Comparison of Cobas EGFR Mutation Test and PANAMutyper R EGFR Assay in the Detection of EGFR Mutations in Plasma from Non-small Cell Lung Cancer Patients

K. Lee, Y. Lee, S. Lim, M. Kwon, H. Park, H. Woo

Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, Korea.

**Introduction:** Detection of *EGFR* gene mutation is essential in non-small cell lung cancer (NSCLC) patients at initial diagnosis for the proper selection of therapy. Sensitivity to tyrosine kinase inhibitors (TKIs) is associated with sensitive mutations, deletions in exon 19 and a point mutation in exon 21 (L858R, L861), exon 18 (G719X), exon 20 (S768I). T790M is associated with acquired resistance after initial response to TKIs. In this study, we assessed the usefulness of the allele specific PCR in the detection of *EGFR* gene mutation using plasma. **Methods:** From 2017 to 2018, a total of 44 formalin-fixed paraffin embedded (FFPE) specimens derived from lung (n=20), pleural fluid (n=9), lymph node (n=7) and other tissues (n=8), and 44 plasma specimens were obtained from 42 NSCLC patients who were at diagnosis before therapy (n=26), under follow-up after therapy (n=18) including TKIs. Mutation status of *EGFR* in FFPE was tested using the peptide nucleic acid (PNA) based PANAMutyper R *EGFR* assay (PANAGENE, Daejeon, Korea). Plasmas were tested using Cobas *EGFR* Mutation Test v2 (Roche Diagnostics, Mannheim, Germany) and PANAMutyper R *EGFR* assay. We investigated concordance between the Cobas and the PANAMutyper in the detection of *EGFR* mutations using plasma specimens, and compared plasma results to FFPE results. We also investigated the frequency of *EGFR* mutations in NSCLC, and association of *EGFR* status with clinical characteristics. **Results:** The mutation concordance between Cobas and PANAMutyper using plasma was 88.6% (kappa 0.77). The concordance rates between PANAMutyper using FFPE and Cobas using plasma and PANAMutyper using plasma were 75.0% (kappa 0.51) and 68.2% (kappa 0.41), respectively. Among 44 FFPEs tested, *EGFR* mutations were detected in 27 (61%) FFPEs, while mutations were revealed in 20 (46%) plasmas by Cobas and 15 (34%) plasmas by PANAMutyper. Detected *EGFR* mutations types and frequencies observed in FFPE were as follows: exon 19 deletion (34%), L858R (18%), L861Q (2%), G719X (2%), T790M with L858R or with exon 19 deletion (16%), and exon 20 insertion (5%). *EGFR* mutations were present only in plasmas but not in FFPEs in three patients at diagnosis and in three patients under follow-up. These three patients under follow-up underwent re-biopsy of tissue, and T790M was positive on re-biopsy. *EGFR* mutations were more commonly detected in never-smokers (n=25) (P=0.022 in tissue, P=0.005 in plasma), while age and sex, tumor stage were not significantly associated with mutations. **Conclusions:** *EGFR* mutations were better detected in FFPEs than plasma specimens, but *EGFR* mutations detected only in plasma would be helpful to diagnose residual cancer or relapse. The Cobas test detected *EGFR* mutation more than PANAMutyper assay on the same plasmas.

#### ST014. Comprehensive Genomic Profiling of Thyroid Neoplasm by Next-generation Sequencing of Fine Needle-Aspiration Biopsy Material Preserved in Cytolyt

H.J. Park, I. Baek, H. Tran, S.A. Alperstein, J.J. Heymann, M. Siddiqui, Q. Pan, W. Song

Weill Cornell Medicine / New York-Presbyterian, New York, NY.

**Introduction:** Fine Needle Aspiration (FNA) is a minimally invasive, cost effective, standard method to collect tissue for evaluation of thyroid nodules. FNA specimens may be collected in Cytolyt solution for cytological examination. This study evaluated if thyroid neoplasm can be



genetically characterized by next generation sequencing (NGS) of thyroid FNA specimens preserved in Cytolyt. **Methods:** OncoPrint Comprehensive Assay (OCA) version 2 was used to profile 143 cancer-related genes with 4 variation classes including Single-Nucleotide Variants (SNVs), Insertions and Deletions (Indels), Copy Number Variants (CNVs), and fusions (RNA only). DNA and RNA were extracted from 96 FNA patient specimens with malignant (n=21, Bethesda category VI), indeterminate (n=52, Bethesda categories III, IV and V), or benign (n=23, Bethesda category II) in cytological diagnoses. In addition, 21 of 96 nodules were resected, and NGS results by Ion AmpliSeq Cancer Hotspot Panel (50-Genes) of the resection specimens was reviewed to check concordance. **Results:** DNA from 71 and RNA from 52 specimens were interpretable. Uninterpretable specimens may be the results of degradation of nucleic acid after storage (Median: 41, Range: 5-98 days) at room temperature. Alterations with clinical significance were identified from papillary carcinoma (*BRAF*<sup>V600E</sup> (n=16, 59.3%), and *EML4ex2-ALKex13* fusion), medullary carcinoma (*HRAS*<sup>G13R</sup> and *RET*<sup>L629\_D631delinsH</sup>), follicular adenoma (*HRAS*<sup>Q61K</sup>), and nodular hyperplasia (*HRAS*<sup>G13R</sup> and *HRAS*<sup>Q61K</sup>). In addition, *BRAF*<sup>K601\_S605del</sup> was detected in a case for which a consensus diagnosis could not be reached, even after resection. *EML4ex2-ALKex13* fusion was confirmed by RT-PCR and Sanger sequencing. Paired testing between OCA and 50-Genes demonstrated 100% mutation concordance. **Conclusion:** FNA specimens preserved in Cytolyt solution are an adequate source of material for NGS testing. Use of these specimens for NGS provides an accurate genetic profile of thyroid neoplasm, including both novel and recurrent genetic alterations. These findings have immediate clinical implications in guiding surgical management.

#### ST015. TruSight Oncology 500: Measuring Tumor Mutation Burden with Targeted Sequencing

J. Ju, T. Jiang, S. Zhang, G. Berry, Y. Fu, S. Kaplan, J. Yao, S. Johansen, B. Crain, M. Salmans, P. Le, A.C. Jager, C. Zhao, S. Bilke, T. Pawlowski  
Illumina, San Diego, CA.

**Introduction:** Tumor Mutation Burden (TMB) has emerged as an important biomarker for cancer therapy selection after recent studies have shown the correlation between TMB and the effectiveness of checkpoint inhibitor immunotherapies. TruSight Oncology 500 (TSO 500) is a targeted next generation sequencing assay developed by Illumina for measuring TMB in formalin-fixed, paraffin-embedded (FFPE) tumor samples. TSO 500 targets a 523 DNA gene panel and is designed to be a tumor only workflow. As the assay does not require matched normal samples, the removal of technical noise and germline variants are critical steps in accurately measuring TMB. **Methods:** The TSO 500 assay is performed with unique molecular identifiers (UMIs), and sequenced on the Illumina NextSeq 550 platform. Data analysis is performed using an internal pipeline for detecting variants at 5% allele frequencies. For technical noise removal, we have developed a new variant calling algorithm that not only utilizes the information from UMIs, but also leverages sample specific error profiles to ensure a uniform variant calling performance across samples of different FFPE qualities. To accurately remove germline variants from TMB calculations, we developed a hybrid strategy that integrates information from large-scale public databases with the measured coverage and variant allele frequency of each variant. Ultimately, the number of somatic variants in coding regions with allele frequency above 5% is divided by total target coding regions with a coverage of at least 50x to calculate TMB. To assess the performance of the assay, we analyzed 117 unique FFPE tumor/normal samples across 5 tissue types with TSO 500 and additionally a subset (N = 55) with whole exome sequencing. **Results:** For technical noise removal, we assessed the number of false positive variants in the healthy normal FFPE samples. On average we observed 0.92 false positives per sample independent of sample quality ( $R^2=0.008$ ) with >87% samples containing fewer than 2 false positive variants. In addition, we tested a set of FFPE and cell line mixing samples with variants close to 5% and achieved a sensitivity of 98.7%. In germline variant filtering, we reached a specificity above 99.5%, which leaves fewer than 5 germline variants on average per megabase, and 90% sensitivity in identifying somatic variants that are used for TMB

calculation. Combined, the TMB measurements generated by TSO 500 highly correlated with those of paired tumor/normal whole exome sequencing ( $R^2 = 0.95$ ,  $N = 55$ ). Finally, with a TMB threshold of 10, we reached 92% classification accuracy ( $N = 117$ ) in distinguishing TMB high and TMB low samples. **Conclusions:** Our results demonstrate the ability of TSO 500 to robustly measure TMB in FFPE samples.

#### ST016. Analytical Validation of the OncoPrint Comprehensive Assay v3 with FFPE and Cell Line Tumor Specimens in a CAP-accredited and CLIA-certified Clinical Laboratory

A. Yuki, J.L. Chitwood, H. Sidhu, B. Kong, V.L. Dasalla, J. Black, S. Salazar, J. Williamson  
Thermo Fisher Scientific, West Sacramento, CA.

**Introduction:** Next generation sequencing (NGS) technology has demonstrated research advancements with the potential to improve future patient outcomes. As whole genome or exome sequencing can be cost prohibitive for clinical or research laboratories, targeted NGS panels are a practical means of assaying tumor genetics for clinically-relevant mutations. The OncoPrint Comprehensive Assay v3 (OCAv3) is a pan-cancer research NGS panel for detecting mutations, copy number variants (CNV), and fusions in 161 genes. We present here an analytical validation of the OCAv3 panel in a CAP and CLIA-accredited laboratory, demonstrating its effectiveness for testing formalin-fixed paraffin-embedded (FFPE) tumor samples and cell lines. **Methods:** FFPE samples (n=99 from 9 different tumor types: colon, lung, breast, brain, melanoma, pancreas, prostate, stomach and uterus) and cell lines for specific tumor variants (n=11) were sequenced. Libraries were prepared following OCAv3 kit instructions with 10 ng of input for DNA and RNA samples. Purified libraries were templated and sequenced with an Ion Chef and S5 XL system. Read alignment to hg19 and variant calling were performed using Torrent Suite and Ion Reporter software. Limit-of-detection (LOD) was determined by serially diluting variant cell lines with a variant-neutral cell line to a minor allele frequency (MAF) of 5% for single nucleotide variants (SNV) and insertions/deletions (INDEL), or 2 copies of a CNV. Sanger sequencing (SNV, INDEL), clinical NGS on another platform (SNV, INDEL, fusions), *in situ* hybridization (CNV, fusions) and qPCR (fusions) were used for orthogonal testing and comparative results used to calculate accuracy, specificity and sensitivity (orthogonally confirmed positives and negatives versus false positives and negatives). Samples with known variants were used to measure reproducibility (4 replicates with 4 technologists on 4 different sequencers) and repeatability (3 replicates with 1 technologist within 24 hours). **Results:** LOD for SNV and INDEL was 250 reads and 5% MAF, and 7 copies for CNV. Sensitivity, specificity and accuracy for SNV (77 tested), INDEL (13), CNV (16) and fusions (25) were all >99%. Repeatability was >99% for all SNV, CNV and fusions examined and 90% for INDEL. Reproducibility was 96%, 97%, >99% and 95% for SNV, INDEL, CNV and fusions, respectively. The positive and negative predictive values for all SNV, INDEL, CNV and fusions examined were >99%. **Conclusion:** Our validation shows targeted sequencing with the OncoPrint Comprehensive Assay v3 panel (For Research Use Only. Not for use in diagnostic procedures) produces data of sufficient sensitivity, specificity and accuracy in a variety of FFPE-derived tumor tissue using low amounts of DNA and RNA input.

#### ST017. Nextgen Digital Spatial Molecular Pathology: Digital IHC Coupled to Automated Gene and Protein Expression Profiling Measuring Complex Signatures within the Context of the Tumor Microenvironment

E. Immler<sup>1</sup>, M. Marron<sup>1</sup>, R. Nagle<sup>2</sup>, P. Shepard<sup>3</sup>, J. Yeakley<sup>3</sup>, B. Seligmann<sup>1</sup>  
<sup>1</sup>BioSpyder Technologies, Inc., Tucson, AZ; <sup>2</sup>University of Arizona, Tucson, AZ; <sup>3</sup>BioSpyder Technologies, Inc., Carlsbad, CA.

**Introduction:** Measurement of complex gene expression molecular signatures from fixed tissue has been problematic, especially given the desire to correlate those measurements to existing histology techniques within the context of the tumor microenvironment. We report the development and use of an *in situ* targeted sequencing assay to carry out gene expression analysis on antibody or H&E stained slides. A pan-cancer profile of 5,207 genes was obtained from selected focal areas within the tissue section using the TempO-Seq Digital Spatial Molecular Pathology (DSMP) platform, demonstrating that spatial resolution results

in biomarker specificity with a system any molecular pathology lab can use. **Methods:** The homogeneous TempO-Seq assay (Yeakley *et al.*, PLOSone, 2017; DOI:10.1371/journal.pone.0178302) was adapted to an *in situ* format carried out on an automated Bond (Leica) slide stainer followed by antibody or H&E staining, before performing IHC analysis and automated recovery of probes from a pan cancer assay of 5,207 genes for sequencing from focal areas on a digital imaging (CellSensus) platform. Alignment and data analysis was carried out using the automated TempO-SeqR software package. **Results:** Replicate areas of cancer, stroma, and normal histology of 130 to 30 µm diameter within breast and prostate cancer formalin-fixed, paraffin-embedded (FFPE) sections were profiled directly from antibody or H&E stained sections. The staining not only permitted targeting of the tissue to be profiled, but provided images that validated that the profiling data was tissue specific and not cross-contaminated. Among other questions, we address the statistical power and repeatability of the assay how stroma and high grade PIN adjacent to cancer differs from non-adjacent stroma and high grade PIN, as well as differences in cancer across a tumor. **Conclusions:** We demonstrate that the TempO-Seq DSMP platform can obtain complex gene expression profiles within the context of the tumor microenvironment histology, and that this spatial resolution provides biomarker specificity allows for histologically "pure" tissue profiles to be easily obtained. We demonstrate that DSMP can be used to address at the molecular level whether adjacent high grade PIN can be classified as cancer *in situ*, and to define molecular differences between adjacent and non-adjacent tissue, and between different areas of cancer within the tumor. We demonstrate a platform that carries out the TempO-Staining by an automated slide stainer, and combined IHC analysis/automated profiling on a digital imaging platform (CellSensus), followed by PCR and sequencing. This is a DSMP platform any molecular pathology lab can use to obtain complex molecular profiling and multi-omic data.

#### ST018. Clinical Implementation of Precision Medicine in the Classification of Medulloblastomas: Concordance, Conflict, Recurrence, and Reclassification

B. Liechty<sup>1</sup>, S. Patel<sup>2</sup>, D. Pendrick<sup>3</sup>, M. Snuderl<sup>2</sup>, J. Garvin<sup>3</sup>, M. Ko<sup>3</sup>, M. Mansukhani<sup>3</sup>, S. Hsiao<sup>3</sup>, H. Fernandes<sup>3</sup>

<sup>1</sup>Columbia University Medical Center/New York Presbyterian Hospital, New York, NY; <sup>2</sup>New York University Langone Medical Center, New York, NY; <sup>3</sup>Columbia University Medical Center, New York, NY.

**Introduction:** Medulloblastomas are high grade pediatric brain tumors. Four molecularly defined subtypes of medulloblastomas are associated with different biological behavior. WNT pathway tumors demonstrate favorable outcomes, group 3 tumors have the most aggressive behavior, and SHH and group 4 tumors display a less predictable behavior. Clinical implementation of RNA expression and methylation array profiling may provide supplemental information for more accurate classification and therapeutic management of medulloblastomas.

**Methods:** Histologically diagnosed medulloblastomas were subject to whole transcriptome and paired tumor/normal whole exome sequencing (WES) at Columbia University Medical Center and methylation array profiling at New York University Medical Center. Transcriptome data was interrogated by gene set enrichment analysis (GSEA) and manual inspection for differentially expressed genes. Germline variants were filtered through the use of patient-matched DNA. Somatic mutations in the tumor were further clarified by subtracting all variants in matched normal specimen with allelic fractions >5% from variants with allelic fractions >10% in the tumor. Methylation array data was evaluated using the CNS tumor classifier developed by the German Cancer Research Center. **Results:** Twenty-six tumors from 21 patients with at least one histological diagnosis of medulloblastoma were studied. Of 5 patients with multiple tumors, 3 had recurrence of medulloblastomas, while 2 had new primaries with findings consistent with glioma. 1 WNT pathway tumor with concordance by GSEA and methylation array profiling, was found to have a pathogenic variant in CTNNB1. 7 SHH pathway tumors were identified with high confidence, and cases with methylation data were concordant (n = 4). Truncating PTCH1 mutations were seen in 2 cases, while 1 case was found to have a homozygous deletion of PTCH1, and 1 case had a germline pathogenic variant in TP53. GSEA and methylation identified two group 3 tumors with high level expression of MYC. One group 4

medulloblastoma was identified with no conflicts. Eleven cases had conflicting subclassifications by at least one modality between group 3 and group 4 medulloblastoma. One group 4 tumor by GSEA had no match by methylation, and 1 tumor diagnosed as medulloblastoma by histology had molecular signatures favoring reclassification as a glioma.

**Conclusions:** The combination of methylation array profiling, transcriptome sequencing, and WES allows for identification of WNT and SHH pathway tumors, and provides supplemental information for identifying tumors incorrectly classified by histology. Distinguishing group 3 and group 4 tumors remains difficult, but high level expression of MYC is seen in all medulloblastomas which classify without conflicts.

#### ST019. Clinical Validation of a Combined DNA and RNA Target-capture Next Generation Sequencing (NGS) Test for Solid Tumors on FFPE Specimens

S.P. Strom<sup>1</sup>, M. Li<sup>1</sup>, L. Lam<sup>1</sup>, R.T. Jones<sup>1</sup>, J. Liu<sup>1</sup>, W. Scaringe<sup>1</sup>, J. Gao<sup>1</sup>, K. McDonnell<sup>2</sup>, S.B. Gruber<sup>2</sup>, H. Gao<sup>1</sup>

<sup>1</sup>Fulgent Genetics, Temple City, CA; <sup>2</sup>University of Southern California, Los Angeles, CA.

**Introduction:** Many types of genetic alterations are relevant for the management and treatment of individuals with solid tumors. New combined methods enable the detection of a wide variety of these alteration types, including fusion genes, known mutations, hotspots, truncating variants across whole genes, mutation burden, novel insertions and deletions, amplifications, and variant transcript isoforms. This study utilized control cell lines as well as previously evaluated patient samples to establish clinical grade sensitivity, limit of detection, and specificity for this testing. **Methods:** The Illumina TruSight Tumor 170 (TST) reagents and workflows were used to enrich DNA and RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue sections for 170 targets, including 55 fusion genes. The BaseSpace suite of applications was used for alignment and variant calling. A custom pipeline was designed to assess tumor mutation burden. GenomOncology's Clinical Workbench and Knowledge Management System were used to aid in technical assessment, annotation, filtering of variants, and clinical reporting.

**Results:** Cell-line base accuracy studies showed a high correlation of variant allele frequencies compared to expected values and replicates; all 15 expected gene fusions and transcript variants were detected. Average coverage across patient specimens was >1,000x coverage, with >99% coverage of coding bases at 250x being standard. Clinically relevant mutations, including amplifications, were detected in all clinical specimens, including 6 different amplifications. Limit of detection studies successfully identified variants between 5-10% VAF. The BaseSpace application did repeatedly detect clear false positive variant calls, requiring manual inspection of aligned sequence reads (BAM file) prior to reporting variants clinically. **Conclusions:** This combined workflow, which is available for clinical testing as the Solid Tumor Molecular Profile test, is highly powered to detect clinically relevant alterations across many different types of solid tumor.

#### ST020. Molecular Epidemiology of CREBBP and EP300 Mutations in Solid Tumors

J.P. Solomon, S. Gupta, A. Zehir, M.F. Berger, M.E. Arcila, M. Ladanyi, S. Dogan

Memorial Sloan Kettering Cancer Center, New York, NY.

**Introduction:** CREBBP and EP300 encode two related proteins, CREB-binding protein and p300, that act as histone acetyl transferases and transcriptional coactivators. Often mutated in leukemia and lymphoma, CREBBP and EP300 alterations are less commonly seen in carcinomas. Here, we explore the molecular epidemiology of CREBBP and EP300 alterations in a pan-cancer cohort. **Methods:** Mutational profiles of 26,784 tumor samples from 24,518 patients were analyzed by hybridization-based exon-capture next-generation sequencing (MSK-IMPACT) from January 2014 to May 2018. We analyzed the frequency and types of CREBBP and EP300 mutations and their associations with overall survival (OS), highlighting lung adenocarcinoma (LUAD, N=2838), lung small cell carcinoma (LUSC, N=188), colorectal adenocarcinoma (CRC, N=2154), urothelial carcinomas of the upper tract (UTUC, N=132) and bladder (BLCA N=538), endometrioid uterine adenocarcinoma (EUC, N=380), and cutaneous

squamous cell carcinoma (CSQCC, N=80).

**Results:** *CREBBP* and *EP300* mutations respectively occurred in 23% and 14% of UTUC, 20% and 26% of CSQCC, 15% and 5% of LUSC, 14% and 11% of BLCA, 10% and 13% of EUC, 7% and 4% of CRC, and 4% and 2% of LUAD. These alterations were significantly associated in CRC, BLCA, UTUC and EUC (Bonferroni adjusted  $p < 0.001$ , 0.013,  $< 0.001$ , and  $< 0.001$ , respectively). For *CREBBP*, the proportion of truncating mutations ranged from 15% in CSQCC to 63% in UTUC, and for *EP300*, from 11% in LUSC to 47% in UTUC. The tumor mutation burden (TMB) was relatively higher in *CREBBP* and/or *EP300* mutated tumors. For example, the median TMB in *CREBBP* mutated CRC and EUC was 55 vs. 7 (Student t-test,  $p < 0.001$ ) and 60 vs. 8 (Student t-test,  $p < 0.001$ ), respectively. The TMB in *EP300* mutated LUAD was also relatively higher (14 vs. 6, Student t-test,  $p < 0.001$ ). In CRC, *CREBBP* variants were more common in primary tumors (112/1304, 9% vs 31/902, 3%, Fisher exact  $p = 0.0001$ ), and were associated with better OS (Log-rank, primary tumors  $p = 0.001$ ; all samples  $p = 0.0007$ ). *CREBBP* alterations were also associated with better OS in primary BLCA and UTUC (Log-rank  $p = 0.041$  and 0.005). For CSQCC, mutations in either *CREBBP* or *EP300* were associated with better OS (Log-rank  $p = 0.015$ ), while neither *CREBBP* nor *EP300* impacted OS in EUC. In LUAD, *EP300* mutations were more common in metastatic tumors (43/1131, 4% vs 27/1828, 1.5%, Fisher exact test,  $p = 0.0001$ ) and were associated with worse OS (Log-rank, primary tumors  $p = 0.02$ ; all samples  $p = 0.001$ ). *CREBBP* mutations did not impact OS in LUAD. **Conclusion:** The impact of *CREBBP* and *EP300* mutations on outcome in solid tumors may depend on tumor origin and phenotype, suggesting that the biological roles of *CREBBP* and *EP300* mutations may be defined by their cellular context.

#### ST021. Prevalence of EGFR Mutations in Indian Lung Cancer Patients

R. Kataria<sup>1</sup>, S. Verma<sup>1</sup>, V. Kumar<sup>1</sup>, P. Chauhan<sup>1</sup>, J. Sharma<sup>1</sup>, A. Verma<sup>1</sup>, G. Kumar<sup>1</sup>, A. Kumar<sup>1</sup>, S. Mishra<sup>1</sup>, R. Pal<sup>1</sup>, S. Arora<sup>1</sup>, S. Kumar<sup>1</sup>, L. Kini<sup>1</sup>, S. Sharma<sup>1</sup>, A. Dewan<sup>1</sup>, K. Sharma<sup>1</sup>, E. Jain<sup>1</sup>, Narendran<sup>1</sup>, S. Pandey<sup>1</sup>, J. Mukherjee<sup>2</sup>, A. Pandita<sup>2</sup>

<sup>1</sup>CORE Diagnostics, Gurgaon, India; <sup>2</sup>CORE Diagnostics, Palo Alto, CA; <sup>3</sup>University of California, San Francisco, CA.

**Introduction:** Epidermal growth factor receptor (*EGFR*) gene mutations play a significant prognostic and predictive role in non-small cell lung cancer (NSCLC) patients mainly in adenocarcinoma when it comes to personalized therapy. In the current study, we sought to investigate the frequency and patterns of *EGFR* mutations in a cohort of histopathologically proven lung cancer patients from India. This cohort included patients from stages I-IV of lung cancer, and both treatment naïve and treated patients. **Methods:** The study entailed histological verification of lung cancer, and thence the extraction of DNA from formalin fixed, paraffin embedded (FFPE) tissue samples. A tumor content of 10% was the cut-off for performing the *EGFR* mutation analysis. The DNA was subjected to mutation analysis using a realtime ARMS-PCR based assay that encompasses exons 18, 19, 20, and 21. **Results:** A total of 2692 lung cancer patient samples were screened for *EGFR* mutations. Of these, 840 (31%) (Male: Female ratio = 1.1:1; Age range = 20-85 years) presented with a mutation of the *EGFR* gene. The most common *EGFR* mutations encountered were in Exon 19 (deletions) at 53%, while in 6% cases, it was expressed with another mutation. Exon 21 mutation (L858R) was seen in 28% cases, with 2.7 % cases showing co-expression with other mutation. The exon 20 (T790M) was seen in 2.3% cases, while in 6.1% cases it was co-expressed with another mutation. The majority of the *EGFR* mutations (47%) were detected in the age group of 60-80 years. **Conclusion:** This is the largest study to report the frequency of the *EGFR* mutations in the Indian population. This pattern of *EGFR* mutation in lung cancer patients was comparable to that reported from other parts of the world. A high incidence of exon 19 deletion mutation was found in the *EGFR* gene. Further analysis with clinical correlation would enable better understanding of the role of *EGFR* mutations in the lung cancer patient treatment and outcome.

#### ST022. Detection and Quantitation of Human Papilloma Virus Type 16 in Oropharyngeal Squamous Cell Carcinomas

K. Vadlamudi, H. McGuff, Y. Wang, S.D. Rowan, S.W. Redding, W.B. Furmaga, H. Fan

University of Texas Health San Antonio, San Antonio, TX.

**Introduction:** High risk human papilloma virus (HPV) type 16 is associated with oropharyngeal squamous cell carcinoma (OPC). To study if HPV16 DNA is present in OPC tumors, we developed and validated two TaqMan real-time PCR assays targeting the HPV16 E6 and E7 regions, which can both detect and quantitate the HPV16 load in tumors. The tumor suppressor gene p16 is overexpressed in OPC, and immunohistochemistry (IHC) staining of p16 in tumor tissue is commonly used as a surrogate marker for OPC. In this study, results were also correlated between HPV16 viral status and p16 expression in OPC tissues. **Methods:** Formalin-fixed and paraffin-embedded (FFPE) tissue collected from 63 cases of OPC from the south Texas region was included in this study. Five unstained slides were cut from each FFPE block, 2 slides were used for p16 IHC, and remaining 3 used for HPV16 PCR assays. Except one case with limited tissue, the FFPE section tissue size ranged from 5-750 mm<sup>2</sup>. The tumor cell percentage within the tissue sections of these OPC ranged 5-90% (median 45%). Macrodissection was performed on 15/63 cases to enrich tumor cells. DNA was extracted by Qiagen EZ1 DNA Tissue Kit and amplified by specific primers and TaqMan probes on the ABI 7900HT Real-Time PCR System. A control gene cyclophilin, was amplified along with patient DNA to monitor assay efficiency. SiHa cell line DNA, known to contain 5 copies HPV16 E6 per triploid cell was used to produce standard curves. Quantitative HPV16 load was expressed as HPV16 E6 or E7 copies/cell, or relative quantitation (RQ) to the SiHa cell line. Assays' linearity was determined by testing Ca Ski cell line which known to contain about 600 copies HPV16 E6/cell. **Results:** Both HPV16 E6 and E7 assays were linear across 6 orders of magnitude and had the sensitivity to detect 1 copy/PCR or 1% HPV16 in serially diluted SiHa DNA. All 63 cases were with cyclophilin amplification indicated suitable DNA for the study. HPV16 was detected in 29/63 (46%) cases by both E6 and E7 PCR assays. There was good agreement between HPV16 PCR and p16 IHC methods in 29/63 positive and 31/63 negative cases. The p16 IHC assay detected 3 additional positive cases. The HPV16 load ranged 1-253 copies/cell by E6 PCR (median 20), and 1-153 copies/cell by E7 PCR (median 18) on OPC tumors. Comparing to SiHa cell line, the RQ of HPV16 load in OPC tumors ranged 1-149% by E6 PCR (median 11%), and 1-95% by E7 PCR (median 11%). **Conclusions:** The E6 and E7 PCR assays are a rapid, reliable, and sensitive method to qualitatively and quantitatively detect HPV16 in OPC tumors. The same method can also be used for other HPV16 related tumor studies.

#### ST023. Cross-Platform Comparison of NGS and MALDI-TOF for Detecting RAS/RAF Mutations in Circulating Tumor DNA from Metastatic Colorectal Cancer Patient Plasma

W. Guo

Zhongshan Hospital Fudan University, Shanghai, China.

**Introduction:** Evaluating tumor RAS/RAF status is essential for treatment selection and prognosis evaluation in metastatic colorectal cancer (mCRC) patients. Analyzing circulating tumor DNA (ctDNA) in mCRC patients has many advantages because of its non-invasive nature, e.g. it provides an alternative for RAS/RAF assessment when tissue is not available or allows assessment of clinical response between cycles of treatment. However, since the abundance of ctDNA is generally low, the detection of ctDNA using different platforms or methods needs to be carefully assessed before adapting new technology into clinical application. **Methods:** Sixty mCRC patients under different treatment status were recruited. A cross-platform comparison between MALDI-TOF (UltraSEEK) and next generation sequencing (NGS; amplicon-based) was done by examining *KRAS/NRAS/BRAF/PIK3CA* mutation frequency in ctDNA extracted from plasma. Discordant results between two platforms were examined by droplet digital PCR (ddPCR). All results were compared to the mutation status determined by ARMS-based PCR from tissue. **Results:** In the comparison between NGS and MALDI-TOF, 65 hotspots for *KRAS/NRAS/BRAF/PIK3CA* are common to both platforms. 33/60 (55.0%) and 36/60 (66.0%) of the patients were reported to be



positive by NGS and MALDI-TOF, respectively. The concordance rate between two platforms was 73.21%. Discrepancy between two platforms was examined by ddPCR to determine the true positive or true negative nature of the sample. The PPA of NGS and MALDI-TOF calculated according to 65 hotspots was 93.94% and 90.91%; and NPA was 99.97% and 99.63 %, respectively. Overall ctDNA detection rate was 53.57% and the final determination of plasma mutation status was then compared to tissue results. In 20 treatment naïve patients, the consistent rate between plasma and tissue was 90.0% (18/20). In 33 primary lesion resected patients, chemotherapy at the time of plasma acquisition didn't affect consistent rate between plasma and tissue (treated versus untreated, 72.22% vs 80.00%,  $P>0.05$ ). Longitudinal testing of plasma in 4 cases showed that ctDNA was effective to monitor the response to treatment. **Conclusion:** ctDNA detected by NGS and MALDI-TOF was shown to be a reliable method that reflects tumor burden and informs treatment response. Although challenges exist in detecting low frequency variants in plasma, appropriate selection of a technology allows reliable examination of clinical utility in upcoming clinical studies.

#### ST024. Clinical Implementation of Mutational Signature Analysis

L. Lawrence, C. Kunder, H. Stehr, J. Zehnder

Stanford University, Stanford, CA.

**Introduction:** Mutational signatures have been described in the literature and a few centers have implemented pipelines for clinical reporting. Here we describe our experience validating a mutational signature caller for use with clinical tumor sequencing. **Methods:** Clinical specimens from 1,495 samples were analyzed for the presence of mutational signatures using deconstructSigs in R. Samples included predominantly solid tumors (93%), with the remaining 7% consisting of poorly differentiated, hematolymphoid, and melanocytic neoplasms. Variants with 20 or more variant reads from the Stanford Actionable Mutational Panel (STAMP, a targeted sequencing panel covering fewer than 200 genes) were analyzed. Cosmic signatures were used as mutational signature controls. **Results:** Signature 10 (associated with POLe mutation) achieved perfect separation of cases and controls in samples appearing hypermutated, with the caveat that only 4 true positive cases were present in our cohort. Signature 4 (associated with tobacco smoking) was an insensitive (14.9%) but highly specific (95.0%) indicator of a pulmonary primary at a cutpoint of 0.089 (the proportion of analyzed variants attributable to signature 4). The proportion of variants attributed to signature 7 (associated with ultraviolet radiation) as an indicator of a cutaneous primary was similarly insensitive (16.4%), but again highly specific (98.5%) at a cut-off of 0.77. Ten of twenty-four (10/24) malignancies with unknown primaries displayed mutational signatures characteristic of a site concordant with the clinically suspected primary site. The difference between the number of variants analyzed in concordant and discordant groups was statistically significant (median concordant=8 variants, median discordant=5 variants;  $p=0.01974$ , Mann-Whitney). **Conclusions:** Mutational signatures represent an opportunity for orthogonal testing of primary site, which may be particularly useful in supporting cutaneous or pulmonary sites in poorly differentiated neoplasms. Here we demonstrate that mutational signatures are suitable for implementation with targeted sequencing data yielding highly specific results, though sensitivity is limited. Tobacco smoking, ultraviolet radiation, and POLe mutational signatures are the most appropriate signatures for immediate implementation. There appears to be a dose-response relationship between the number of variants analyzed and the ability of mutational signature analysis to correctly suggest a primary site in malignancies of unknown primary. Even relatively small numbers of variants, however, appear capable of providing evidence to support a clinically suspected primary.

#### ST025. Implementation and Validation of the Moffitt Solid Tumor Actionable Result (STAR) Assay

E.L. Roberts, T. Boyle, A. Magliocco, D. Qin, R. Kothapalli, J.

Rodriguez, L. Moscinski

H. Lee Moffitt Cancer Center, Tampa, FL.

**Introduction:** Moffitt Cancer Center has clinically validated and implemented the Moffitt Solid Tumor Actionable Result (STAR) assay for comprehensive evaluation of patient cancer specimens. The Moffitt STAR panel is a next generation sequencing (NGS) assay capable of DNA and

RNA analysis of 170 genes for alterations (e.g. single nucleotide variants (SNVs), insertions and deletions (indels), amplifications (CNVs), and fusions/splice-variants). **Methods:** Patient and reference samples (N=96) were used to establish quality metrics for the validation. Extractions are performed from formalin fixed paraffin embedded samples using the Qiagen Allprep DNA/RNA kit. Libraries are prepared utilizing the Illumina TruSight Tumor 170 (TST 170) kit. Sequencing is performed using an Illumina NextSeq 550 sequencer. Sequence analysis and variant calling is performed with the TST170 App utilizing the HIPAA-compliant Basespace Enterprise platform. FASTQ, BAM, and VCF files are transferred to the Pierian Diagnostics Clinical Genomics Workstation bioinformatics pipeline to generate raw specimen reports. Filters were selected during the validation for optimal sensitivity and specificity by variant type. Post-implementation of Moffitt STAR, reports are analyzed, edited, and signed out by Moffitt pathologist providing a report of tiered variants in the patient electronic medical record. **Results:** Accuracy analysis from 18 tests of the Acrometrix Oncology Hotspot control revealed >99% accuracy for substitutions and indels. SNVs were detected in coding regions with 96% positive percentage agreement (PPA, sensitivity), 100% negative percentage agreement (NPA, specificity). Ninety-nine percent positive predictive value (PPV) and 100% negative predictive value (NPV) with the filter set at 5% variant allele frequency and 250X depth. With the same filters, indels were detected with 89% PPA, 100% NPA, 77% PPV, and 100% NPV. Fusions/splice variants were detected with 95% PPA and 98% PPV for variants with a limit of detection of 7 copies/ng and exclusion of one fusion, EGFR-SEPT14 due to sequence repetitiveness at the breakpoint. CNVs had 100% reproducibility between runs. Analysis of 21 clinical specimens showed 98% mapping of high quality variants at 500X depth. Results of over 200 pre and post-implementation clinical specimens were compared with available known results and revealed expected results, along with many previously unknown findings. **Conclusions:** Comprehensive mutation testing allows for a better understanding of a patient's cancer with accurate identification of genetic alterations. Overall, the Moffitt STAR NGS assay has excellent coverage and detects genetic alterations with high concordance with known results and allows for discovery of previously unidentified mutations.

#### ST026. A Functional DNA Repair Assay Platform to Stratify Melanoma and Select the Best Therapeutic Option

S. Sauvaigo<sup>1</sup>, M. Benkhia<sup>2</sup>, F. Braisaz<sup>2</sup>, S. Libert<sup>1</sup>, F. de Fraipont<sup>2</sup>, C.

Aspor<sup>3</sup>, S. Mouret<sup>4</sup>, J. Plumas<sup>4</sup>, F. Bouquet<sup>5</sup>, M. Leccia<sup>2</sup>

<sup>1</sup>LXRepair, Grenoble, France; <sup>2</sup>CHU Grenoble Alpes, Grenoble, France;

<sup>3</sup>EFS, Grenoble, France; <sup>4</sup>EMR EFS-UGA-INSERM U1209- CNRS, La

Tronche, France; <sup>5</sup>Institut Roche, Boulogne-Billancourt, France.

**Introduction:** Combined targeted therapies and immune checkpoint blockade have tremendously modified the management of melanoma patients and increased long term benefit for a subset of them. Identifying biomarkers that can predict the clinical benefit of a therapy and help select the best treatment sequences or combinations for each patient is required to guide treatment prescription. DNA repair mechanisms protect cells from the carcinogenic effects of UV radiations and are part of a large functional network regulated by kinases. Additionally, DNA repair defects are responsible for the accumulation of mutations possibly associated with high tumor mutation burden. **Methods:** We present a unique strategy to stratify melanoma based on a multiplex functional DNA repair analysis that investigate simultaneously repair pathways responsible for removal of photoproducts, oxidative damage and alkylated bases. A series of lesion-containing plasmids immobilized on dedicated biochips were used as substrates for an *in vitro* repair reaction. **Results:** We showed that the samples (established cell lines or tumors) classification based on the mutation profile usually matched the samples classification based on the DNA repair profile. However some samples present discrepant DNA repair profile (WT-, BRAF- or NRAS-like) showing that samples clustered on the basis of mutations in driving genes are in fact heterogeneous. Treatment of melanoma cells by vemurafenib (BRAFi), cobimetinib (MEKi) or the drug combination led in about 60% of samples to inhibition of DNA Repair associated with the expected signaling pathway inhibition. However strong upregulation of some DNA repair activities was also observed in 30% samples suggesting a possible activation of parallel signaling pathways.

Several DNA repair defects were identified in clinical samples possibly at the origin of elevated mutational load. Interestingly preliminary results showed that depending on the genotype considered, there might be a correlation between DNA repair activities and patient survival.

**Conclusion:** The ultimate goal of our study is to determine an approach to reliably identify responders to targeted therapies. Further analysis on a larger cohort could confirm these results and allow establishing correlations between DNA repair signature, other candidate biomarkers and clinical parameters, and evaluate the potential of such a diagnostic approach for the clinical management of melanoma patients.

#### ST027. Ultra-Rapid EGFR Mutation Assessment in Lung Adenocarcinoma without Prior DNA Extraction

M.E. Arcila, P.A. Salazar, I. Rijo, D. Elezovic, N. Rekhtman, D.J. Buonocore, O. Lin, J.E. Chaff, M. Ladanyi, K. Nafa  
Memorial Sloan Kettering Cancer Center, New York, NY.

**Introduction:** Institutions providing care for lung cancer patients are increasingly adopting large panel next generation sequencing (NGS) assays to comprehensively assess tumor samples for all clinically relevant genetic alterations in a single assay. Despite clear advantages, rapid, more narrowly targeted testing for EGFR mutations remains essential to meet standards of turnaround time (TAT) or when tissue is limited. We describe our clinical validation of a fully automated system to enable ultrarapid EGFR mutation assessment while maximizing the residual material available for subsequent, more comprehensive NGS testing.

**Methods:** Testing methodology involved the clinical validation of the Idylla EGFR mutation assay, using an integrated, real-time PCR-based system (Idylla - Biocartis) for qualitative detection of 52 mutations in exons 18, 19, 20, and 21. While the system is designed for detection directly from formalin-fixed, paraffin-embedded (FFPE) tissue sections without the need for prior DNA extraction, we assessed broader utility to also encompass stained cytology smears and touch preparations, residual minimal cytology material in CytoLyt, extracted DNA and aliquots of pre-capture NGS libraries prepared for the 468 gene MSK-IMPACT large panel NGS assay. All samples were concurrently tested by MSK-IMPACT and a sizing assay for rapid detection of insertion/deletions (indels) in EGFR and ERBB2. The accuracy and overall performance of the Idylla EGFR mutation assay was assessed and clinical workflows redesigned.

**Results:** Sixty-two samples (50 mutation positive, 12 negative) harboring 96 mutations were tested, encompassing 25 FFPE tissue sections, 15 extracted DNA, 9 cell pellets from residual cytology material in CytoLyt, 2 stained slides from cytology smears, 2 stained touch preparations and 9 NGS libraries. Overall, we achieved 100% accuracy in mutation detection compared to the reference methods with excellent reproducibility. Sensitivity studies established the limit of detection between 2.5-5% variant frequency (VF) depending on the input. Minimal input studies performed on extracted DNA, showed  $\geq 50\text{ng}$  (~8,000 cell equivalents) were required to consistently detect a mutation known to be present at 2.5%VF. The technical hands on time averaged 4 minutes and time from set up to instrument report generation was 150 minutes.

**Conclusion:** The incorporation of ultrarapid methods for targeted marker assessment, such as the Idylla system, simplifies laboratory workflows and enables rapid treatment decisions. It offers fast and reliable analysis from numerous sources beyond FFPE, including stained cytologic samples and residual limited cytologic material after thin preparations, without the need for prior DNA extraction.

#### ST028. Confirmation of Novel Gene Fusions Detected by Next-Generation Sequencing using Enriched RNA Libraries

N.T. Ngo, H. Nguyen, A. Almradi, C.E. Hill, L. Zhang  
Emory University School of Medicine, Atlanta, GA.

**Introduction:** RNA-based next generation sequencing (NGS) is a highly sensitive and specific method for detecting gene fusions in formalin-fixed paraffin embedded (FFPE) tissue. Well characterized fusions found by NGS do not typically require confirmation by an orthogonal method. However, potential novel fusions not reported in public databases should undergo confirmatory testing, for purposes of quality control and documentation. Here we report improvements on the method to confirm gene fusions. **Methods:** We use the ArcherFusionPlex NGS Sarcoma Assay to detect fusions involving a panel of 26 genes commonly

rearranged in soft tissue tumors. Anchored multiplex polymerase chain reaction (PCR) allows for detection of virtually any fusion gene partner. The cDNA library preparation entails RNA extraction, reverse transcription (RT) and two rounds of PCR, each followed by bead purification. The resulting cDNA library is highly enriched for targeted sequences. We sought to confirm potential novel fusions by a PCR-based method. We designed one set of primers for each gene partner to simultaneously detect fusion cDNA and wild-type cDNA for each gene. The cDNA from originally extracted total RNA (after RT) or enriched library prepared for Archer NGS was used as template and underwent two rounds of PCR (using the same primers in both rounds), followed by fragment size analysis by capillary electrophoresis. Controls included cases with well-characterized gene fusions and splenic RNA. **Results:** We confirmed 2 novel fusions: GATA6-FOXO1 in a mesenchymal neoplasm of eyelid and HMG2-KSR2 in a uterine high grade sarcoma. GATA6-FOXO1 was detected at very low levels by NGS (7 unique start sites, 14 reads). We detected the fusion and wild-type FOXO1 in the enriched library with one PCR round, and in the total RNA only after two PCR rounds. Wild-type GATA6 was only detected in the enriched library. HMG2-KSR2 was detected at intermediate levels by NGS in 2 specimens (8 and 33 unique start sites, 117 and 197 reads respectively). The fusion was only detected in the enriched library. Wild-type HMG2 and KSR2 were detected in the enriched library after one PCR round and in the total RNA only after two PCR rounds. **Conclusions:** Fusion transcripts may exist at very low levels in a sea of total RNA. This presents the main challenge to designing a confirmatory assay to detect a novel fusion. Utilization of the enriched cDNA library or performing multiple rounds of PCR on the cDNA template from the original total RNA helped to overcome this challenge. Our results indicate using enriched cDNA is more sensitive for detecting novel fusions. A potential improvement on our method would be a nested PCR assay with multiple primer sets.

#### ST029. Assessment of Significant Components in Multigene Testing for Breast Cancer in Clinical Laboratories

P. Gao, R. Zhang, J. Li  
Beijing Hospital, Dongcheng Qu, Beijing, China.

**Introduction:** Breast cancer (BC) is one of the major causes of cancer-related deaths in women around the world. Rapid progress in multigene testing via next-generation sequencing (NGS) has boosted the development of novel drugs to better match BC patients with effective therapies. Accurate experimental detection ability, consistent variant interpretation results, and abundant targeted drug information have been considered as significant components in precision medicine, which should be detailed evaluated to figure out the problems and to provide reliable clinical report for patients. **Methods:** Mimicking clinical samples were provided to 101 laboratories, each with a mock clinical information associated with BC. Laboratories were required to use their routine methods to perform the whole NGS pipeline including from detection to interpretation process. Detection results were evaluated based on predefined marking criteria, discordant reasons in variant interpretation and targeted drug recommendation process were also analyzed. **Results:** Overall, variant detection capability across laboratories was considered to reach an acceptable level with a correct detection rate of 89.0% (81/91) in 91 feedbacks. 78.9% (15/19) of the errors in detection process were false-positives or false-negatives. Excluding these wrong detection results, diverse databases used, different-based variant interpretation standardizations were the principal reasons which contributed to conflicting variant interpretation results. The targeted drug recommendation process was in a state of confusion. Only one laboratory recommended 81.3% (39/48) of the correct targeted drugs whereas 53.1% (43/81) of the laboratories reported massive invalid or insufficient drug information. Reasons for invalid drug information mainly lied in failures in updating the databases timely and inconsistency with the detected mutations or the given clinical information. For the targeted drug selection process against multiple detected variants, most laboratories would obey different factors including variant clinical significance, variant allele frequency (VAF) and variant position in the signal pathway to firstly give targeted drugs, whereas others would list all the targeted therapeutics against every detected mutation with no priority.

**Conclusions:** The whole NGS pipeline were analyzed in detail in our

study. Variant detection abilities were satisfactory whereas improving providing proper therapeutic recommendations across laboratories was urgently needed. Hence, the standardized targeted drug report strategy should be established to give accurate and exhaustive reference information for clinicians, which could better inform the precision treatment for BC patients.

#### ST030. Culture of Circulating Tumor Cells (CTCs) using Three-dimensional Culture and Conditional Reprogramming Methods

C. Park, Y. Jeong, S. Park, D. Kim, C. Jeon

Daegu Catholic University Medical Center, Daegu, South Korea.

**Introduction:** Circulating tumor cells (CTCs) presented rarely in circulation of tumor patients, and knowing their properties are important in managing cancer patients. However, culture of CTCs is not easy in the peripheral blood. We used three-dimensional environment and conditionally reprogrammed method to increase the culture positive rates.

**Methods:** We collected 30 peripheral bloods of cancer patients who diagnosed as colorectal cancer (CRC, n=22) or breast cancer (BRC, n=8) in Daegu Catholic University Medical Center between Apr 2017 and Jan 2018. The venous blood was collected using STRECK Cell-Free DNA BCT tube. The blood samples were centrifuged to isolate mononuclear cells portion, lysed RBCs with Roche RBC lysis buffer. We used Corning Matrigel to create three-dimensional condition, and Rho kinase inhibitor(Y-27632) with mouse fibroblast as feeder cell to reprogram the isolated cells. After 30 days of culture, cells were recollected, extracted for RNA, and amplified for the expression of *GAPD*, *hTERT*, *MAGE A1-6* by real-time PCR. Patient cancer stage (16 patients' stage  $\leq$  II, 9 patients III, 5 patients IV) and PCR results were compared. **Results:** All cultured 30 blood samples disclosed some cells or few colonies. Their positive rates of *hTERT* and *MAGE A1-6* gene were 53% (n=16) and 30% (n=9) respectively. The positive rates of either and both gene were 63% (n=19) and 20% (n=6). The *hTERT* results correlated significantly with T stage (r=0.42) but not with N and M stage. The *MAGEA1-6* results correlated significantly with T, N and M stage (p<0.05).

The *hTERT* and *MAGE* positive results showed the best correlation with M stage (r=0.67, p<0.05). **Conclusions:** The three dimensional and reprogrammed method was effective for the CTC culture in the blood. More than 30% of CTC positive rates were estimated in our study. The *MAGE A1-6* gene was a good marker for the cancer cell verification after culture because it significantly correlated with their T, N and M stage.

#### ST031. ISO Certification of a Complete Next Generation (NGS) Sequencing Workflow for BRCA1/2 Analysis

S. Marchini, S. Pileggi, L. Mannarino, I. Craparotta, L. Paracchini, L. Beltrame, S. Ballabio, F. Donati, M. D'Incalci

Mario Negri, IRCCS, Milan, Italy.

**Introduction:** Molecular diagnostic tests for germline pathogenic variants in the tumor suppressor genes *BRCA1* and *BRCA2* allows identification of patients with hereditary forms of breast (BC) and ovarian cancer (EOC), as well as assessment of individual risk for developing such cancers. Identification of *BRCA1/2* mutation is of great clinical importance as affected individuals are referred to genetic counseling, vigilant surveillance, and potentially life-saving preventative treatments.

Furthermore, with recent development and approval of PARP inhibitors, the mutational status of *BRCA1/2* is also used to drive targeted therapy decisions. **Methods:** In our laboratory we sought to establish a Next-generation Sequencing (NGS) test to sequence full-length coding regions of the *BRCA1/2* genes. The complexity in the mutations and their clinical implications necessitates a diagnostic tool that offers a robust and easy workflow, combined with accurate and reproducible results as well as up-to-date interpretation for variant findings. In this abstract we describe the process to setup, validate, quality control and accredit an NGS workflow. **Results:** Based on the GeneReader platform, the Center for Biomedical Analysis and Clinical Genomics (CABGeC) at Department of Oncology, IRCCS "Mario Negri" Institute for Pharmacological Research, Milan Italy has been recently recognized as a certified laboratory (UNI EN ISO 9001:2015) for the identification of germline pathogenic variants in the *BRCA1/2* genes. Preliminary inter run and intra run tests were performed to confirm panel reproducibility in the correct variants identification and calling (100%). Over the last six months of activity, 42

cases of EOC have been analyzed, and 5 pathogenic germline variants (two cases were mutated in *BRCA1* and three in *BRCA2*) were identified. Orthogonal Sanger sequence validation confirmed identified mutations.

**Conclusions:** We have successfully implemented an NGS test for *BRCA1/2* using the GeneReader NGS System. We established sample and process control from extraction through variant annotation and interpretation. Our experience can be leveraged by other laboratories new to NGS or *BRCA* testing.

#### ST032. Analytical Validation of the Oncomine Breast cfDNA Assay v2

W. Liu<sup>1</sup>, C. Spittle<sup>1</sup>, C. Galderisi<sup>2</sup>, J. Li<sup>1</sup>

<sup>1</sup>MolecularMD, Cambridge, MA; <sup>2</sup>MolecularMD, Portland, OR.

**Introduction:** The Oncomine Breast cfDNA Assay v2 is designed to detect mutations in breast cancer in 10 genes including *PIK3CA*, *AKT1*, *ESR1*, *TP53* and *ERBB2* (~152 hotspots) and copy number variants in *CCND1*, *ERBB2* and *FGFR1*. We performed analytical validation studies for the detection of hotspot mutations and copy number variants using commercially available cfDNA controls, fragmented cell line DNA dilutions, plasma-derived cfDNA samples and formalin-fixed, paraffin-embedded (FFPE) DNA samples. **Methods:** The assay enables mutation detection using two PCR reactions, one to attach the unique molecular tags and the second to amplify the library. Wild type plasma cfDNA samples (n=5) and normal FFPE samples (n=5) were used to assess limit of Blank (LoB). Limit of Detection (LoD) and precision for single-nucleotide variants (SNVs) and CNV detection in plasma cfDNA was determined using serial dilutions of fragmented cell line DNA or commercial cfDNA controls. The LoD and precision for SNV detection in FFPE samples was determined using serial dilutions of DNA extracted from FFPE embedded cell lines or commercial FFPE controls. Libraries were prepared according to manufacturer's instructions and sequenced using Ion Chef and S5 XL with 20 libraries per chip (Ion 540). Data was analyzed using Torrent Suite Software and Ion Reporter 5.6.0. Concordance studies were performed using another next generation sequencing (NGS) panel or droplet digital PCR (ddPCR). **Results:** No SNVs or CNVs were detected in 5 wild type cfDNA (10ng) and 5 normal FFPE DNA (14ng) samples for specificity analysis. The LoD for SNV detection in cfDNA was mutation dependent and ranged between 0.3%-1% MAF using 6.5 ng cfDNA. The LoD ranged between 1-5% MAF with 1ng cfDNA. The LoD for FFPE samples was 1-3% MAF using 14ng DNA input. Studies are on-going to determine the LoD for CNV. The assay showed good inter-assay reproducibility with positive and negative samples consistently reported with comparable MAF. Concordance between the NGS panels was >90% for mutations. Discordant results are being investigated using ddPCR. **Conclusions:** Our study demonstrates that the Oncomine Breast cfDNA Assay v2 allows for high-throughput and sensitive detection of hotspot mutations and CNV in cfDNA and FFPE DNA samples. The tolerance for low DNA input and low error rate makes it especially suited for liquid biopsy analysis as well as small tissue biopsies and poor quality FFPE samples.

#### ST033. Assessment of Pre-analytical Effects on RNA Sequencing

A. Beams, N. Valencia, N. Riccitelli, R. Pollner

Navigate BioPharma Services, Carlsbad, CA.

**Introduction:** RNA sequencing provides unparalleled accuracy, sensitivity, and coverage for both targeted and whole-transcriptome expression analysis. Numerous off-the-shelf RNA sequencing assays have been developed for a wide range of sample types and genetic targets, however the performance of these panels is highly dependent on the initial quality of RNA used in the library preparation. The AmpliSeq Transcriptome Human Gene Expression assay was developed specifically to address the challenges associated with sequencing low-quality RNA. Herein, we evaluated the performance of the AmpliSeq Transcriptome Human Gene Expression assay for accuracy, sensitivity, specificity, and reproducibility in samples of varying RNA quality. **Methods:** RNA was isolated from either whole blood or formalin-fixed, paraffin-embedded (FFPE) samples using the Qiagen RNeasy or Qiagen AllPrep FFPE extraction kits, respectively. RNA shearing was performed on a Covaris LE220 using custom protocols optimized for the desired shearing lengths. RNA quality was assessed using the Agilent BioAnalyzer and NanoString gene expression measurements. Libraries were prepared according to the



manufacturer's protocol and sequenced on the IonChef/IonS5xL system. Raw read counts used in subsequent analyses were reported with the IonTorrent v.5.2 software. **Results:** Robust expression data was observed for a wide range of RNA inputs and qualities. Under optimal conditions, the AmpliSeq Transcriptome produced quantitative results down to 0.1 ng input, with <2% variation in measured expression between replicates, and >95% specificity at a limit of blank of three transcripts detected.

Expression profiles obtained with the AmpliSeq Transcriptome panel aligned closely with results from microarray and NanoString-based studies in both fresh and formalin-fixed samples. A net reduction in library yields correlated with RNA fragmentation levels and fixation, however sufficient material for sequencing was obtained from as little as 1 ng of highly fragmented RNA. In contrast, the presence of small-molecule inhibitors abrogated the benefits of high-quality RNA, and additional purification steps were required prior to library preparation. Significant bias in total signal was observed across different extraction methodologies.

**Conclusions:** Accurate and precise whole-transcriptome profiles were obtained for both high-quality, unfixed RNA and formalin-fixed, fragmented RNA over a range of inputs. Although uncontrollable pre-analytical effects can impact library preparations, a variety of approaches are available to mitigate these influences. Overall, the AmpliSeq Transcriptome panel provides access to robust sequencing data for all qualities of RNA, exemplifying the expression-profiling power of next-generation RNA sequencing.

#### ST034. Performance Evaluation of Asuragen QuantideX NGS RNA Lung Cancer Panel by ACL Laboratories

S. Spirtovic, L.J. Mazur, B. Baltadjieva, M. Patel, D. Yu, L. Sok, M. Mihalov

ACL Laboratories, Rosemont, IL

**Introduction:** The QuantideX NGS RNA Lung Cancer Kit is intended for the detection of clinically-relevant content for RNA targets common to lung cancer including; 107 specific RNA fusions, MET exon 14 skipping, 3'-5' imbalance ratios for 5 RNA expression markers involved in oncogenic translocations. Guidelines strongly advise using multiplex or next generation sequencing (NGS) to optimize patient therapy (NCCN, 2017). ACL Laboratory evaluated performance QuantideX NGS RNA Lung Cancer panel and feasibility of replacing orthogonal assays (fluorescence *in situ* hybridization or FISH) currently used for lung CA testing. **Methods:** One hundred and thirty-four formalin-fixed, paraffin-embedded (FFPE) clinical samples containing lung tumors were tested by both methods. Five slides bearing a 5 µm-thick section was obtained from each sample. The average tumor cellularity was 57%. For analytical performance assessment ACL used; commercial reference material cat # HD784 from Horizon Discovery and cat # 0710-0129 from SeraCare. Method 1: Samples were tested by; Vysis ALK Break Apart FISH Probe IVD Kit and Vysis 6q22 ROS1 Break Apart FISH Probe. SeraCare and Horizon Discovery fusion reference standards were determined and confirmed by digital PCR at manufacturer site. Method 2: QuantideX NGS RNA Lung Cancer Kit, Asuragen. All FFPE samples were extracted with the MaxWell instrument and Maxwell RSC RNA FFPE Kit (Cat. # AS1440) from Promega. NGS libraries were prepared using 50-100 ng RNA per rxn with Lung Cancer kit (RUO) Asuragen (Cat # 49602). Sequencing was performed using the MiSeq DX instrument (RUO mode) and MiSeq System Reagent Kit v3. Somatic rearrangements were assessed using Asuragen, QuantideX Reporter v2.0. **Results:** ACL reported 1.5% failure rate for FFPE specimen type – Asuragen observed failure rate was (<7%). Uniformity of coverage in 134 interrogated samples produced average yield of 16,487 copies well above 400cp minimum cutoff. Precision study using fusion standard from SeraSeq and Horizon Discovery was 99.2% (264/266). Accuracy when compared NGS to droplet digital PCR (ddPCR) all samples correlated 100%. ALK FISH and NGS comparison generated 4 discrepant samples. One sample was determined to be false positive by FISH. Two samples were re-run by NGS. Repeated result was reviewed and all QC metrics for run and samples were consistent with very clear negative result. Forth sample was QNS. The analytical sensitivity at 95% confidence was determined as follows: MET-1%, ALK, ROS -5%, RET, NTRK1-10%.

**Conclusions:** Overall, these results demonstrate that QuantideX NGS RNA Lung Cancer panel performed well in comparison to FISH and

ddPCR assays. This allows a laboratory to accurately detect actionable lung cancer fusions with a single NGS assay in one seamless workflow, greatly improving efficiency, effectiveness using very challenging sample size.

#### ST035. Performance Evaluation of Illumina TruSight Tumor 15 Panel by ACL Laboratories.

S. Spirtovic, L. Mazur, B. Baltadjieva, M. Patel, E. Katz, L. Sok, T. Kozak, M. Mihalov

ACL Laboratories, Rosemont, IL

**Introduction:** The TruSight Tumor 15 Panel is a highly multiplexed targeted resequencing assay for detecting somatic mutations across hundreds of mutational hotspots in cancer genomes. It provides a streamlined workflow for DNA extracted from formalin-fixed paraffin embedded (FFPE) samples including data generation and analysis. ACL Laboratory evaluated the performance of the TST15 next generation sequencing (NGS) panel and its feasibility to replace a number of orthogonal assays currently used for oncology testing. **Methods:** 139 FFPE clinical samples contained solid tumors were tested by two methods. Four slides bearing a 5 µm-thick section was obtained per sample (average tumor cellularity was 52%). For analytical performance assessment ACL used; QMRS (Horizon Discovery, cat. No. HD200) and ATCC cell lines; CRL5883, HCT116, A375, CRL5824. Method 1: Samples were tested by in-house assays based on; real time PCR (clamp or melting curve), Pyro or Sanger Sequencing. QMRS (Horizon Discovery) standard was determined and confirmed by digital PCR at manufacturer site. Method 2: TruSight Tumor 15 Panel Kit, Illumina. FFPE samples were extracted with the Promega MaxWell instrument and Promega Maxwell RSC RNA FFPE Kit (Cat. # AS1440). NGS libraries were prepared using 10-20 ng DNA per rxn with TruSight Tumor 15 kit (RUO) Illumina (Cat # OP-101-1001). Sequencing was performed using the MiSeq DX instrument in RUO mode and MiSeq System Reagent Kit v3. Somatic gene variants were assessed using Illumina software; Local Run Manager, MiSeq Reporter and Variant Studio 3.0. **Results:** Depth and uniformity of coverage of 139 FFPE clinical samples and QMRS Positive QC were as follow; a total of 234 variants were detected and all of them had RD above >500x and 95% were above >2,000x range, proving very robust performance of TST15 assay. Cell lines dilution levels were tested 20x to determine limit of detection (LoD) for selected mutations. The analytical sensitivity at 95% confidence was determined as follows: single-nucleotide variant (SNV): 4- 5%, deletion; 4-5%, insertion; 7.5% variant frequency. Comparison of 139 tumor samples demonstrated a concordance of 100% for SNVs and small insertion/deletions (indels) between methods. Precision study using QMRS standard (Horizon Discovery) was 99.8% (239/240). One low frequency mutation was called false WT. On-board Illumina MSR software missed 9 (4.9%) known positive variant calls, and provided a limited amount of QC metrics. ACL used customized Variant Studio 3.0, which showed 100% correlation with in-house orthogonal methods. **Conclusions:** Overall, these results demonstrate that NGS TST15 panel performed well in comparison to orthogonal assays. This allows a laboratory to accurately detect actionable mutations with a single NGS assay in one seamless workflow, greatly improving economy and efficiency of testing.

#### ST036. Comparison of Three Next-generation Sequencing Platforms in Fusion Detection: FusionPlex by Archer, OncoPrint by ThermoFisher, and AmpliSeq by Illumina

X. Qu<sup>1,2</sup>, I.M. Coleman<sup>2</sup>, C. Yeung<sup>2</sup>, C. Morrissey<sup>3</sup>, P.S. Nelson<sup>2</sup>, M. Fang<sup>2</sup>

<sup>1</sup>Seattle Cancer Care Alliance, Seattle, WA; <sup>2</sup>Fred Hutchinson Cancer Research Center, Seattle, WA; <sup>3</sup>University of Washington, Seattle, WA.

**Introduction:** Recent technical advances have enabled multi-target and novel fusion detection using next generation sequencing. As the techniques are adopted by clinical labs, assay performance comparison is urgently needed. Prostate cancer is suitable for this purpose as it frequently harbors gene fusions, often with heterogeneous breakpoints. Here, we present the results of three assays performed on the same samples, by comparing fusion calls, breakpoints, and the quantitation of fusion variant frequency. **Methods:** RNA was extracted from cryopreserved metastatic tumors of men with castration-resistant prostate cancer (n=24, University of Washington Prostate Cancer Donor Autopsy

Program). Samples were tested on: 1) Archer platform - libraries were prepared in house using the FusionPlex solid tumor kit, sequenced on MiSeq, and analyzed using Archer Analysis 5.1; 2) ThermoFisher platform - performed by the Lifelab using the OncoPrint Comprehensive Assay kit and Ion S5 XL sequencer; and 3) Illumina platform - performed by Illumina using AmpliSeq for Illumina comprehensive panel v3 and MiSeq. A total of 35 genes are targeted by all three panels. **Results:** Among the 24 samples, 13 were tested by three platforms: Illumina showed *TPRMS2/ERG* in 4 samples; ThermoFisher confirmed the *TPRMS2/ERG* calls and identified 2 additional samples with *TPRMS2/ETV4*; Archer confirmed the *TPRMS2/ERG* and *TPRMS2/ETV4* results, and also identified *ETV1* fusion in one of the samples with *TPRMS2/ETV4*. Among the remaining 11 samples, 10 were tested using Illumina and Archer: *TPRMS2/ERG* was detected by both in 8 samples, while Archer also called out *ETV1* fusion in the 2 remaining samples. One sample was tested using ThermoFisher and Illumina but did not show any fusion. Of the *TPRMS2/ERG* positive samples, 3 had multiple *TPRMS2/ERG* variants with 5 to 7 different breakpoints that were consistently identified by all three platforms. The comparison of variant frequency assessment via raw reads, unique reads, and unique start sites yielded provocative results: the raw read counts were highly correlated between ThermoFisher and Illumina ( $R^2 > 0.98$ ); and between raw reads of ThermoFisher/Illumina and unique start sites of Archer ( $R^2 > 0.75$ ). **Conclusions:** When both partners were targeted, fusions were consistently detected by FusionPlex (Archer) and OncoPrint (ThermoFisher). The anchorage PCR chemistry of FusionPlex enabled the detection of fusions with novel partners, exemplified by the detection of *ETV1* fusions in this study. Fusion variant frequency may help with quantification of clone size and warrants further investigation.

#### ST037. WITHDRAWN

#### ST038. A Turnkey Solution for NGS-based Detection of Somatic Mutations in Cancer

M. Yee<sup>1</sup>, A. Fan<sup>1</sup>, K. Lim<sup>1</sup>, Y. Ng<sup>1</sup>, R. Demirdjian<sup>2</sup>, T. Zhang<sup>4</sup>, O. Scully<sup>4</sup>, Y. Yu<sup>4</sup>, P. Ariyaratne<sup>1</sup>, W. Lye<sup>1</sup>, R. Luo<sup>1</sup>, E.J. Wee<sup>1</sup>, W. Huang<sup>1</sup>, M. Francillette<sup>3</sup>, E. Rouleau<sup>3</sup>, L. Lacroix<sup>3</sup>, G. Michel<sup>2</sup>, C. Lee<sup>1</sup>

<sup>1</sup>Vela Diagnostics Singapore Pte Ltd, Singapore; <sup>2</sup>Vela Diagnostics Germany GmbH, Hamburg, Germany; <sup>3</sup>Gustave Roussy, Paris, France; <sup>4</sup>Vela Genomics, Singapore.

**Introduction:** Next-Generation Sequencing (NGS) has revolutionized cancer research, diagnosis and therapy. However, the application of NGS in routine clinical settings still poses many challenges, especially in workflow automation, data analysis and results interpretation. Herein, we developed an automated sample-to-result NGS workflow targeting mutational hotspots in 80 genes relevant to multiple cancers. **Methods:** The Sentosa SQ system uses a liquid handling robot (Sentosa SX101) for automated DNA extraction of formalin-fixed, paraffin-embedded (FFPE) samples, PCR setup and library preparation including sequencing adaptor ligation, purification, normalization, and pooling of samples. Template preparation and sequencing are then performed on the Ion Torrent NGS platform. After primary analysis (base-calling), sequencing data is automatically transferred from the sequencer to the Sentosa SQ Reporter software for secondary analysis (alignment and variant calling). Finally, the interpretation of the detected variants for pathogenicity, clinical significance classifications, current therapies and clinical trials, are provided via a cloud-based software, Therakey. Complementing the automated workflow is a flexible 80-gene mutation hotspot panel (Sentosa OncoKey Select) consisting of 3 primer pools. Primer pool 1 contains clinically targets relevant to the highly prevalent colorectal cancer (CRC) and non-small cell lung cancer (NSCLC), whilst primer pool 2 and 3 contains pan-cancer targets. Evaluation of the assay was done using Horizon reference material and 30 NSCLC and CRC FFPE samples. **Results:** The Sentosa SQ system required only 3 hours of hands-on time and a total turnaround time of 25 hours to process 15 samples and 1 control using the OncoKey Select panel. With a minimal sample input of 10ng DNA per primer pool, we were able to achieve a median coverage of  $\geq 300\times$  for  $>90\%$  of the 350 amplicons on the Horizon reference material. When only the first primer pool was used, a median

coverage of  $\geq 500\times$  for  $>90\%$  of the 130 amplicons with a limit of detection of 5% variant frequency could be achieved. Both configurations of the assay also performed consistently well on FFPE samples. Lastly, clear and concise Quality Control, Pathology and Interpretation reports for each sample are provided by the Sentosa SQ Reporter and Therakey softwares. **Conclusion:** This highly automated NGS workflow is a viable turnkey solution for cancer mutation detection in routine diagnostics.

#### ST039. Clinicopathologic and Molecular Features of Undifferentiated Round Cell Sarcomas of Bone and Soft Tissues, including BCOR-CCNB3 and CIC-DUX4 Test Results

B. Rekhi, P. Kembhavi, S. Mishra, O. Shetty, J. Bajpai  
Tata Memorial Hospital, Mumbai, India.

**Introduction:** Lately, undifferentiated round cell sarcomas have been described, including *BCOR-CCNB3* and *CIC-DUX4* positive sarcomas. **Methods:** In this study, 42 cases of undifferentiated round cell sarcomas were critically analyzed, including 23 cases tested for *BCOR-CCNB3* and *CIC-DUX4* fusions, by PCR technique. **Results:** Twenty-two (52.3%) tumors occurred in males and 20 (47.6%) in females; in the soft tissues (30; 71.4%) and bony sites (12; 28.5%), most commonly over trunk, (8/30; 26.6%) and upper extremities (7/30; 23.3%). Most frequently involved bones were femur (4/12) and tibia (3/12). Histopathologically, most tumors (24/42, 57.1%) displayed round to oval cytology with a prominent myxoid stroma in 12/42 (28.5%) and necrosis in 20/42 (47.6%) tumors. By immunohistochemistry, tumor cells were variably positive (mostly 'dot-like' or cytoplasmic with incomplete membranous pattern) for CD99/MIC2, in 35/42 (83.3%) tumors, Flt1 in 25/42 (59.5%), WT1 in 14/17 (31.1%) and calretinin in 3/12 (25%) tumors. INI1/SMARCB1 was completely lost in 2 tumors. Four out of 22 (18.1%) cases were positive for *BCOR-CCNB3* fusion, while 3 of 22 (13.6%) cases displayed *CIC-DUX4* fusions. CCNB3 immunostaining was diffusely positive in 10/38 (26.3%) cases, including 3 / 4 (75%) cases of *BCOR-CCNB3* positive sarcomas. Most patients (10/33) (30.3%) were treated with surgical resection and chemotherapy. During follow-up (34 cases; 80.9%), over a duration of 3 to 45 months (median=8), 4 (11.7%) patients developed recurrences, 11 (32.3%) developed metastases and 14 (41.2%) patients died-of-disease. The median overall survival (OS) was 17 months and the median disease-free survival (DFS) was 6 months. Among various clinicopathologic parameters, there was a significant correlation between distant metastasis and DFS. (p value = 0.008). **Conclusions:** Undifferentiated round cell sarcomas are rare tumors; occur more frequently in the extremities, with *BCOR-CCNB3* positive cases slightly more frequent than *CIC-DUX4* positive, in our population. CCNB3 immunostaining is useful for identifying *BCOR-CCNB3* positive sarcomas. Overall, undifferentiated round cell sarcomas are associated with a relatively aggressive clinical course, as compared to Ewing sarcomas, despite similar treatments.

#### ST040. KRAS Mutations in Tissue Samples from Cologuard-Positive Patients

K. Murphy<sup>1</sup>, C.L. Lastufka<sup>1</sup>, K. Wicks<sup>1</sup>, A.R. Lewis<sup>1</sup>, K. Weatherhead<sup>1</sup>, A. Fratto<sup>1</sup>, C.A. Rittle<sup>1</sup>, A. Piper<sup>2</sup>, T. Ritter<sup>2</sup>, C. Fourment<sup>2</sup>, C.A. Roberts<sup>1</sup>

<sup>1</sup>ProPath, Dallas, TX; <sup>2</sup>Texas Digestive Disease Consultants, Southlake, TX.

**Introduction:** The Cologuard test is an FDA-approved, stool-based colon cancer screening test. The assay combines an immunohistochemical assay for human hemoglobin, methylation analysis of two genes – *NDRG4* and *BMP3*, and mutation analysis of the *KRAS* gene. The results are combined to produce a composite score and reported as either positive or negative. The goal of this study was to assess the likely contribution of *KRAS* mutation analysis to the interpretation of the Cologuard composite score. **Methods:** We identified 79 patients who tested positive by the Cologuard test and had tissue collected during colonoscopy. Our cohort included 35 males and 44 females with an average age of 73 years (range 53 to 91). The diagnoses of these patients were 68 (86.1%) tubular adenoma/tubulovillous adenoma, 7 (8.9%) sessile serrated/tubular/tubulovillous adenoma, 3 (3.8%) high grade dysplasia, and 1 (1.3%) adenocarcinoma. We analyzed 168 formalin-fixed, paraffin-embedded (FFPE) tissue biopsy specimens from the 79 patients for *KRAS* mutations. Tissues were microdissected from unstained

slides and DNA was extracted using Maxwell reagents (Promega). KRAS codon 12 and 13 mutation analysis was performed by polymerase chain reaction (PCR) followed by pyrosequencing. **Results:** Of the 79 patients with a positive Cologuard test and tissue available for testing, 13 (16.5%) had at least one tissue specimen that tested positive for a KRAS mutation. KRAS mutations were detected in 10/68 (14.7%) of tubular adenoma/tubulovillous adenoma, 1/7 (14.3%) of sessile serrated adenoma, 1/3 (33.3%) of high grade dysplasia cases, and 1/1 (100%) of adenocarcinoma cases. Four different KRAS mutations were identified: G12V, G12D, G12S, and G13D. Interestingly, 4 of the 13 (30.8%) KRAS mutation-positive cases demonstrated 2 different KRAS mutations either within the same tissue sample or between different tissue samples from the same patient. **Conclusions:** Our data suggest that it is likely that KRAS mutation analysis contributes relatively little to the overall sensitivity of the Cologuard test.

**ST041. Evaluation of the Biocartis Idylla Rapid Near-to-Patient EGFR Mutation CE-IVD Marked Tissue Test: Correlation to an FDA Approved Orthogonal Method using 79 Clinical Formalin-Fixed, Paraffin-Embedded Tissue Samples**

M. Kohlman<sup>1</sup>, B. Lentricchia<sup>2</sup>, H. Brown<sup>3</sup>, S. Jamieson<sup>3</sup>, A. Kohlman<sup>1</sup>

<sup>1</sup>AstraZeneca, Gaithersburg, MD; <sup>2</sup>AstraZeneca Pharmaceuticals, Waltham, MA; <sup>3</sup>AstraZeneca, Cambridge, United Kingdom.

**Introduction:** Turnaround times for an EGFR mutation determination from formalin-fixed paraffin-embedded (FFPE) tissue in the US can take more than two weeks, mostly determined by the testing method. Idylla (Biocartis, Mechelen, Belgium) offers a single-use self-contained cartridge system requiring minimal molecular laboratory expertise and hands-on time to operate, enabling an analysis time of less than 3 hours from FFPE sample to result. Targeted EGFR tyrosine kinase inhibitor (TKI) therapy requires an accurate and robust test to select patients for treatment. It was our intent to determine if the Idylla EGFR mutation CE-IVD marked tissue test has the potential for analytical performance equivalent to the FDA approved cobas EGFR Mutation Test v2 (Roche Molecular Systems, Pleasanton, CA). **Methods:** A cohort of 79 FFPE tissue samples from advanced NSCLC patients was assembled from commercial vendors (n=61) and two non-interventional clinical studies ASSESS (NCT01785888) and IGNITE (NCT01788163) (n=18). Samples with pre-determined EGFR mutation negative and mutation positive results were included in the test cohorts. One 5 µm FFPE section from each of the samples was analysed by the CE-IVD marked version of the Idylla EGFR Mutation Test and the CE-IVD version of the cobas EGFR Mutation Test v2 according to manufacturers' instructions. In addition, reference standards from Horizon Diagnostics were tested: HD200, HD850, HD300 and HD141, representing 5 different EGFR mutations with allelic frequencies ranging from 1-24.5%. **Results:** Four reference samples were tested in triplicate on the two platforms. Both platforms detected mutations according to the manufacturers' assay specifications in all 5 EGFR mutation mixtures. Notably, Idylla could detect 4 mutations at a 1% allele frequency whereas the cobas test could not. Next, 79 clinical samples were tested by both platforms. Idylla returned a final invalid rate of 3.8% (3/79) after re-testing six initially invalid samples. In contrast, the invalid rate for the cobas test was 8.8% (7/79) after 15 initially invalid samples were retested. Common EGFR mutations (exon 19 deletions or L858R) were detected in 49 of the 76 samples by Idylla and 48 of 72 samples by the cobas test, respectively. For the 72 cases that returned valid results for both platforms, the overall percentage agreement for these common mutations individually or combined was 100%. Of the 4 samples with valid Idylla results but invalid cobas results one sample was positive for an EGFR-TKI actionable mutation (L858R). **Conclusions:** The CE-IVD marked Idylla EGFR Mutation Test provided comparable data to the cobas EGFR Mutation Test v2. Most significantly, the Idylla platform had a lower invalid test rate and much shorter time to result.

**ST042. Clinically Significant Germline Variants Detected by Mutation Profiling of Non-small Cell Lung Cancer in Patients with Multiple Nodules Harboring Different Somatic Mutations**

E.P. Fellowship, D. Dolderer, N.T. Ngo, C.E. Hill, L. Zhang  
Emory University Hospital, Atlanta, GA.

**Introduction:** Approximately 15% of lung cancers occur in non-smokers, suggesting that there may be genetic susceptibility factors. We found distinct germline mutations in 3 patients with lung adenocarcinoma in the cancer mutation profiling tests. The approaches to confirm germline variants will be discussed. **Methods:** Cancer mutation profiling was performed on formalin-fixed paraffin embedded (FFPE) tissues from all separate lung nodules of 3 patients using Illumina TruSight Tumor Next-Generation Sequencing (NGS) 26 gene panel for the MiSeq/NextSeq. Multiplex polymerase chain reaction (PCR) based SNaPshot mutation test was performed on one lung nodule which extracted DNA failed to pass quality control parameters to run NGS. Confirmation of germline mutation was done on two of the cases, one in our institution by using TruSight Tumor NGS on a benign lymph node, and the other patient at a referral laboratory on peripheral blood mononuclear cells. **Results:** Three germline variants in the TP53, APC, and EGFR genes were identified. Case #1 is a 55 year-old never-smoker female with family history of tumors from the brain, breast and esophagus in her siblings, presented with two lung nodules with two different EGFR exon 19 deletion mutations and TP53 P152L germline mutation (confirmed with blood cells). Case #2 is a 73 year-old former smoker female with a history of breast cancer, presented with two lung nodules harboring MET exon 14 skipping mutation and KRAS G12C mutation respectively, found to have APC L1129S presumable germline mutation with allele frequency approximately 50% in each nodules (not yet confirmed). Case #3 is a 69 year-old never-smoker female with a history of breast cancer and a sibling with lung cancer, presented with 3 lung nodules, one with EGFR G719S mutation, another with EGFR L858R and TP53 R273H somatic mutations, and the third with EGFR exon 19 deletion, all found to have an EGFR T790M germline mutation (confirmed with benign lymph node). **Conclusions:** We report 3 cases of multiple lung adenocarcinomas; each carries a germline variant that have been found in patients with increased risk of developing other forms of cancer. Identifying clinically significant germline sequence variant can be challenging in cancer mutation profiling tests designed to detect somatic mutations. Although ideally cultured fibroblasts from skin should be used to confirm germline variants, alternative approaches may be sufficient depending on the clinical situation.

**ST043. Clinical Validation of a Custom-designed Next-generation Sequencing-based FusionPlex Panel for Salivary Gland Tumors**

N.V. Guseva, A. Bhattarai, A.A. Stence, R.R. Sompallae, K. Sompallae, R.A. Robinson, A. Rajan, A. Fillman, S. Saba Sedaghat, A.D. Bossler, D. Ma  
University of Iowa Hospitals and Clinics, Iowa City, IA.

**Introduction:** Salivary gland tumors (SGT) represent 3-6% of head and neck tumors. Accurate diagnosis can be challenging due to the diversity of these tumors, morphologic overlap between different tumor types, and the limited immunohistochemistry for differential diagnosis. It is even more challenging for fine needle aspirate (FNA) diagnosis. Recurrent genomic rearrangements resulting in gene fusions are identified in increasing numbers of SGTs, which could serve as diagnostic and prognostic markers. Here we describe a custom-designed, next generation sequencing (NGS)-based assay utilizing Anchored Multiplex PCR enrichment to detect gene fusions involving 14 genes, and hotspot mutations in PRKD1 and HRAS associated with salivary gland tumors. **Methods:** Fourteen genes (ETV6, EWSR1, HMGA2, HRAS, MAML2, MYB, MYBL1, NFIB, NTRK3, NUTM1, PLAG1, PRKD1, PRKD2, PRKD3) were selected based on extensive literature review. An RNA-based panel was designed using the Archer Assay Designer. REH cell line (with ETV6/RUNX1) and 7 previously tested tumors by different platforms (3 lung adenocarcinoma, 1 thyroid carcinoma, and 3 sarcomas) were included as controls. Forty-six SGTs including 18 adenoid cystic carcinoma (ACC), 19 mucoepidermoid carcinoma (MEC), 7 secretory carcinoma (SC), 1 pleomorphic adenoma (PA), and 1 polymorphous low-grade adenocarcinoma



(PLGA) were tested. Six cases with corresponding FNA smears were tested in parallel. 200 ng total RNA was used with gene-specific primers (GSP) and Archer FusionPlex reagents to generate targeted NGS libraries. Sequencing was performed on Illumina MiSeq and data were analyzed using the Archer Analysis platform. Limit of detection (LOD) was determined by mixing studies diluting fusion/ single nucleotide variant (SNV)-positive RNA into RNA from variant-negative tumor.

**Results:** Expected gene fusions were identified in each tumor type: *NFIB/MYB* (ACC), *ETV6/NTRK3* (SC), *MAML2/CRTC* (MEC), and a novel *PLAG1* fusion (PA). *PRKD1* p.E710D and *HRAS* p.Q61E were detected in 1 PLGA and 1 thyroid carcinoma, respectively. Specific fusions were confirmed by RT-PCR followed by Sanger sequencing and *HRAS*, and *PRKD1* mutations were confirmed by a DNA-based NGS assay. LOD was defined as 10% tumor content for fusion and 1% of mutant reads for SNV. There was 100% concordance between formalin-fixed, paraffin-embedded (FFPE) and FNA materials. **Conclusions:** This novel custom-designed NGS panel could detect fusions and SNV from various SGT types. Multiple fusion variants including novel rearrangements were identified without prior knowledge of the fusion partners, which would expand our understanding of salivary gland tumors. The assay works well on FFPE and FNA materials. It is beneficial for the diagnosis of challenging cases and has prognostic as well as therapeutic applications.

#### ST044. A Comparison of the Performance Characteristics of the Illumina TruSeq Stranded mRNA Kit and TruSeq RNA Sample Preparation v2 Kit for Gene Fusion Detection

R.N. Wehrs, J.I. Davila, N. Fadra, R.A. Jackson, A.A. Nair, E.A. Zimmerman Zuckerman, X. Wu, L.M. Peterson, J.H. Blommel, J.S. Voss, K.C. Halling  
Mayo Clinic, Rochester, MN.

**Introduction:** In this study we compared the performance characteristics of Illumina's TruSeq RNA Sample Preparation v2 kit (a non-stranded RNA-seq method) and the TruSeq Stranded mRNA Library Prep kit for the detection of gene fusions. **Methods:** Total RNA was extracted from solid tumor tissue (fresh/frozen, cell cultures and cell lines) or peripheral blood using a Qiagen miRNeasy Micro and Mini kit, respectively. RNA was quantified using a Qubit RNA BR assay kit and quality was assessed via RNA Integrity Number (RIN) using an Agilent 2100 Bioanalyzer. Samples with RIN values of  $\geq 5$  were advanced onto cDNA synthesis and library prep using the TruSeq non-stranded kit and TruSeq stranded Kit with Janus (Perkin Elmer) and Biomek (Beckman Coulter) liquid handlers. The TruSeq stranded kit was only evaluated using Mayo Clinic-developed adapters/indexes. The post-analytic library yield was assessed with the Bioanalyzer and Qubit DNA HS assay kit and libraries with a concentration of  $\geq 2$  nM were used for Next Generation Sequencing. Paired-end, 101 bp-read sequencing was performed on a HiSeq 2500 (Illumina) in Rapid Run mode. Data analysis was performed using Map-Seq, a Mayo Clinic-developed suite of alignment, fusion detection and filtering programs to detect fusions on a pre-selected set of 571 genes and associated transcripts. A fusion call was made when  $\geq 5$  supporting reads were identified. **Results:** A total of 54 positive and 6 negative samples were used to evaluate the intra-assay and inter-assay reproducibility, accuracy, reference range, limit of detection and analytical specificity of the assays. In the initial studies, samples evaluated with the TruSeq Stranded kit yielded a 6-10 fold higher final library concentration than the non-stranded kit which led to higher library input for sequencing, over-clustering and false positive calls. Both the TruSeq stranded and non-stranded kit detected the expected positive fusions cases. False positive fusion calls were reduced by decreasing the number of library prep enrichment PCR cycles from 15 to 10 and the loading concentration from 8pM to 6pM. Additionally bioinformatic analysis revealed that all false positive calls had a high number of 'split reads' and a low number of 'spanning reads' and that these false positive calls could be avoided by setting a threshold of  $\geq 3$  supporting spanning reads for reporting fusions. **Conclusions:** Initial analyses revealed that the higher efficiency of the TruSeq stranded kit caused a high number of low-level false positive gene fusion results as compared to the non-stranded RNA-seq kit. However, by incorporating changes in our PCR cycling, sequencing input and

bioinformatic fusion calling algorithm we have been able to avoid false positive fusion calls.

#### ST045. Development and Validation of an RNA Sequencing Assay for the Detection of Gene Fusions in Formalin-fixed Paraffin Embedded Tumors

R.A. Jackson, J.I. Davila, N. Fadra, A.A. Nair, J.S. Voss, R.N. Wehrs, M.A. Atiq, B.W. Eckloff, K. Rumilla, B.R. Kipp, R.B. Jenkins, J. Jen, K.C. Halling  
Mayo Clinic, Rochester, MN.

**Introduction:** RNA-seq analysis enables the detection of gene fusions, which can be used to establish diagnosis and guide targeted treatments for cancer patients. The most commonly used RNA-seq library preparation method, TruSeq, is limited to high quality RNA obtained from fresh tissue or cell cultures. However, it is much easier to transport and store FF formalin-fixed, paraffin-embedded (FFPE) tissue which provides access to a vast archive of specimens that can be easily transported from other institutions, increasing clinical utility. To this end, we have developed an RNA-seq gene fusion detection assay designed specifically for use on FFPE and other low quality RNA specimens. **Methods:** Total RNA was extracted from FFPE tissue using the Qiagen miRNeasy FFPE kit. RNA quality (DV200>30%) was determined using the Agilent 2100 Bioanalyzer and quantity was measured with the Nanodrop 2000. A custom 2360 gene panel was designed using Agilent SureDesign Software. Panel genes were chosen by evaluating cancer pathways, cancer databases and currently available next generation sequencing (NGS) tests at Mayo Clinic and other institutions. Using the Agilent SureSelect XT RNA Direct kit, cDNA synthesis and end repair were performed manually, while NGS libraries were prepared on a Biomek FXp Liquid Handler (Beckman-Coulter). Paired-end, 101bp sequencing was completed on the HiSeq 2500 (Illumina) in rapid run mode and data was analyzed using Mayo Clinic's custom bioinformatics pipeline, Map-RSeq. Twenty-three FFPE tumors with 16 different known fusions identified by other methods, e.g. RT-PCR or fluorescence *in situ* hybridization (FISH), 7 tumors with unknown fusion status and 12 normal tissues were analyzed. Five FFPE samples with 4 fusions were compared to paired fresh frozen samples on the same run to evaluate performance characteristics between sample types with this assay and between the FFPE and the fresh frozen version of the assay. **Results:** The expected fusions were detected in all 23 samples. No fusions were found in the unknown fusion status tumors or normal samples. Fusions were identified in all 4 of the fresh frozen samples; however, only 1 out of 4 concurrently run FFPE samples reached our cut-off of >25 million reads precluding further analysis. It was determined that when FFPE and fresh frozen samples are sequenced together, the majority of the reads belong to the fresh frozen samples, so subsequent runs were performed with only FFPE samples. **Conclusion:** We have developed and performed preliminary experiments to evaluate the accuracy of an RNA-seq assay for the detection of fusions in FFPE tissue. This method is being optimized to be a high throughput and fully automated assay for the detection of gene fusions with high sensitivity and specificity.

#### ST046. Multi-institutional Evaluation of the 2017 AMP, ASCO and CAP Standards and Guidelines for Interpretation and Reporting of Sequence Variants in Cancer

D. Sirohi<sup>1</sup>, R. Schmidt<sup>1</sup>, D.L. Aisner<sup>2</sup>, A. Behdad<sup>3</sup>, B.L. Betz<sup>4</sup>, N. Brown<sup>4</sup>, J.F. Coleman<sup>1</sup>, C.L. Corless<sup>5</sup>, G. Deftereos<sup>1</sup>, M.D. Ewalt<sup>6</sup>, H. Fernandes<sup>6</sup>, S.J. Hsiao<sup>6</sup>, M.M. Mansukhani<sup>6</sup>, S.S. Murray<sup>7</sup>, N. Niu<sup>8</sup>, L. Ritterhouse<sup>8</sup>, J.P. Segal<sup>8</sup>, L.V. Furtado<sup>1</sup>  
<sup>1</sup>University of Utah/ARUP Laboratories, Salt Lake City, UT; <sup>2</sup>University of Colorado School of Medicine, Aurora, CO; <sup>3</sup>Northwestern University, Chicago, IL; <sup>4</sup>University of Michigan Health System, Ann Arbor, MI; <sup>5</sup>Oregon Health and Science University, Portland, OR; <sup>6</sup>Columbia University, New York, NY; <sup>7</sup>University of California, San Diego, CA; <sup>8</sup>University of Chicago, Chicago, IL.

**Introduction:** In 2017, the Association for Molecular Pathology (AMP), American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) jointly released guidelines for the interpretation and reporting of sequence variants in cancer. Application of these guidelines may vary widely among pathologists, however, and may lead to conflicts

of potential clinical significance. This study was undertaken to evaluate the performance of the 2017 AMP/ASCO/CAP guidelines across different laboratories and to assess current practices and perceptions surrounding these guidelines. **Methods:** Twenty participants from 10 institutions that routinely perform next generation sequencing assays for solid tumors contributed a total of 63 de-identified variants, spread across different genes, variant categories, and tumor types. From these, 51 variants were selected and distributed to all participants for classification using the new guidelines. Agreement was assessed using observed agreement and chance-corrected agreement (Cohen's kappa with linear weighting). Intra-institutional percentage agreement was calculated where possible. Participants were invited to take a web-based survey regarding their perceptions of the guidelines. **Results:** Overall observed agreement between participants was 84% with expected and chance-corrected agreement statistics of 73% and 42%, respectively. Most disagreements occurred between Tiers I vs II or Tiers II vs III, although 5 disagreements spanned Tiers I-IV. Intra-institutional agreement ranged from 34%-100%. To resolve disagreements, a summary of compiled variant classifications and additional pertinent information about each variant were redistributed to all participants. Seven participants updated their classification choices with 1 to 4 variants reclassified per participant. Seventy-five percent (n=15) of the participants responded to the survey with 20% (n=3) having already implemented the guidelines in clinical practice. The main perceived limitations included the complexity of the guidelines, discordance between clinical actionability and pathobiological relevance, lack of familiarity of clinicians with the new classifications, and uncertainty when applying criteria to potential germline variants. **Conclusions:** This study demonstrates noteworthy discordance between pathologists for variant classification in solid tumors when using the new AMP/ASCO/CAP guidelines. Participants also perceived that the guidelines failed to address classification of some variant types (eg. oncogenic variants that may be germline or may not have clinical actionability). This study highlights a need to address such limitations prior to mainstream clinical adoption.

#### ST047. Establishing the Impact of *STK11* Canonical Splice Site Variants Identified by NGS Panel Testing in Non-Small Cell Lung Cancers (NSCLC): Prognostic and Therapeutic Implications

D.J. Seward<sup>1</sup>, L.L. Donnelly<sup>1</sup>, H.M. Sarausky<sup>1</sup>, J. Armstrong<sup>1</sup>, M. Cameron<sup>1</sup>, P. Deming<sup>2</sup>, K. Hampel<sup>1</sup>, N. Sidiropoulos<sup>1</sup>

<sup>1</sup>University of Vermont Medical Center, Burlington VT; <sup>2</sup>University of Vermont, Larner College of Medicine, Burlington VT.

**Introduction:** *STK11*, also called *LKB1*, encodes an important tumor suppressor, regulating many key signaling networks such as the AMPK and mTOR pathways. Clinically, germline *STK11* loss of function (LoF) variants are pathognomonic for Peutz-Jeghers syndrome. Somatic LoF mutations are found in several cancer types, including NSCLCs, where they are associated with worse prognosis. Ongoing work suggests tumors lacking *STK11* exhibit increased expression of the cytokines IL-33, IL-6 and CXCL7. IL-6 is known to promote neutrophil recruitment, leading to reduced T-cell infiltration and T-cell dysfunction within the tumor microenvironment. Ultimately this may explain why *STK11* LoF predicts insensitivity to immune-modulatory therapies. These observations underscore the importance of documenting *STK11* status in NSCLC patients, both for prognostic and potential therapeutic purposes. Germline to our work, the functional impact of variants occurring at *STK11* canonical splice-sites (CSS) cannot be determined using DNA sequencing alone. Given that *STK11* CSS are relatively common, expected in 1-3% of all NSCLCs (~4000/yr), directly evaluating *STK11* mRNA is essential to predicting phenotype. The goal of our study was to evaluate *STK11* CSS variants detected in NSCLCs, characterize the resultant mRNA species, and evaluate functional impact. **Methods:** CSS variants were identified by next generation sequencing (NGS) assays performed on NSCLCs. Preliminary impact on *STK11* mRNAs was evaluated using a mini-gene splicing assay. Briefly, *STK11* wild-type exons and portions of flanking introns were cloned between synthetic exons within a reporter vector. Point mutagenesis was employed to create the CSS variants observed in clinical samples. Cell lines were transfected with WT or variant constructs and RNA evaluated for splicing defects using RT-PCR. Clinical samples were evaluated by RT-PCR followed by Sanger sequencing to

assess *STK11* splicing *in vivo*. **Results:** Upon evaluating novel somatic *STK11* CSS alterations, we report variant-specific splicing outcomes including exon skipping, intron read-through, and cryptic splice site usage. Our results support the assertion that predicting splicing outcomes based on primary sequence alone is not currently possible. Further we show that not all iterative CSS variants result in *STK11* LoF. **Conclusions:** Mounting data support a central role for *STK11* in modulating tumor cytokine profiles. In the near future, reporting *STK11* functional status is likely to be a prerequisite for initiation of immune-modulatory therapies. Our results demonstrate the necessity of direct *STK11* mRNA analysis prior to designating CSS variants as LoF. Further, we demonstrate the feasibility of reflex *STK11* mRNA analysis when CSS are detected by NGS methods.

#### ST048. Validation of the ArcherDx VariantPlex Solid Tumor Assay for the Molecular Analysis of Clinical Tumor Samples

K.D. Davies, J. Haney, M. Seager, C.A. Lawrence, D.L. Aisner  
University of Colorado, Anschutz Medical Campus, Aurora, CO.

**Introduction:** Next-generation sequencing (NGS)-based assays have become critical for the molecular analysis of clinical tumor specimens due to the need to assess mutational status of multiple genes simultaneously. Here we describe the analytical validation of the ArcherDx VariantPlex Solid Tumor assay, an NGS library preparation system that amplifies selected regions in 67 genes, for the detection of single nucleotide variants (SNVs) and insertions/deletions (indels) in solid tumor samples. **Methods:** Ninety-one samples, including clinical solid tumor samples, commercially available reference materials, and a cell-line mixture, were investigated as part of the validation. These samples contained 654 variants detected by orthogonal testing in regions in common with the VariantPlex assay. For clinical samples, total nucleic acid (TNA) was extracted from formalin-fixed, paraffin-embedded (FFPE)-processed tumor material. Library preparation was achieved per the manufacturer's instructions with minor modifications. Libraries were sequenced via either the Illumina MiSeq or NextSeq instruments. Analysis of raw sequence data for variant calling was performed by the ArcherDx Analysis algorithm version 5.1.2.2. **Results:** Assay sensitivity was 99.5% when considering variants  $\geq 10\%$  variant allele frequency (VAF). However, sensitivity was only 88.0% for variants  $< 10\%$  VAF. Thus, the limit of detection for the assay was determined to be 10% VAF. Taking into account assay metrics including read depth, read complexity, VAF, strand bias, and sequence direction bias, only one false positive was encountered (assay specificity = 99.9%). Sequencing depth (unique reads) across all assay regions, calculation of which was achieved via an add-on to the analysis algorithm, was found to be DNA quality-dependent. The assay demonstrated a high degree of reproducibility, both in wet-bench steps (inter-assay, intra-assay, and inter-technician) and in analysis steps. **Conclusions:** The ArcherDx VariantPlex Solid Tumor assay demonstrates high sensitivity and specificity in detection of SNVs and indels at  $\geq 10\%$  VAF. Metrics generated by the analysis algorithm are critical in the removal of assay artifacts. The assay is suitable for the molecular analysis of clinical solid tumor specimens.

#### ST049. Clinical Targeted Next-generation Sequencing Panel Testing in Non-small Cell Lung Cancer: Single Institution Experience at a High Scale National Reference Laboratory

K. Barber<sup>1</sup>, W. Mendonca<sup>1</sup>, N. Harada<sup>1</sup>, J. Bissonnette<sup>2</sup>, A. Jhuraneey<sup>1</sup>, E. Antoniou<sup>1</sup>, B. Tandon<sup>1</sup>, L. Liang<sup>1</sup>, R.T. Klein<sup>3</sup>, J. Zhao<sup>1</sup>

<sup>1</sup>BioReference/GenPath, Elmwood Park, NJ; <sup>2</sup>GeneDx/BioReference, Gaithersburg, MD; <sup>3</sup>GeneDx/BioReference, Elmwood Park, NJ.

**Introduction:** Clinical targeted next-generation sequencing (NGS) panels are emerging as a mainstream diagnostic test in the routine clinical laboratory setting for comprehensive genomic profiling in non-small cell lung cancer (NSCLC). The National Comprehensive Cancer Network (NCCN) guidelines recommend that biomarker testing in NSCLC be done as part of a broad molecular panel containing, at a minimum, the following genes: *EGFR*, *BRAF*, *KRAS*, *MET*, and *HER2* (ERBB2) (NCCN NSCLC, 4.2018). In a recent NGS study of NSCLC, 82.4% of samples harbored at least one gene alteration (31.4% *KRAS*, 22.4% *EGFR*) (Fumagalli et al., 2018). Herein, we report one high scale national reference laboratory's experience with clinical targeted NGS panel testing in NSCLC. **Methods:**

We performed a retrospective analysis of the 5,145 cases of NSCLC specimens (containing at least 10% tumor burden) profiled using the GenPath OncoSight 18-gene lung tumor NGS panel from January 2015 to April 2018. Somatic mutational landscape was compared for NSCLC subtypes including adenocarcinomas and squamous cell carcinomas. **Results:** Overall, 4,117 cases (80.0%) were found to have at least one somatic alteration. A total of 151 cases (2.9%) were deemed quantity/quality not sufficient (QNS). Disease-associated alterations were detected in 17/18 genes included in the panel (no abnormalities were identified in *FGFR1*). Clinically actionable hotspot alterations in *KRAS*, *BRAF*, and *EGFR*, as well as alterations in *MET* and *HER2*, together accounted for 43.7% of the total disease-associated alterations. When assessed by subtype, the most frequently altered genes among adenocarcinomas include, *TP53* (42.9%), *KRAS* (32.0%), *EGFR* (13.9%) and *BRAF* (4.5%), and the most frequently altered genes in squamous cell carcinomas include, *TP53* (79.2%), *PIK3CA* (8.9%) and *KRAS* (7.0%). The other genes on the panel collectively accounted for the remaining 3.5% of the total disease associated alterations detected among NSCLC cases studied. **Conclusion:** This data demonstrates significant clinical utility of NGS panel testing in NSCLC. Potentially actionable findings were noted among multiple genes, with a very low QNS rate. The hotspot alterations in *KRAS*, *EGFR* and *BRAF*, as well as the alterations in *MET* and *HER2*, account for more than one third of all the alterations detected and result in clinical utility to the patient. Rates of detection using this assay are consistent with similar smaller studies reported in the literature (Fumagalli *et al.*, 2018). Although *TP53* alterations are not referenced in NCCN guidelines, several clinical trials are now available for NSCLC patients with a *TP53* alteration, suggesting that limiting an NGS panel to just the minimum of recommended genes by NCCN could miss alterations that may provide benefit.

**ST050. MLH1/PMS2-deficient, BRAF-mutated, and Calretinin-positive Colorectal Carcinoma Presents at Advanced Stage and is Associated with Poor Differentiation and Poor Prognosis**

W. Zhang<sup>1,2</sup>, X. Guo<sup>1,2</sup>, S. Chung<sup>1</sup>, Q. Liu<sup>1</sup>

<sup>1</sup>Montefiore Medical Center, Bronx, NY; <sup>2</sup>Mount Sinai Health System, New York, NY.

**Introduction:** Colorectal carcinoma is the third leading cause of cancer deaths in the United States. It has been well reported that *BRAF* mutation is associated with poor prognosis in colorectal cancer. However, there are also reports indicating that even within MLH1/PMS2-deficient *BRAF* mutated tumors, there may be different subgroups, with different prognostic implications. Calretinin, a highly conserved calcium binding protein, is expressed in colon cancer, but is absent from normal human enterocytes. Here, we present two cases of MLH1/PMS2-deficient, *BRAF*-mutated high-grade colorectal carcinomas that show strong diffuse staining for calretinin. **Methods:** The first case is a 57-year-old man admitted for abdominal pain. The second case is a 77-year-old man admitted for abdominal pain and dark stool. Both patients showed large obstructing tumors in the ascending colon by colonoscopy. CT scan revealed multiple nodules in the liver and bilateral lungs in both cases. Both colon tumors and liver nodules were biopsied. Immunostains for AE1/3, CK7, CK20, CDX2, chromogranin, synaptophysin, CD45, calretinin, and mismatch repair proteins (MMR) were performed as well as *BRAF* mutation analysis. **Results:** Colon biopsies showed high-grade carcinoma without morphologically recognizable differentiation. The tumors in both cases were positive only for AE1/3 and calretinin, but negative for all other markers. MLH1 and PMS2 were lost in both cases, however, MSH2 and MSH6 were retained. Both tumors were also proved to be *BRAF* mutated. Liver biopsies showed similar morphology to the colonic tumors. Both patients were diagnosed as stage IV colorectal carcinoma. The first patient died three weeks after the diagnosis. The other patient underwent palliative ileo-colonic bypass surgery, and is currently 1 month post diagnosis and receiving chemotherapy.

**Conclusions:** Both tumors are MLH1/PSM2-deficient *BRAF*-mutated with diffusely strong expression of calretinin, but negative for CK20 and CDX2, which indicates a loss of colonic differentiation. A study on human colon cancer cell line has raised the possibility that calretinin is involved in maintaining the cells in an undifferentiated state. Calretinin has also been shown to affect cell proliferation and migration. Even though a large-scale

necessary to prove our hypothesis, we propose that calretinin is only expressed in poorly and/or undifferentiated colorectal carcinomas, and may be associated with an early metastatic potential and poor prognosis. A calretinin immunostain and a next-generation sequencing (NGS) cancer hotspot panel on a large cohort are in progress for further mechanistic characterization.

**ST051. Analytical Validation of a DNA Dual Strand Approach for an FDA-approved NGS based Praxis Extended RAS Panel for FFPE Metastatic Colorectal Cancer Samples**

A. Iyer, N. Udar, R. Haigis, M. Porter, S. Smith, S. Dhillon, J. Lu, P. Wenz, K. Gutekunst  
Illumina, Inc., San Diego, CA.

**Introduction:** Next generation sequencing (NGS) has moved quickly to clinical applications for precision medicine. The Praxis Extended RAS Panel Assay is the first FDA-approved NGS *in vitro* diagnostic for evaluating RAS mutations in colorectal cancer (CRC) to determine patient eligibility for treatment with Vectibix (panitumumab). *KRAS* exon 2 (codon 12-13) mutations are an established predictive biomarker of resistance to anti-EGFR therapy for mCRC. Multiple clinical trials have established the value of extended RAS testing to include exon 2 (codons 12-13), exon 3 (codons 59-61), and exon 4 (codons 117-146) of both *KRAS* and *NRAS*. The clinical benefit of Vectibix is restricted to patients who have no tumor mutations in extended RAS genes. The Praxis Extended RAS Panel Assay is a qualitative *in vitro* diagnostic test using targeted high throughput parallel sequencing for the detection of 56 mutations in RAS genes in DNA extracted from formalin-fixed, paraffin embedded (FFPE) CRC tissue. We demonstrate the analytical validation of DNA input, limit of detection (LOD), and precision/reproducibility of the Praxis Extended RAS Panel Assay. **Methods:** DNA from FFPE tissue was qualified by measuring amplifiability relative to a control DNA (delta Cq; dCq) that evaluates both DNA quality and quantity. The panel utilizes TruSeq Custom Amplicon technology with a dual strand approach that targets each DNA strand independently to distinguish artifacts commonly found in FFPE DNA, with sequencing on the MiSeqDx instrument. Mutations are reported only if found on both strands. **Results:** For DNA Input, 12 observations of 5 CRC FFPE specimen mixes, each containing 2 panel mutations, one at low (approx. 8%) and one at high (approx. 14%) mutation frequency (MF) were collected across 3 days at dCq 4-5 and 5-6. dCq 4-5 showed 100% (60/60) sample pass rate and 100% (120/120) expected mutations. dCq 5-6 showed 92% (55/60) sample pass rate and 95% (114/120) expected mutations, confirming the minimum DNA Input as dCq ≤ 5, which is approximately 25 ng of intact DNA. 12 observations of each of these 5 CRC FFPE specimen mixes (dCq 4-5) were also collected over 3 days with 3 reagent lots and demonstrated a low total standard deviation range from 0.011 to 0.029. Across all lots/days, 99.7% (359/360) of observations showed the expected mutation. The LOD study used 3 CRC FFPE specimen mixes (dCq 4-5), each with 2 panel mutations at approx. 10%, 5%, 2.5%, 1.25% and 0% MF, run in duplicate with 2 reagent lots, 5 operators on 2 days for 20 observations, overall 600 observations (20 obs × 5 levels × 3 mixes × 2 lots). The LOD claim is 5%. **Conclusions:** The Praxis Extended RAS Panel is a sensitive, precise, reproducible assay for precision medicine in determining eligibility for treatment with Vectibix.

**ST052. Optimization of Testing Methods in Detecting MET Amplification, Expression, and Activation for Targeted MET TKI Treatment in Non-small Cell Lung Cancer Patients**

H. Gong, J. Rowland, J. Wahl, E. Pacia, S. Tangri, J. Lameh  
Navigate BioPharma Services, Carlsbad, CA.

**Introduction:** MET amplification and protein expression are known to cause dysregulation of the MET/HGF pathway in non-small-cell lung cancer (NSCLC) patients. Several MET targeting inhibitors have already shown promising results in treatment of patients. Identifying patients with these molecular aberrations is critical to ensuring clinical benefits from MET targeted treatments. This study compares the different testing methods used to detect MET amplification and expression for selection of patients for MET therapies. **Methods:** Formalin fixed paraffin embedded (FFPE) NSCLC specimens were tested in the fluorescence *in situ*



hybridization (FISH) and immunohistochemistry (IHC) assays. Two assays were employed to measure MET amplification by FISH. Assay A assessed the average MET genomic copy number (GCN) up to a cap of 16 and was evaluated by two independent technologists who analyzed 30 nuclei each. Assay B assessed the average MET GCN over 50 viable tumor nuclei located in regions of MET amplification, and did not cap MET signals. MET protein expression was measured by immunohistochemistry (IHC) using the Ventana SP44 antibody. HistoScore (H-score) was calculated by a semi-quantitative assessment of both the intensity of staining (0, 1, 2, or 3) and the percentage of positive cells. MET overexpression was defined as  $\geq 50$  the median value for the H-score.

**Results:** MET FISH positivity was detected in 19.6% NSCLC specimens using assay A with a qualitative cut-off of average MET GCN  $\geq 5$ ; while MET amplification was observed in 29.6% of specimens tested by assay B. Patients with an average GCN of 5 or greater showed favorable response to treatment using assay A. The learning from assay A was used in the optimization of the assay to an enhanced version (assay B), leading to a more stringent scoring methodologies and optimization of cut offs that will potentially help better detect MET alterations in patients suitable for MET therapies. H-score did not predict patient response to MET targeted therapy as seen in the previous trial, however, in patients whom  $\geq 50\%$  tumor cells expressed MET protein at 3+ responded favorably to MET targeted therapy. **Conclusion:** It is critical to define appropriate qualitative cut-offs for MET FISH assays that depend on specific scoring criteria. The IHC data suggest that H-scores should be carefully used to measure protein expression. Combination of H-score with percentage staining in 3+ category of MET IHC was important for predicting patient response. Our data indicate MET FISH and IHC assays can be used to select patients for MET targeting therapies. However, development of optimal assays to select right patients requires a deep understanding of technology limitations and the biomarker biology.

#### ST053. Personalized ddPCR Mutation Assays Targeting Patient Specific ctDNA: A Tool to Monitor Treatment Responses to Mutation-Specific T-cell Transfer Immunotherapy in Epithelial Cancer Patients

L. Xi, H. Chung, J. Ho, T.H. Pham, N. Zacharakis, S.A. Feldman, P.F. Robbins, S.A. Rosenberg, M. Raffeld  
National Cancer Institute, National Institutes of Health, Bethesda, MD.

**Introduction:** Tumor-infiltrating lymphocyte (TIL)-mediated immunotherapy has shown great promise as a curative treatment modality, particularly in melanoma where durable remissions have been achieved in up to 1/3 of patients with stage IV melanoma. Recent studies in other solid tumors have begun to show promising results as well. Complete responses using RECIST criteria may take many months, and biomarkers of response are critically needed to separate responding patients from non-responders. We have previously shown that circulating tumor DNA (ctDNA) can be used to follow and potentially predict response to this therapy in BRAF V600E positive melanomas (Xi L *et al.*, 2016). Here we show that random somatic mutations identified by exome sequencing, and further selected for immunogenicity, can be used to follow response and disease activity in a variety of gastrointestinal and breast cancers. **Methods:** Exome sequencing was performed in 10 patients with gastrointestinal or breast cancers, and in 1 patient with melanoma. Mutations were modeled for immunogenicity, and TILs were enriched for activity against selected neoantigens prior to treatment as previously described (Tran E *et al.*, 2015). 1-3 mutation targets per patient, including at least one target used for TIL treatment, were selected and tumor specific custom droplet digital PCR (ddPCR) mutation assays were designed (a total of 30 assays for the 11 patients). Genomic DNA isolated from each paired primary tumor and normal tissue was used to confirm the specificity of each assay. 116 serum samples were collected from the 11 patients at 5 or more time points pre and post treatment and cell-free DNA (cfDNA) was isolated. ddPCR was performed on the cfDNA using the custom-designed assays, and temporal changes in ctDNA targets were correlated with each patient's clinical response to TIL immunotherapy. **Results:** Twenty-nine of the 30 custom ddPCR assays confirmed the presence of the corresponding mutation in the primary tumor from each patient, and the absence of the mutation in normal tissue. Twenty-six of the 29 validated ddPCR assays identified its target in at least two serum samples from the corresponding patient during his/her

treatment course, but not from other controls. Furthermore, dynamic changes in ctDNA were highly correlated with the clinical course of each patient. **Conclusions:** Personalized custom ddPCR assays designed to target tumor specific mutations in circulating plasma DNA can be used as biomarker assays to follow patient treatment responses to cellular immunotherapy. This approach may be applicable to other therapies as well, and particularly useful in tumors that have no alternative biomarkers.

#### ST054. Importance of Amplicon Size for Detecting Microsatellite Instability in Liquid Biopsies

M. Campan<sup>1</sup>, M. Bask<sup>2</sup>, J. Pettersson<sup>2</sup>, L. Du<sup>2</sup>, T.I. Long<sup>2</sup>, A. Barzi<sup>2</sup>, L. Dubeau<sup>2</sup>, P.M. Ward<sup>2</sup>

<sup>1</sup>University of California, Los Angeles CA; <sup>2</sup>University of Southern California, Los Angeles CA.

**Introduction:** We previously reported on the feasibility of detecting microsatellite instability in liquid biopsies from patients with colorectal cancer and on the potential of this approach to monitor treatment responsiveness using the Promega MSI analysis kit v1.2. Amplicon size is especially important when working with liquid biopsies because circulating cell free DNA consists mostly of approximately 170bp fragments corresponding to the length of a nucleosomal loop. We sought to compare the performance of the MSI v1.2 kit to that of the recently developed, but not yet available commercially Promega MSI kit v2.0 on some of the same subjects used in this earlier study to evaluate the implications of differences in amplicon and biomarker sizes on detectability of microsatellite instability in liquid biopsies. Version 2.0 targets 4 microsatellite biomarkers also targeted by version 1.2 (Bat-25, Bat-26, NR-24 and Mono-27) but the size of the amplicons is smaller with version 2.0 (75-120bp compared to 94-154bp). Version 2.0 also includes 4 biomarkers with longer poly-A repeats (Bat-52, Bat-56, Bat-59 and Bat-60) with amplicon sizes between 101bp and 190bp. **Methods:** Five subjects with microsatellite unstable tumors with successive liquid biopsies that had shown conversion from microsatellite unstable to microsatellite stable status with the MSI kit v1.2 following treatment, and two subjects with microsatellite unstable tumors with no instability detected in liquid biopsies with version 1.2, were retested with version 2.0. Two samples were serially diluted to evaluate assay sensitivity. Two subjects with no detectable amplicons in their liquid biopsies with version 1.2 were also included. **Results:** There was full concordance between the diagnoses made with the 2 kits. The magnitude of the signals obtained with version 2.0 was increased 6.5-fold (range 1.6-21.7) compared to version 1.2, improving the lower limit of detection from 200pg input DNA with version 1.2 to 100pg with version 2.0. The 2 liquid biopsies from which no detectable amplicons could be generated with version 1.2 showed robust biomarker profiles with version 2.0. The Bat-60, Bat-59 and Bat-52 biomarkers, with amplicon sizes of 110, 117 and 132bp, were also detectable using an input of 100pg while the 140bp Bat-56 amplicon was only seen with 200pg input DNA. **Conclusions:** The Promega MSI v2.0 kit increases sensitivity of detecting microsatellite instability in liquid biopsies compared to version 1.2 primarily due to differences in amplicon sizes and not due to differences in lengths of poly-A repeats.

#### ST055. DNA Sequencing of Human, Epstein-Barr Virus, and *Helicobacter pylori* Genomes to Classify and Monitor Gastric Adenocarcinoma

K. Greene<sup>1</sup>, C.H. Edgerly<sup>1</sup>, S. Elmore<sup>1</sup>, M.O. Meyers<sup>1</sup>, M.S. Lee<sup>1</sup>, R.L. Dominguez<sup>2</sup>, D.R. Morgan<sup>3</sup>, R. Betancourt<sup>1</sup>, N.D. Montgomery<sup>1</sup>, M.L. Gulley<sup>1</sup>

<sup>1</sup>University of North Carolina, Chapel Hill, NC; <sup>2</sup>Western Regional Hospital, Santa Rosa de Copan, Honduras; <sup>3</sup>Vanderbilt University, Nashville, TN.

**Introduction:** Classification of recurrent or advanced gastric adenocarcinoma increasingly relies on gene tests, such as *ERBB2* amplification and Epstein-Barr virus (EBV) status, to inform therapy selection. Furthermore, cell free DNA levels of such tumor markers may reflect tumor burden and efficacy of therapy. We devised sequencing assays to simultaneously quantify human gene variants and oncogenic pathogen genomes, and we applied the assays in cancer tissue and in serial plasma specimens from gastric cancer patients. **Methods:** Targeted DNA sequencing (ArcherDx libraries, Illumina

NextSeq v2 reagents) was done on paraffin-embedded tumor tissues (QIAamp DNA Micro extraction kit) from 40 US gastric cancer patients and on 63 matched plasma specimens (Promega Maxwell RSC LV ccfDNA extraction kit) at active disease or post-therapy timepoints. An additional 25 gastric cancer tissues from Honduras were also sequenced. Reads were generated from up to 67 human cancer genes to detect mutations, insertion/deletions (indels), and CNVs, and to quantify levels of EBV and *Helicobacter pylori* genomes after aligning reads to the respective reference genomes, collapsing PCR replicates using unique molecular identifiers, filtering, and applying noise reduction algorithms. Comparator methods include 1) viral load by Q-PCR that was validated against NGS viral read counts in lymphoma patient specimens, and 2) Her2 (ERBB2) expression and fluorescence *in situ* hybridization (FISH) amplification status. **Results:** Robust sequencing data was generated from biopsy, resection and plasma specimens. Nineteen of 25 Honduran cancers harbored at least one somatic tumor marker, and 35/40 US tumors had somatic markers that could be monitored in plasma. EBV and/or *H. pylori* genomes were commonly detected in tumor tissues, and co-infection was more prevalent in Honduran than US cancer cohorts (48% vs 0%). *ERBB2* amplification by sequencing was concordant with Her2 status as tested by orthogonal methods. Known tumor markers were detected in plasma of 40% of active cancer patients, and serial levels mirrored efficacy of therapy with no variants detectable in plasma at remission timepoints. EBV read counts per million total reads were proportional to viral loads by Q-PCR. *H. pylori* DNA was not detected in plasma. **Conclusions:** Modern sequencing technology can simultaneously genotype human DNA and quantify pertinent infectious agents in either tissue or plasma, streamlining identification of actionable targets including oncogenic pathogens, and promoting research on microbial-human interactions underlying tumorigenesis and response to treatment.

#### ST056. Clinical Utility of Comprehensive Genomic Profiling in Pediatric Brain Tumors

J. Ji, K. Kaneva, M.C. Hiemenz, R.J. Schmidt, A. Margol, N. Robison, G. Dhall, D. Tian, D. Hawes, J.A. Cotter, A.R. Judkins, J.A. Biegel  
Children's Hospital Los Angeles, Los Angeles, CA.

**Introduction:** Recent large-scale genomic studies have revealed a variety of novel, clinically significant molecular variants in pediatric brain tumors. We developed a comprehensive DNA- and RNA-based next generation sequencing (NGS) panel, OncoKids, to detect diagnostic, prognostic, and therapeutic markers across various pediatric malignancies, including brain tumors. OncoKids is used in conjunction with chromosomal microarray analysis (CMA) for routine clinical molecular diagnostics. **Methods:** To date, OncoKids has been employed for 97 consecutive pediatric brain tumors, including low and high-grade gliomas, embryonal tumors and ependymomas. Clinical CMA data was analyzed in parallel with OncoKids for 61% (59/97) of these cases.

**Results:** OncoKids demonstrated pathogenic mutations in 63% (61/97) of cases. A total of 85 single-nucleotide variant (SNV) and small insertion/deletions (InDels) of strong clinical significance (Tier I) or potential clinical significance (Tier II) in 29 genes were reported in 47 cases. Diagnostic and prognostic Tier I mutations included mutations in *H3F3A*, *HIST1H3B*, *FGFR1*, *ACVR1*, and *TP53* in high-grade gliomas; *BRAF V600E*, *FGFR1*, and *NF1* mutations in low grade gliomas; *KDM6A*, *PTCH1*, *SMARCA4*, and *TP53* mutations in medulloblastomas; *CTNNB1* exon 3 mutations in craniopharyngiomas; and *SMARCB1* mutations in rhabdoid tumors. A combination of *H3F3A G34R*, *ATRX*, and *TP53* Tier I mutations which characterize a specific subtype of high grade glioma was identified in four histologically challenging but distinct neuroepithelial tumors. Clinically significant RNA fusions were identified including *KIAA1549-BRAF* in 11 pilocytic astrocytomas, *PTPRZ1-MET* in two high grade gliomas, *YAP1-MAML2* in one malignant neoplasm of the posterior fossa without further histologic classification, and *FGFR2-SHTN1* in one ganglioglioma. Amplifications of *EGFR*, *MYCN*, *MET*, and *MDM4* were detected in three high grade gliomas. A highly mutated pattern was observed in two high grade-gliomas; both of the patients had a germline mutation in mismatch repair genes. Potential targeted therapies with *BRAF*, *FGFR*, *mTOR*, *MET*, *IDH1* or *ALK* inhibitors, were suggested in 34% (21/61) of cases. OncoKids analysis also revealed potential germline mutations in cancer

predisposition genes for 26% (25/97) of cases. CMA demonstrated clinically significant copy number alterations in 13 of 26 tumors with non-informative OncoKids results, e.g., loss of chromosome 3 including the *VHL* gene in hemangioblastoma and *i(17q)* in medulloblastoma. **Conclusions:** Our results demonstrate significant clinical utility of integrating NGS-based genomic profiling into routine clinical testing for pediatric brain tumors.

#### ST057. Validation of Antibody Panels for High-plex Immunohistochemistry Applications

D.A. Hinerfeld, K. Barker, H. Metz, C. Merritt, L. Dennis, J. Beechem  
Nanostring Technologies, Seattle, WA.

**Introduction:** Characterization of the spatial distribution and abundance of key proteins within tissues enables a deep understanding of biological systems. However, it has proven difficult to perform such studies in a highly-multiplexed manner on formalin-fixed, paraffin-embedded (FFPE) tissue sections. There has been significant progress in developing technologies with expanded capabilities to analyze higher numbers of proteins, however, the validation of these technologies and their associated affinity reagents remains a significant barrier to adoption. We have developed a validation pipeline that ensures optimal sensitivity and specificity for high-plex antibody panels for the analysis of FFPE sections using the NanoString Digital Spatial Profiling (DSP) platform. The DSP is designed to simultaneously analyze up to 96 proteins by detecting oligos conjugated to antibodies that can be released via a UV-cleavable linker.

**Methods:** Antibodies targeting immuno-oncology proteins were tested for specificity and sensitivity by immunohistochemistry on FFPE human tissues, as well as human cell line pellets to evaluate binding specificity in both unconjugated and oligo-conjugated antibodies. The sensitivity and dynamic range were tested using FFPE cell pellets with target-specific positive and negative cells at different ratios. An interaction screen was performed to evaluate potential deleterious effects of multiplexing antibodies, and a human tissue microarray (TMA) containing normal and cancer tissues was employed to assess assay robustness. The reproducibility of the panel on DSP was tested on serial FFPE tumor specimens by correlating the expression of all markers across 24 spatially-registered regions of interest (ROI) as well as the ability to reveal biological heterogeneity within lymphoid tissue by characterizing the expression of 40+ proteins in a spatial grid of 100µm x 100µm ROIs. **Results:** Immunohistochemical analysis of unconjugated and oligo-conjugated antibodies displayed indistinguishable staining patterns on control tissues and cell lines. Mixed cell pellet assays revealed strong correlations between observed counts and positive cell numbers. Antibody interaction studies showed similar count values for antibodies alone or in combination, and TMA hierarchical clustering analysis demonstrated expected patterns of expression across tissue types. Analysis of all markers across 24 registered regions of interest across serial FFPE sections were highly correlated. Spatial analysis of lymphoid tissue revealed high levels of biological heterogeneity across multiple germinal centers. **Conclusion:** These results demonstrate the validation and application of high-plex protein panels to accurately interrogate the immune biology within FFPE tissue using the NanoString DSP platform.

#### ST058. Novel Liquid Biopsy (ctDNA) Reference Material Development and Characterization using CRISPR/Cas9-engineered Cell Lines

S. Saddar, S. Thakker, Q. Zheng, S. Kondapalli, V. Mani, D. Sengupta, G. Roma  
AccuRef Diagnostics, Milpitas, CA.

**Introduction:** Circulating tumor DNA (ctDNA) also known as liquid biopsy has emerged as a clinically useful non-invasive biomarker for somatic cancer mutations. With recent advances in Next-Generation Sequencing (NGS) and digital PCR (dPCR), it is now possible to detect low allele frequency mutations with greater precision. However, accurate mutational profiling from ctDNA remains challenging because of low amount of ctDNA in plasma, and frequent observation of mutant alleles with very low allelic frequency. The accurate quantification of the number of mutant fragments in a sample remains critical. Because of these reasons, there is a need for a reproducible source of ctDNA-mimetic reference material for assay development, limit-of-detection (LOD) assessment, quality assurance, and proficiency testing. Current commercially available ctDNA

reference standards are developed using two principal methods, each with limitations. Sonication of cancer cell line DNA does not mimic naturally-occurring ctDNA fragment properties (e.g. size distribution and DNA blunt ends), and synthetic spiked-in oligonucleotides do not provide the genomic complexity of cell line-based materials. Here, we demonstrate the development of a novel ctDNA-mimetic reference material based on nucleosomal-digestion of CRISPR/Cas9 engineered cell lines in synthetic plasma to mimic patient samples. Since mutational profiling from ctDNA has been successfully applied to detect *EGFR* mutations in NSCLC patients, we have selected a panel of 10 frequently mutated *EGFR* regions to demonstrate this concept. **Methods:** We engineered 10 actionable cancer mutations in the *EGFR* gene using CRISPR/Cas9 technology. Mutations were confirmed by Sanger sequencing and illumina NGS assay. A nucleosomal digestion protocol was then utilized to fragment the gDNA into lengths that mimic true patient ctDNA. Targeted MAFs of 0%, 0.1%, 1.0%, and 5.0% were then created by mixing digested ctDNA and were confirmed by dPCR, and NGS. Fragment size distribution was assessed by BioAnalyzer and compared to human extracted ctDNA.

**Results:** Using proprietary nucleosomal digestion, we achieved fragmentation profiles centered at ~166bp. Targeted allele frequencies of were obtained with high accuracy using dPCR and NGS. The synthetic plasma extraction behaved similarly to extracted ctDNA from human plasma specimens. **Conclusion:** We have developed a low allelic frequency *EGFR* ctDNA mimetic reference material in synthetic plasma with high accuracy and reproducibility. This reference material is ideal for optimization of NGS or dPCR clinical workflow. CRISPR/Cas9 engineered cell lines are biologically relevant materials and allow for a reproducible and renewable source of ctDNA reference standards for assay development and quality control.

#### ST059. Detection of *ALK*, *RET*, *ROS1* Rearrangements by NanoString in Brazilian Patients with Non-small Cell Lung Cancer

L. Novaes, F. de Paula, A. Evangelista, L. Mattos, P. De Marchi, C. Silva, L. Ferro, R.M. Reis

Barretos Cancer Hospital, Barretos Spaulo, Brazil.

**Introduction:** Non-small cell lung cancer (NSCLC) is the most common type of lung cancer with low survival rates. The presence of *ALK*, *RET*, or *ROS1* rearrangements in NSCLC patients guides treatment with targeted therapy (crizotinib) in the most advanced cases. The response to crizotinib in *ALK*- and *ROS1*-positive varies according to the fusion partners. The NanoString platform is useful to identify these rearrangements and to detect the fusion partner using routine collected samples such as formalin-fixed paraffin-embedded tissues (FFPE) showing a great potential as a diagnostic approach in the routine of personalized medicine. This study aims to evaluate the frequency of *ALK*, *RET*, and *ROS1* rearrangements and the potential of NanoString to be employed as a diagnosis method for detection of these rearrangements in a Brazilian patients with NSCLC. **Methods:** In a series of more 500 NSCLC patients diagnosed at Barretos Cancer Hospital, including 496 FFPE and 50 fresh-frozen tissue the NanoString platform was used to detect *ALK*, *RET* and *ROS1* rearrangements. The rearrangements were also validated by fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC). Moreover, the presence of rearrangements in all samples will be associated with clinicopathological features.

**Results:** All samples were previously tested for *EGFR* and *KRAS* mutations. Only cases wild type for *EGFR* and *KRAS* (n=270) were considered for NanoString analysis. Among fresh-frozen samples (n=50), 6 cases were positive for *ALK* rearrangements, including 3 cases with *ALK-MLL4* translocation and 3 cases with unknown partner. A subset (n=27) of FFPE samples were analyzed so far, with 5 positive cases for *ALK* rearrangements, which were confirmed by IHC. *RET* rearrangement (unknown partner) was observed in 1 case and 1 *ROS1* (*EZR-ROS1*partner) fusion was identified in 1 case. **Conclusion:** The NanoString platform is able to identify the *ALK*, *RET*, and *ROS1* rearrangements in both FFPE and fresh-frozen NSCLC samples. The identification of the fusion partners may result in better tailored therapy for Brazilian patients.

#### ST060. Characterization of the Tumor Microenvironment using a Novel High-plex Protein Imaging Technology

D.A. Hinerfeld, S.E. Church, A. Pavenko, A. White, A. Sullivan, S. Warren, J. Beechem

Nanostring Technologies, Seattle, WA.

**Introduction:** Spatial characterization of the immune contexture in the tumor microenvironment (TME) is essential for understanding the relationship between immune and tumor cells. However, it has proven difficult to perform such studies in a highly multiplexed manner using limited samples. To address this unmet need, we have developed an imaging and tissue-sampling platform designed to simultaneously analyze up to hundreds of tumor and immune proteins in distinct TME locations on a single formalin-fixed, paraffin-embedded (FFPE) tissue section. This novel technology, called Digital Spatial Profiling (DSP) was applied to the characterization of immune cold and inflamed Non-Small Cell Lung Cancer (NSCLC) tumors. **Methods:** Six FFPE NSCLC tumor specimens were subjected to DSP to determine the spatial expression of 30+ immune-related proteins. Following antigen retrieval, sections were stained with a cocktail of 30+ DNA barcoded antibodies in combination with fluorescently labeled antibodies (CD45, CD3, Pan-CK, DNA) which were used to define the immune-enriched regions of the tumor. Using the fluorescent signal as a guide, regions of interest (ROI) were delineated based on immune-enriched (CD45+), tumor-enriched (Pan-CK+) or mixed (both Pan-CK+ and CD45+). Each ROI was illuminated by UV excitation of the defined ROI, which releases the antibody-bound DNA barcodes for downstream quantitation on the NanoString nCounter System. Each sample was assessed with 24 ROIs, as well as, a sequential slide from the same sample with UV illumination of the entire slide as a comparison. **Results:** We show that analysis of distinct immune-enriched, tumor-enriched and tumor-immune mixed regions of NSCLC tumors provides unique immune contexture that is different than whole slide protein or RNA analysis. We also show distinct differences in immune and tumor protein expression in immune hot and cold tumors. This suggests DSP could ultimately be used to identify unique spatial biology and immune markers to identify prognostic and predictive biomarkers.

**Conclusions:** Using this novel approach, we demonstrate multiplexed protein analysis of defined regions of interest within a tumor enabling systematic interrogation of the immune status in the tumor microenvironment. We demonstrate the ability of this technology to reveal immune biology that can point to novel biomarkers or therapeutic targets.

#### ST061. GNAS Expression Improves Survival Prognostication of SHH Medulloblastoma Molecular Subgroup

L. Leal<sup>1</sup>, L. Sussuchi<sup>1</sup>, A. Feijó<sup>1</sup>, F. de Paula<sup>1</sup>, F. Saggiore<sup>2</sup>, J. Stavale<sup>3</sup>, S. Malheiros<sup>3</sup>, B. Mançano<sup>1</sup>, B. Luu<sup>4</sup>, L. Neder<sup>2</sup>, M. Taylor<sup>4</sup>, R. Reis<sup>1</sup>

<sup>1</sup>Barretos Cancer Hospital, Barretos, Brazil; <sup>2</sup>University of São Paulo, São Paulo, Brazil; <sup>3</sup>Universidade Federal De São Paulo, São Paulo, Brazil;

<sup>4</sup>Sick Kids, Toronto, Ontario, Canada.

**Introduction:** Medulloblastoma is the most frequent malignant brain tumor in children. Currently, four distinct medulloblastoma molecular subgroups have been identified: MB<sub>SHH</sub>, MB<sub>WNT</sub>, MB<sub>GRP3</sub> and MB<sub>GRP4</sub>. For medulloblastoma molecular classification, the NanoString is a high-throughput platform, highly sensitive, robust and useful for analysis of formalin-fixed, paraffin-embedded (FFPE) tissues. Although a 22-gene panel employing the NanoString technology was previously successfully developed for medulloblastoma molecular subgrouping, MB<sub>SHH</sub> may be sectioned into 2 distinctive subgroups according clinical and molecular characteristics. Our aim was to apply the 22-gene panel for medulloblastoma molecular subgrouping with further key cancer-related genes in order to improve classification and subclassification of Brazilian MB<sub>SHH</sub> using NanoString. **Methods:** FFPE samples from 131 medulloblastoma cases from four reference centers in Brazil were enrolled. Gene expression was assessed using the 22-gene panel previously developed by Dr. Taylor's group for medulloblastoma molecular subgrouping plus *GNAS*. Raw data was normalized by HK, followed by class prediction with Prediction Analysis of Microarrays (PAM). MB<sub>SHH</sub> subclassification was performed by *GNAS* expression. The molecular profile was associated with patients' clinical outcome. **Results:** The medulloblastoma cases were distributed into MB<sub>SHH</sub> (n=61), MB<sub>WNT</sub> (n=21), MB<sub>GRP4</sub> (n=26) and MB<sub>GRP3</sub> (n=23). The lowest and the



highest overall survival (OS) was found for MB<sub>GRP4</sub> and MB<sub>WNT</sub>, respectively (mean of OS was 118.4 and 158.6 months, respectively). High and low expression of the tumor suppressor gene *GNASIN* MB<sub>SHH</sub> cases was associated with better and worse OS ( $p=0.02$ ), respectively.

**Conclusions:** We implemented the NanoString platform for molecular classification of a medulloblastoma Brazilian series as an effective diagnostic tool for personalized medicine. The 22-gene panel for molecular classification of medulloblastoma associated with *GNAS* expression may improve the prognostication of survival in MB<sub>SHH</sub> patients.

#### ST062. Distinct Genetic Signature of Mucinous Micropapillary Breast Carcinoma from its Invasive Non-mucinous Counterpart

M. Gurav, O. Shetty, N. Gardi, S. Desai, T. Shet  
Tata Memorial Hospital, Mumbai, India.

**Introduction:** The present study is designed based on the concept that a subset of mucinous breast carcinoma is actually mucinous variant of the aggressive invasive micropapillary carcinoma (IMPC). The presence of mucin halts the extreme angioinvasion in IMPC and converts it into an indolent tumor viz: Mucinous micropapillary carcinoma (MuMPC). The aim of the study is to define whether these histologically similar entities IMPC and MuMPC harbor similar or distinct patterns of exome profile.

**Methods:** The study included 20 paired samples with 10 cases of each of IMPC and MuMPC (fresh frozen tumor tissue and corresponding peripheral blood). Paired end (150bp) whole Exome sequencing (WES) was performed on Illumina HiSeq 2500 platform with V4 chemistry and 100x average coverage. Raw paired end reads in FastQ format were aligned to hg19 reference genome using bwa mem (v.0.7.16a). Unusual flag information was removed using SAM tools fixmate (v.1.6-1) followed by reads sorting and duplicate removal using Picard tools (v.2.10.0). SAMtools mpileup (v.1.6-1) was used to locate non-reference positions in tumor and germline samples. Somatic variant calling was performed using VARSCAN2 somatic (v.2.4.3) and further annotated with latest version of ANNOVAR to restrict analysis to coding variants. The candidate genes are being validated using Sanger Sequencing, qPCR and immunohistochemical analysis. **Results:** On Somatic analysis, total of 253 and 352 variants were detected in IMPC and MuMPC respectively with 14 of these being common among the two subsets. IMPCs revealed mutational profile comparable to common breast carcinoma exhibiting high frequency of activating mutations of *PIK3CA* (4/10; 40%), *MAK3K* (3/10; 30%), *TP53* (2/10; 20%) along with 1 case *AKT1* mutations. Interestingly unlike IMPC cases, none of the MuMPC cases showed *PIK3CA* or *MAP3K* mutations. *GATA3* mutations (3/10; 30%) were specifically seen in MuMPC followed by mutations in *CCDC168*, *GPS2*, *THSD7A*, *SHC1*, *KMT2B*, *FAM47C* (2/10; 20% each). One case of MuMPC also showed mutations in *HDAC9* along with *HIST1H3G* and *HIST1H2BK* mutations. **Conclusions:** The above mutations indicate that IMPC and MuMPC share few mutations that are responsible for the micropapillary pattern. The absence of *PIK3CA* mutations and presence of *GATA3* mutations appear to be the event causing mucin production in micropapillary carcinomas. Investigation into these pathways that cause mucin production will offer us exciting avenues for therapy in future.

#### ST063. Development of a Next Generation Sequencing Panel for Glioma Classification

N. D'Haene, B. Meléndez, L. Lebrun, M. Remmelink, S. Rorive, O. Blanchard, N. De Nève, C. Van Campenhout, I. Salmon  
Erasme Hospital, Université libre de Bruxelles, Brussels, Belgium.

**Introduction:** The 2016 updated World Health Organization classification system of gliomas integrates molecular parameters and histology to define tumor entities and to provide a more accurate diagnostic and prognostic information to the previous histology-based classification. The increasing number of markers and tests to be performed for a correct diagnostic procedure makes gene-targeted next generation sequencing (NGS) a powerful tool in routine pathology practice. This is a cost-effective and sensitive approach for detecting multiple genetic alterations from a small amount of formalin-fixed, paraffin-embedded (FFPE)-extracted DNAs. We designed and validated a 14 gene-NGS panel for detecting mutations and copy number variations in gliomas, including a reliable method for

detecting the 1p/19q codeletion. **Methods:** For validation of the NGS glioma panel we used 52 glioma samples, 2 reference mutated DNAs and 12 non-tumor samples. The panel was implemented in our institution and prospectively applied to 52 samples. NGS was performed by using the Ion Torrent AmpliSeq technology. The Cancer Hotspot Panel (CHP) was used to validate the mutations identified in glioma samples. The criteria defined for detection of 1p/19q codeletion by NGS was based on loss of heterozygosity analyses of single nucleotide polymorphisms. *In situ* hybridization (ISH) techniques were used to validate 1p/19q codeletion or *EGFR* amplification. **Results:** A specificity of 100% and a sensitivity of 94.5% was achieved for mutation detection, with a good correlation for the allelic frequencies between our glioma panel and the CHP. The 1p/19q codeletion showed 100% of specificity and was validated in all except one case (17/18, sensitivity of 94.4%). This exceptional case showed two different populations, as showed by fluorescent ISH. *EGFR* amplification was reliably detected by NGS and the results were validated by chromogenic ISH. **Conclusions:** Our glioma NGS panel is an accurate and sensitive method to detect mutations and copy number alterations including the 1p/19q codeletion in gliomas, in a rapid and cost-effective approach, allowing the correct classification of gliomas.

#### ST064. Mutational Profiling in Advanced Non-small Cell Lung Cancer (NSCLC) Patients: A Tertiary Care Study of 1,052 Cases from Eastern India

P. Gupta, K. Saha, S. Vinarkar, S. Banerjee, P. Santra, S.S. Chowdhury, B. Pal, R.K. Srimali, B. Biswas, D. Dabkara, S. Ganguly, G. Mukherjee, D. Midha, M. Parihar, N. Arora, D.K. Mishra  
Tata Medical Center, Kolkata West Bengal, India.

**Introduction:** Lung cancer is one of the most common cause of mortality all over the world. The Globocan estimate of lung cancer in India with an age standardized incidence rate is 6.9 per 100,000 of our population. NSCLC is the most common subtype of Lung cancer. With the availability of targeted therapy drugs, the survival in cancer has increased from a median overall survival of 11 months to an overall 5 year survival rate of 17.8% over the last 10 years. Due to these advancements, molecular testing in lung cancer patients has become mandatory and is a part of all treatment guidelines. **Methods** Retrospective data was compiled of 1,052 formalin-fixed, paraffin-embedded (FFPE) samples from 1017 NSCLC patients (including 35 rebiopsy, 100 cytology samples) referred for *EGFR* mutation analysis in the Molecular Genetics department at Tata Medical Center, Kolkata, India over a period of three years. These cases were evaluated using Sanger Sequencing (n=499), real time Therascreen V2 PCR Kit/Diatech kits (n=384) and AmpliSeq Cancer Hotspot Panel V2 on the Ion Torrent platform (n=169). The main objective of this study was to study the incidence of *EGFR* mutations in our patients and also to analyze the additional mutational status using 50 gene next generation sequencing (NGS) panel. All the NSCLC cases were classified according to the WHO 2015 classification. **Results:** Mean age of the patients in our study was 55.9 years with a M:F ratio of 2.73 :1 (745 : 272). Overall, of the 1,017 diagnostic samples evaluated, 29.2 % (297) cases were *EGFR* mutated (28.5% cases on Sanger sequencing (141/494), 28.3 % by Real time PCR (102/360) and 33.1% by NGS (54/163). The most common mutation seen was deletion 19 (n=160; 53.8%) followed by L858R in exon 21 (n=89; 29.9%), G719X (n=13), insertion 20 (n=11), S768I (n=6), T790M (n=4) and other rare mutations (n=14). Double mutations were found to be in 18 cases with L858R mutation being the commonest to occur (n=9). 163 diagnostic cases were analyzed by next generation sequencing amongst which the most common mutation was found to be *TP53* (38.7%) followed by *EGFR* (33.1%), *KRAS* (17.5%), *KIT* (5.5%), *CDKN2A* (5.5%), *CTNNB1* (4.9%), *ERBB2* (3.06 %), *BRAF* (3.06%), *STK11* (2.4%), *MET* (2.4%), *PIK3CA* (1.8%), *PTEN* (1.2%), *IDH1* (1.2%), *APC*, *RAS*, and *NRAS* 0.06% each. There were 35 patients who were analyzed on rebiopsy in which T790M mutation was positive in 57.1% (n=20). **Conclusion:** *EGFR* is found to be frequently mutated in the Eastern subgroup of Indian patients. Mutational profiling of patients leads to personalized care and treatment in such patients which leads to improved survival. Next generation sequencing allows parallel sequencing of multiple genes which can lead to availability of complete molecular profile of the patient in shorter time and better sensitivity.

# ST065. Optimization of a Next Generation Sequencing Panel to Reduce DNA Input and Neoplastic Content Requirements

M. Soucy, K. Kelly, B. Sisson, G. Ananda, A. Hesse, H. Reddi  
The Jackson Laboratory, Farmington, CT.

**Introduction:** The use of next generation sequencing (NGS) technologies has rapidly made its way into the clinical laboratory setting especially focusing on cancer. Here, we evaluated the impact of implementing a reduction of DNA input from 200ng to 50ng as well as a reduction of our neoplastic content requirement from 50% to 30% tumor into our current workflow for a laboratory-developed clinically validated NGS assay, ActionSeq. **Methods:** For the neoplastic reduction evaluation, previously reported samples with known neoplastic content at 50% were titrated with a well characterized tumor normal control NA12878 to create representative samples at varying neoplastic contents (45%, 30%, 25%, etc.) In total, 22 samples with varying neoplastic contents were compared to the 8 samples originally processed at 50% for variant concordance. In addition to these samples, 30 known samples previously processed and analyzed at 200ng were prepped and re-sequenced at an input of 50ng. Concordance was assessed between the two preparations. Optimizations for lower input concentration into the assay included increasing pre-cap PCR to 6 cycles with an hour of adapter ligation to ensure sufficient yield to proceed into the hybridization capture. **Results:** Results for the comparative analysis between the samples originally prepped at 200ng to 50ng showed a concordance of 96.7% when looking at all detected variants and a 100% concordance when looking at clinically reported variants. **Conclusions:** Based on the success of the validation, the reduction of DNA concentration and neoplastic content was implemented into our ActionSeq protocol. By lowering DNA input concentration and neoplastic content, we are able to accommodate samples, which yield lower DNA input and adapt to the industry's standards. While the sensitivity of the assay increased, we did observe a slight decrease in specificity, but over our established cut-off of 96%. The ActionSeq Panel will continue to be an essential tool to be offered in our clinical genomics laboratory.

# ST066. Genetically Defined Subgrouping of Medulloblastomas; A Comparative Study of Real-time PCR and Nanostring Technology Based Gene Expression

S. Epari, M. Gurav, D. Dhanavade, V. Kadam, P. Gogte, M. Anolkar, O. Shetty  
Tata Memorial Centre, Mumbai, India.

**Introduction:** Various molecular methods have been utilized for classifying medulloblastoma (MB) into genetically defined groups. This is a comparative study of two different methods of gene expression based molecular subgrouping of MBs. **Methods:** Formalin fixed paraffin embedded tumor samples of clinicohistologically diagnosed cases of MBs were further classified into molecular subgroups by 2 techniques viz. Taqman based quantitative real time PCR (qRT-PCR) gene expression profiling (based on customized panel of 12 genes) and Nanostring nCounter technology (based on 26 genes customized panel). The molecular subtype was determined based on gene expression levels of each gene compared with reference gene/s, which was measured using relative quantification (RQ) method. The data was analyzed using Expression Suite software v1.1 and nSolver analysis software 4.0 for qRT-PCR and nCounter technology respectively. The data from both these methods were evaluated for concordance. **Results:** One hundred thirty five cases formed study group, 72 were of classic histology; 17 desmoplastic variant, 13 large cell/anaplastic variant and 3 were of extensive nodularity (MBEN = 3). The rest (n = 30) of the cases were classified as MB-NOS; of which 17 showed definite pattern (13 were paucinodular variant, 3 were with myogenic differentiation and interestingly one showed ganglionic differentiation) and other 13 were truly NOS. Based on the qRT-PCR, cases were classified into 24 WNT-activated, 38 SHH-activated and 73 non-WNT/non-SHH [21 group 3, 45 group 4 and 7 could not be classified further]. Most of the classic histological MB cases showed mixed molecular subgroups with group 4 (40.3%) followed by WNT (26.4%), Group 3 (16.7%), SHH (12.5%) and non-WNT/non-SHH, NOS (4.2%). All except 1 case of histological desmoplastic subtype were of SHH-activated and one was group 4; while 38% cases of large cell/anaplastic MB were group 3 and SHH-

activated each; while 7.8% were of WNT, group 4 and non-WNT/non-SHH type, NOS each respectively. All 3 cases of MBEN were SHH activated. Out of 135 cases evaluated by qRT-PCR, 33 cases (WNT: 7, SHH:5, group 3:10, group 4:9 and non-WNT/non-SHH:2) were analyzed and classified using nCounter technology based MB subtype specific gene signature of 26 genes; rest of the cases are being evaluated. Of these 33 cases, 30 showed concordant molecular subgrouping on qRT-PCR and nCounter based molecular classification whereas 3 (9%) cases were discordant. Two non-WNT/SHH-Group 4 cases and 1 WNT case on qRT-PCR method were classified as group 3 by nCounter technology.

**Conclusion:** The study is ongoing and the final results are awaited.

# ST067. Microsatellite Instability Testing on Solid Tumors

L. Cai, E. Kallam, L. Sayblack, S. Hood, P. Finbow, Y. Mckenzie, M. Eisenberg, A. Chenn, L. Kam-Morgan  
Laboratory Corporation of America Holdings, Durham, NC.

**Introduction:** Deficiency in mismatch repair occurs in approximately 15-20% of sporadic colorectal and endometrial cancers, and to a lesser degree in a variety of other solid tumors. Mismatch repair deficiencies have been associated with disease prognosis and been shown to be linked with potential response to the checkpoint inhibitor Pembrolizumab (KEYTRUDA) in various solid tumors. Defects in the mismatch repair process can be identified by a number of laboratory procedures including immunohistochemical methods to assess expression of the mismatch repair (MMR) proteins (MLH1, MSH2, MSH6 and PMS 2), or by using PCR methods to assess replication fidelity of specific microsatellite sequences in the genome. Tumor samples are indicated to be deficient in MMR when one or more the MMR proteins are not expressed, or to have high levels of microsatellite instability (MSI-H). In this study, the clinical and analytical performance features of Promega MSI assay are evaluated. **Methods:** Genomic DNA was isolated from both tumor and normal specimen of the same individual. Multiplex polymerase chain reaction analysis was used to amplify the BAT-25, BAT-26, NR-21, NR-24 and MONO-27 loci. The fluorescent amplification products were analyzed by capillary electrophoresis and amplification patterns for normal and tumor tissue compared for the detection of novel fragments in tumor DNA, indicative of microsatellite instability. Assay's accuracy, repeatability, reproducibility, analytical sensitivity and stability were evaluated. **Results:** Of the specimens tested during validation, 19 formalin-fixed, paraffin-embedded (FFPE) specimens with known MSI results that were 100% concordant. Repeatability (intra-assay precision) and reproducibility (inter-assay precision) were 100%. This assay can detect 8-12% of mutant in a background of wild-type genomic DNA. The DNA stored at 2-8°C was stable for at least 4 weeks and the sectioned FFPE slides stored at room temperature were stable for at least 1 year. The Promega MSI assay has been offered as a clinical test based on the successful performance features. In a set of 1650 colorectal cancer clinical specimens, 15% having MSI-H, 85% MSI stable; 211 endometrial cancer specimens, 31% having MSI-H, 69% MSI stable and 231 other solid tumor specimens, 5% having MSI-H, 95% MSI stable. Results could not be obtained in 7.4% specimens. This was mainly due to a low amount of amplifiable DNA obtained from specimens. **Conclusions:** The Promega MSI assay is a robust, reproducible and sensitive assay using FFPE specimens for mismatch repair deficiency assessment.

# ST068. Development of whole transcriptome sequencing (RNASeq) for the Detection of Clinically Actionable Gene Fusions from FFPE Solid Tumor Biopsies

D. Bergeron, K. Kelly, H. Chandok, G. Ananda, H. Reddi  
The Jackson Laboratory, Farmington, CT.

**Introduction:** Whole transcriptome or RNA sequencing (RNAseq) currently stands as one of the most powerful tools used in the realm of transcriptomics, yielding great insight into how gene expression relates to disease status. In the clinical laboratory, modern technology and innovative approaches are being coupled to bring transcriptome analysis from benchtop to bedside. One such instance involves using RNAseq in the detection of gene fusions, somatic aberrations highly correlated with tumor phenotype accounting for about 20 percent of human cancer morbidity. Harnessing RNAseq derived fusion data can serve to aid physicians in stratifying cancer risk as well as offering targets for anti-

cancer therapeutics. Here we describe the development and assessment of an in-house RNAseq-based assay for detection of a larger number of gene fusions in solid tumors as an upgrade to our current 53 gene panel.

**Methods:** Thirteen formalin-fixed, paraffin-embedded (FFPE) tumor samples with known fusion status were selected, among which included both cell lines and synthetic controls. Libraries were prepared from purified RNA using the KAPA RNA HyperPrep Kit with RiboErase (HMR) and were sequenced using the Illumina NextSeq. Development and optimizations efforts were concerted on adjusting shearing and PCR conditions as well as altering sequencing loading parameters. Sequencing data was processed through multiple bioinformatic pipelines including SOAPfuse, FusionCatcher, and EricScript. Samples were subjected to clinical quality control (QC) monitoring through the entire testing process.

**Results:** Evaluation of JAX QC metrics revealed that all 13 samples prepped using the developed protocol passed clinical quality cut-offs for library preparation, sequencing, and bioinformatics analysis. This RNAseq approach was successful in identifying all 20 clinically actionable fusions present across each of the different sample types as well as calling multiple fusions within the same sample and variants of unknown significance. Further, when comparing these results against the FusionSeq panel, the RNAseq libraries using our optimized approach were more robust and significantly less expensive to process.

**Conclusions:** Our results demonstrate that an RNAseq-based approach for fusion detection is on par if not superior to commercially offered assays. Our RNAseq libraries produced high quality data ready for interpretation and clinician use. This optimized RNAseq approach allows physicians to discover novel treatment options for their patients and with reduced costing on the front end, more patients can ultimately gain access to this testing. With gene fusions implicated in a variety of cancer types, offering high quality, low cost molecular testing services will serve to best benefit the patient and advance personalized medicine.

#### ST069. Molecular Profiling of Adult Diffuse Gliomas Without 1p19q Co-deletion, IDH and TERT Promoter Mutations Reveals Abundance of TP53 and NF1 Mutations and Additional Chromosome Rearrangements

C.J. Zepeda Mendoza, C.M. Ida, C.E. Praska, J.R. Balcom, J. Parisi, M.E. Jentoft, K.B. Geiersbach, A. Raghunathan, C. Giannini, B.R. Kipp, R.B. Jenkins

Mayo Clinic, Rochester, MN.

**Introduction:** The 2016 World Health Organization (WHO) Classification of Tumors of the Central Nervous System classifies adult diffuse gliomas (ADGs) based on histological and molecular parameters. Mutations in *IDH1* and *IDH2* (*IDH*) as well as 1p/19q co-deletion are the primary genetic markers used in the classification algorithm. *TERT* promoter mutations have been identified as an additional genetic marker that impacts prognosis and further refines ADG classification. ADG lacking 1p/19q co-deletion, and *IDH* and *TERT* promoter mutations (so-called "triple negative"), however, have not been fully characterized.

**Methods:** In this study, we profiled 76 "triple negative" ADG (i.e. *IDH* wild-type, *TERT* wild-type, and intact 1p/19q) within a one-year period using a clinically-validated next generation sequencing (NGS) assay targeting 50 ADG-associated genes. **Results:** Forty-five of the 76 (59%) triple negative ADGs were WHO grade IV, while 24 (32%) were WHO grade III, and only seven (9%) were WHO grade II, with a median age at diagnosis of 44 years. Nine of the 76 triple negative ADGs (11.8%) had no detected mutations, 29 (38.1%) had a single mutation, 21 (27.6%) had two mutations, and only 17 cases (22.4%) had 3-6 mutations. We observed a few emerging potential subgroups of "triple negative" ADGs, including cases with mutations limited to selected genes: *EGFR*-only, *FGFR1*-only, *TP53*-only, and *TP53-NF1*-only. *TP53* mutations were detected in 43.4% of triple negative ADGs, and *NF1* mutations in 31.5%, a significant enrichment compared to *IDH* wild-type and *TERT* promoter-mutant gliomas collected on the same period (30% vs. 17.3%, respectively,  $p=0.04$ ). Given that 77.5% of the cases had zero, one or only two mutations, additional genomic events were expected to contribute to tumorigenesis in these ADGs. Indeed, chromosomal microarray analysis of 25 (33%) triple negative ADGs revealed the occurrence of diverse chromosome aberrations, including whole chromosome gains and losses (+7, -10) and partial losses of chromosome arms (9p-, 12q-, 19q-).

**Conclusion:** Overall, our analysis of triple negative ADGs shows potential additional sub-grouping of ADG, which may offer new avenues for refining the classification of this group of ADGs.

#### ST070. Clinical Validation of a Fusion Transcript Next-generation Sequencing (NGS) Panel for Sarcomas and Solid Tumors with Diagnostic, Prognostic and Therapeutic Value

R. Paolillo<sup>1</sup>, A.R. Oran<sup>1</sup>, D.B. Lieberman<sup>2</sup>, J.N. Rosenbaum<sup>2</sup>, R.T. Sussman<sup>3</sup>

<sup>1</sup>University of Pennsylvania, Philadelphia, PA; <sup>2</sup>University of Pennsylvania Perelman School of Medicine, Philadelphia, PA; <sup>3</sup>University of Pennsylvania Health System, Philadelphia, PA.

**Introduction:** Sarcomas are malignant mesenchymal tumors arising from soft tissue and bone, with over 100 different histologic subtypes. Accurate diagnosis directly affects prognosis and treatment strategy. Approximately 25% of sarcomas are defined by chromosomal translocations.

Fluorescence *in situ* hybridization (FISH) is the current gold standard and most commonly used method for detection of solid tumor and sarcoma fusion transcripts on formalin-fixed, paraffin-embedded (FFPE) or fresh tissues. FISH is difficult to multiplex, and often requires a known fusion partner. These are significant limitations for a diagnostic assay designed for sarcoma, as multiple targets may need to be interrogated, and rearrangements are often complex or involve novel partners. Anchored-Multiplex PCR (AMP)-based next-generation sequencing (NGS) assays directly detect oncogenic fusion transcripts, and offer a clinically viable alternative to FISH. Here, we present the clinical validation of a custom 56-gene NGS fusion transcript panel (FTP) for solid tumors and sarcomas. **Methods:** This FTP uses AMP, with anchored gene-specific and universal primers to detect fusion partners and breakpoints with therapeutic, diagnostic and/or prognostic value. Total Nucleic Acid (TNA) from a total of 63 unique tumor specimens (20 sarcoma, 21 lung adenocarcinomas, 18 gliomas, 3 inflammatory myofibroblastic tumors and 1 prostate tumor) are included in the validation to assess the accuracy, precision, lower limits of detection (LLOD), linear dynamic range, sensitivity and specificity of the assay. All validation samples were previously characterized by either FISH or by a previously clinically validated 18-gene fusion transcript panels. In addition, 12 unknown specimens were run to enrich our validation dataset. **Results:** Of the 63 samples tested, 62 passed sequencing quality control. Of these, 42 samples harbored known rearrangements, and 20 were known negatives. Only a single sample that was positive by FISH was called negative by the panel (1 discordant case), resulting in an overall sensitivity of 98%. A probit analysis was used to determine the LLOD of 10% tumor cellularity and 25 ng total TNA input with 95% confidence. The panel performs reproducibly, evaluated with five samples that were repeated 3 times by the same technologist and 3 samples that were repeated by 3 independent technologists. **Conclusion:** The validated NGS-based Fusion Translocation Panel performs with high accuracy, precision, sensitivity and specificity. This assay provides a multiplexed alternative to FISH and single-gene testing for the detection of fusion transcripts for sarcomas and solid tumors.

#### ST071. Precise Characterization of an FFPE Block Developed Using a Mixture of CRISPR/Cas9 Engineered Cell Lines for use as a Molecular Reference Standard

V. Mani, Q. Zheng, D. Sengupta, S. Kondapalli, S. Thakker, G. Roma, S. Sadder

AccuRef Diagnostics, Milpitas, CA.

**Introduction:** Human cancers display intra-tumor heterogeneity in which hundreds of genes, and thousands of gene mutations, have been implicated in oncogenesis. With the ability of Next-Generation Sequencing (NGS) to detect multiple oncogenes simultaneously has enabled the era of precision tumor profiling. Due to the complexity of NGS detection and the deleterious effects of formalin on tissue specimens, there is a significant need for formalin-fixed paraffin embedded (FFPE) molecular reference standards. It can be employed for assay development, quality assurance to validate assay performance and understand cross-site and/or inter-operator reproducibility. FFPE cell blocks and derivative products are thus ideal reference standards for this application, since they represent a biologically-relevant, reproducible, and



renewable source of reference materials. Here we have developed a quantitative multiplex FFPE cell block containing a mixture of 10 CRISPR/Cas9 engineered cell lines featuring oncogenic mutations across multiple genes, including *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, *NRAS*, *HRAS*, and *KIT*. **Methods:** Using CRISPR/Cas9 desired mutations were created and confirmed by Sanger sequencing. The FFPE block was prepared by mixing the cell line with wild-type cells at pre-defined allelic frequencies, treated with neutral-buffered formalin and embedded in paraffin. The block was sectioned into FFPE cell scrolls at 20µm thickness. Inter-block consistency was tested from the top, middle, and bottom of the block by digital imaging. H&E staining was performed for histological. For genetic analysis, gDNA from FFPE cell scrolls were extracted using the automatic platform and allelic frequencies were verified by dPCR and targeted NGS. **Results:** Engineered, homozygous clones were identified by Sanger sequencing. The derivative FFPE cell block showed a high degree of homogeneity by H&E staining of slide-mounted specimens, with area > 60% cell coverage. The 20µm scrolls consistently produced >400 ng of total extractable gDNA, with inter-block variabilities of < 10%. Allelic frequencies were highly reproducible, falling within 20% of the targeted ratio. Variants detected by NGS were consistent with dPCR results. Extracted gDNA from untreated cell pellets showed higher allelic frequency percentages when compared to FFPE treated gDNA. **Conclusion:** CRISPR/Cas9 is an enabling tool for the generation of precision edited cell lines. These engineered cell lines can be reproducibly incorporated into FFPE-mimetic specimens (i.e. blocks), and represent an ideal source of molecular reference materials, particularly for difficult to find oncogenic variants. FFPE reference standard enable the optimization of the FFPE extraction pre-analytical workflow.

#### ST072. Detection of IDH Mutations by DNA Sequencing and Immunohistochemistry in Diffuse Gliomas

P. Dileep Menon, K. Vadlamudi, H. Fan, Y. Wang, S. Pandeswara, A.R. Gilbert

University of Texas Health San Antonio, San Antonio, TX.

**Introduction:** Isocitrate dehydrogenase (IDH) mutations, *IDH1* or *IDH2*, confer significant diagnostic and prognostic implications for patients with diffuse gliomas, the most common primary brain tumor in adults. In this study, we seek to compare the effectiveness of two methodologies used for detection of IDH alterations in diffuse gliomas: direct Sanger sequencing and immunohistochemistry (IHC) targeting the *IDH1* R132H mutant protein. **Methods:** Diffuse glioma cases from 2000-2017 diagnosed in young adults ranging from 18-60 years old were collected via hospital database search. DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue (each case with >50% tumor burden) using the EZ1 DNA Tissue Kit (Qiagen) and amplified by PCR primers targeting the *IDH1* codon 132 and *IDH2* codon 172 regions. Sanger sequencing using BigDye Terminator Cycle Sequencing Kit on the ABI3130xl Genetic Analyzer (Applied Biosystems) was performed and results were analyzed using DNASTar Lasergene 10 software (DNASTAR, Inc). For the IHC method, FFPE tissue sections were stained with an Anti-IDH1 R132H mouse monoclonal antibody (Clone H09; Dianova; Hamburg, Germany) using the BenchMark ULTRA stainer. **Results:** A total of 40 cases with available FFPE blocks were first Sanger sequenced, but 32.5% (13/40) of samples failed molecular analysis due to poor DNA quality and/or quantity likely related to archived FFPE block age (blocks were collected from years 2000 to 2005). IHC testing was then performed on the remaining 27 cases with successful sequencing data. IDH mutations were found in 89% (24/27) of cases by both methods. Sequencing identified 21 cases with *IDH1* mutation (including 19 with R132H, and one each with R132L and R132S mutations) and 2 cases with *IDH2* mutations (R172M and R172K); an additional positive case was detected by IHC method only. Among the 19 tumors harboring *IDH1* R132H (c.395G>A) mutation, positive results were consistently detected by both sequencing and IHC methods. Interestingly, one case with the *IDH1* R132L (c.395G>T) mutation was also detected by the *IDH1* R132H specific antibody. **Conclusion:** Analysis of diffuse gliomas from young adult patients showed that sequencing is superior to IHC for identifying noncanonical IDH mutations, but IHC is a sensitive method for detecting *IDH1* R132H mutant cases. Our data indicate that the R132H antibody can cross-react with the R132L mutant protein, a rarely reported

observation. Most FFPE blocks collected over 13 years prior to analysis failed sequencing attempts, suggesting that IHC may be a superior testing option in degraded or aged tissue samples – an issue that, to our knowledge, has not been addressed in the English literature and thereby warrants further investigation.

#### ST073. Comprehensive and Sensitive Detection of Somatic Mutations for Monitoring Minimal Residual Disease

S. Sankaran, P. Agrawal, M. Parulekar, A. Hariharan, D. Awasthy, A. Shanmugam, S. Gupta, V. Vittal, A. Ravichandran, S. Katragadda, U. Bahadur, V. Gupta, V. Veeramachaneni  
Strand Lifesciences Pvt. Ltd, Bangalore, India.

**Introduction:** There is growing evidence that somatic mutations in cfDNA extracted from peripheral blood plasma (liquid biopsy) can be used for evaluating Minimal Residual Disease (MRD) as well as response to treatment in different cancer types. The MRD workflow involves testing in two phases: 1) profiling of the tumor tissue and selection of somatic variant(s) that can be used for tracking, and 2) tracking the variant at a later time point using a liquid biopsy. **Methods:** We evaluated different technologies and assays that would be suitable for developing a monitoring product. For evaluation, the data from >200 solid tumor samples across various tumors tested on our comprehensive StrandAdvantage 152 gene somatic cancer panel was analyzed. The overall positive detection rate using the 152 gene panel was >80% comprising of both single nucleotide variants across 36 genes and copy number variants. Loss-of-function *TP53* variants were detected in >55% of cases. On comparison of different commercially available cancer hotspot panels, the Swift 56G panel covered the majority of single nucleotide variants detected (>95%) with a positive detection rate of 79%, covering all the detected *TP53* variants. The added advantage of this panel was the ability to use it on both the formalin-fixed, paraffin-embedded (FFPE) tissue as well as liquid biopsy samples. The 56G Swift panel was validated in our lab using both FFPE tissue as well as blood using characterized control and clinical samples with an input DNA requirement of as low as 20 ng. **Results:** We could achieve 92% sensitivity and 100% specificity with a limit of detection (LOD) of 3% for FFPE tissues. The clinical samples analyzed showed >90% concordance when compared with data from runs using the in-house validated TruSeq Cancer Amplicon Panel (Illumina). The sensitivity, specificity and LOD for liquid biopsy samples were 93.9%, 99.9% and 1.0 % respectively. The LOD for the Liquid Biopsy test was further improved to 0.5% using the noise reduction model for all Cosmic high frequency variants. **Conclusions:** In summary, we have validated a protocol for monitoring residual disease that works well with low sample amounts, across both FFPE tissue and blood samples.

#### ST074. Quality Before Input: Validation of a NGS Assay with Respect to Input and Degradation

R. Paolillo<sup>1</sup>, R.T. Sussman<sup>2</sup>, A.R. Oran<sup>1</sup>, D.B. Lieberman<sup>2</sup>, A. Bigdeli<sup>2</sup>, A. Chitturi<sup>2</sup>, J.J. Morissette<sup>1</sup>

<sup>1</sup>University of Pennsylvania Perelman School of Medicine, Philadelphia, PA; <sup>2</sup>University of Pennsylvania Health System, Philadelphia, PA.

**Introduction:** Targeted next generation sequencing (NGS) has become an integral part of clinical cancer prognosis and therapy guidance. However, small specimens such as needle biopsies often yield insufficient DNA for NGS testing. The DNA from some specimen types, such as formalin-fixed paraffin-embedded tissues, can become deaminated in response to fixation and/or fragmented, reducing the amount of amplifiable DNA templates available. Here we present the preliminary validation data of a custom amplicon-based NGS panel designed for the rapid detection of essential variants in low quantity and poor quality specimens. **Methods:** We designed a customized Accel-Amplicon (Swift Biosciences, Inc.) panel targeting hotspots from 59 genes relevant to therapy selection and resistance as well as the full coding region of *TP53*. To assess the reproducibility and lower limit of input for this assay, we used 4 Horizon reference standards (HDx) with 11 verified variants and variant allele frequencies (VAFs). These HDx had varying DNA quality (uncompromised genomic DNA, mildly, moderately and severely formalin compromised) and were tested at decreasing DNA inputs (25, 10, 5 and 1ng), in triplicate. The detected VAFs were correlated with the reported

VAFs. Analytical sensitivity was evaluated by diluting these 4 HDx into normal DNA of similar quality (50% spike in) for single nucleotide variants and insertions/deletions. **Results:** The assay was able to detect all the 11 variants under all conditions at 25ng of DNA input with high VAF correlation ( $R^2 > 0.95$ ). All variants were also detected in the mildly and moderately formalin compromised HDx at both 10 and 5ng DNA input, with high VAF correlation ( $R^2 > 0.95$ ). In all 25ng input HDx and the 10 and 5ng input HDx for mildly and moderately compromised, variants with VAFs as low as 1 or 2% were detected. The moderately compromised HDx did not perform as well when only 1ng of DNA was input into the assay, with VAFs at 1 and 2% dropping out, and a lower VAF correlation ( $R^2 < 0.85$ ), while VAFs in the severely compromised HDx did not correlate with the expected VAFs at any input under 25ng. Together, these data suggest that the lower limit of input for this assay is dependent on DNA quality. VAFs diluted properly in the 50% spiked triplicates overall, with no variants dropping out. A probit analysis was used to determine the assay's lower limit of detection with 95% confidence. **Conclusion:** Here we focus on a critical aspect of the validation of a 60-gene amplicon NGS panel. The assay is able to perform with little variance when as little as 1ng of high quality DNA is input. These data highlight how DNA quality affects the minimum amount of DNA required to reliably detect rare somatic variants in low quantity and/or poor quality specimens.

#### ST075. Molecular Genetic Profiling of Gliomas in Routine Clinical Practice

E. Hughes, R. Barney, S.J. Deharvengt, J. Kilborn, D. Green, J. Peterson, A. Erskine, L. Tafe, J. Lefferts, G.J. Tsongalis, F.B. de Abreu  
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

**Introduction:** The majority of malignant brain tumors in adults are gliomas, derived from neoplastic glial cells, or neuroglia. Gliomas are classified by the World Health Organization (WHO) as astrocytoma, oligodendroglioma, mixed oligoastrocytoma, and ependymoma. Glioblastoma (GBM), occurs in over half of gliomas and is one of the most difficult cancers to treat, with a 5-year survival rate of 5% and a US incidence of 12-13 thousand per year. Gliomas can also be divided into separate molecular subtypes that correlate with biological etiology, prognosis, and therapeutic response, thus it is important for patient care to correctly identify both molecular and histological subtypes. Here, we share our experience with tumor genetic testing, including somatic DNA mutation, methylation, and copy number analysis as part of routine clinical practice in the care of our glioma patients. **Methods:** Over the past 24 months, DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue sections of 139 gliomas using the AllPrep DNA/RNA FFPE Kit. Genomic profiling was performed using the Ion AmpliSeq Cancer Hotspot Panel v2 for mutation analysis, Affymetrix OncoScan for CNV analysis, and EpiTect MethyLight PCR for MGMT promoter methylation analysis. **Results:** Over a two year period, 139 gliomas were assessed by traditional histopathology, immunohistochemistry, and genetic testing inclusive of DNA mutation analysis, MGMT promoter methylation status, and CNV analysis. In line with expected incidence, 81 (58%) patients were diagnosed as GBM, 16 (12%) patients as either anaplastic astrocytomas or oligodendroglioma, WHO grade III, 24 (17%) patients low-grade astrocytomas or oligodendrogliomas WHO grade II, and 10 (7%) patients as low grade gliomas including ependymoma and pilocytic astrocytoma, WHO grade I. Six (4%) patients were classified as grade III/IV and one patient as grade I/II. Thirteen (9%) patients were of the 'triple positive' molecular subtype that includes the prognostically favorable combination of 1p/19q co-deletion, *IDH1* mutation, and methylated *MGMT* promoter. DNA testing showed that 39 (28%) patients contained a clinically actionable variant in either *CDKN2A*, *IDH1*, *IDH2*, *PIK3CA*, or *SMO*, while a further 60 (43%) had variants with prognostic and diagnostic value. **Conclusions:** Molecular testing provides a high level of patient care for our glioma cohort, allowing categorization into diagnostic molecular subtypes that better predict response to clinical intervention and provides more accurate prognostic value. Over 70% of the gliomas tested had a somatic variant associated with diagnosis, prognosis or therapeutic selection. We encourage and support the continued development of advanced technologies to improve the accuracy of molecular stratification of glioma.

#### ST076. Clinical Validation of *MLH1* Promoter Methylation Testing using the High-throughput MethylationEPIC (850k) Array Platform

J. Benhamida, M. Arcila, T. Bale, K. Nafa, J. Sadowska, L. Villafania  
Memorial Sloan Kettering Cancer Center, New York City, NY.

**Introduction:** Methylation of the *MLH1* promoter is an important biomarker in mismatch repair defective tumors that is traditionally tested by single gene amplification assays. New high-throughput platforms provide the ability to profile genome-wide methylation events. In this study, we demonstrate the feasibility of assaying *MLH1* promoter methylation using the methylationEPIC (850k) platform. **Methods:** Our cohort consisted of 20 cases (N=10 positive and negative *MLH1* samples). The reference assay was a pyrosequencing-based assay that interrogates five *MLH1* promoter CpG loci (in-house assay). For the 850k platform, 250ng of sample DNA was processed and analyzed on the Illumina iScan. The platform assays 866,836 cytosine loci across the genome. We based our *MLH1* assay on four CpG loci within a region associated with *MLH1* repression. Data analysis and normalization was performed with R using the Minfi package. For each locus, the platform measures a methylated signal and unmethylated signal based on the relative amount of cytosine and thymine at the locus (bisulfite conversion effect). Methylation levels were quantified with beta values (the ratio of the methylated signal to total signal) and probe-specific methylation cutoffs were defined using dilutions of a positive formalin-fixed, paraffin-embedded (FFPE) sample. Separation between the beta values of the negative sample and 6.25% dilution sample was observed, suggesting an analytic sensitivity of 5-10%. Probe-specific cutoffs were defined to separate the beta values of the negative sample from the beta values of the 6.25% dilution sample. This method was chosen to ensure that the assay captures all methylation levels  $\geq 10\%$ . Positive and negative *MLH1* promoter methylation was defined as all four probes being above or below their probe-specific cutoffs, respectively. A finding of one to three positive probes was designated as indeterminate. Intra- and inter-assay reproducibility was performed using three positive and three negative samples. Limit of detection was assessed using input DNA amounts between 250ng and 25ng. **Results:** Using the probe-specific cutoffs and criteria for positivity, the assay demonstrated 100% sensitivity and specificity on the 20 samples tested. Inter- and intra-assay reproducibility demonstrated minimal variance across samples. One replicate (a sample at the limit of sensitivity) demonstrated borderline positivity in one probe (indeterminate sample). Detection of methylation was robust down to 25ng of sample input. **Conclusions:** The findings support the use of high-throughput methylation arrays for clinical testing of *MLH1* promoter methylation. Importantly, the methodology can be generalized to assay other gene level methylation events (e.g. MGMT, etc.) across the genome on a single platform.

#### ST077. Analytic Validation of a Clinical Next-generation Sequencing (NGS) Panel for Somatic Mutations in Uveal Melanoma

K.R. Covington<sup>1</sup>, L.E. Meldi-Sholl<sup>2</sup>, J.K. Wilkinson<sup>2</sup>, M.A. Durante<sup>3</sup>, C.L. Decatur<sup>3</sup>, D.A. Vargas<sup>2</sup>, P.M. Kristen<sup>1</sup>, K.M. Oelschlager<sup>2</sup>, J. Harbour<sup>3</sup>, J.F. Stone<sup>2</sup>, F.A. Monzon<sup>1</sup>

<sup>1</sup>Castle Biosciences, Friendswood, TX; <sup>2</sup>Castle Biosciences, Phoenix, AZ; <sup>3</sup>Sylvester Comprehensive Cancer Center and Interdisciplinary Stem Cell Institute, University of Miami, FL.

**Introduction:** Uveal melanoma (UM) is a rare, intra-ocular cancer that metastasizes in approximately 50% of cases. A prognostic 15-gene expression profile (GEP) test was developed to accurately determine metastatic risk from a fine needle aspiration biopsy (FNAB) of the tumor. Several genes recurrently mutated in UM have also been identified. Some are common to melanocytic tumors and drive G-protein coupled receptor signaling, including *GNAQ*, *GNA11*, *CYSTLR2*, and *PLCB4*. Other gene mutations arise later and may help inform metastatic risk including *EIF1AX*, *SF3B1*, and *BAP1*. In an effort to provide as much molecular information about a UM tumor from a single FNAB, a custom UM next-generation sequencing panel (UM NGS) was designed and validated. **Methods:** A custom UM NGS panel was designed to detect hotspot mutations in *GNAQ*, *GNA11*, *CYSTLR2*, *PLCB4*, and *SF3B1*, the first two exons of *EIF1AX*, and all of *BAP1*. A custom bioinformatics pipeline was designed and tested using a pair of FASTQ files for a well-characterized normal control, NA12878. Concordance studies were

performed on 28 UM samples with existing, but blinded, sequencing data, as well as 18 FNABs that were first sequenced with the UM NGS panel followed by Sanger sequencing. Dilution studies, including commercial controls, were performed to determine the limit of detection (LOD). Intra-assay and inter-assay experiments were performed to evaluate concordance in variant detection and variability in observed variant allele frequency (VAF). **Results:** All 32 variants in NA12878 contained in the UM NGS panel were correctly called by the bioinformatics pipeline. Concordance studies on 28 UM samples with existing sequencing data showed that all 43 single nucleotide variants (SNVs) and 7 insertion/deletions (indels) were correctly called. In 18 UM FNABs that were newly sequenced, 24 SNVs and 5 indels were identified. Two of the 24 SNVs had VAFs below the LOD of Sanger sequencing, but the remaining 22 SNVs and 5 indels were concordant between the UM NGS panel and Sanger sequencing. Minimal dilutions were made for 42 SNVs and 8 indels; the mean detected variant allele frequency was 4.5%, suggesting a LOD of 5%. Intra-assay experiments included 4 SNVs and 5 indels performed in triplicate, with 100% concordance of variant calls and a mean difference in VAF (bias) of -2.4%. Inter-assay experiments were performed on 3 different days with 2 operators and resulted in 100% concordance of 5 SNVs, with a bias of 0.7%. **Conclusion:** A custom 7-gene UM NGS panel and associated bioinformatics pipeline can accurately and reliably identify SNVs and indels in DNA derived from UM tumor tissue. Therefore, both gene expression and somatic mutations can be evaluated from a single FNAB of the primary eye tumor.

#### ST078. Analytic Validation of a Clinical Next-generation Sequencing (NGS) Test for BRAF and NRAS Mutations in Cutaneous Melanoma

L.E. Meldi-Sholl<sup>1</sup>, K.R. Covington<sup>2</sup>, D.A. Vargas<sup>1</sup>, J.K. Wilkinson<sup>1</sup>, K.M. Plasseraud<sup>2</sup>, K.M. Oelschlager<sup>1</sup>, R.W. Cook<sup>2</sup>, J.F. Stone<sup>1</sup>, F.A. Monzon<sup>2</sup>

<sup>1</sup>Castle Biosciences, Phoenix, AZ; <sup>2</sup>Castle Biosciences, Friendswood, TX.

**Introduction:** Cutaneous melanoma (CM) is characterized by recurrent mutations in several genes. BRAF mutations occur in ~40-50% of CM cases, primarily in exon 15 at the V600 locus. After BRAF, NRAS is the next most commonly mutated gene, with ~15-20% of CM cases harboring mutations, usually affecting Q61, G12, or G13. Targeted therapies towards mutant BRAF and downstream effectors such as MEK are used as adjuvant therapy and in the treatment of metastatic CM. Additionally, clinical trials are available for patients with BRAF nonV600 mutations and NRAS mutations. Therefore, the identification of BRAF and NRAS mutations in CM are important to inform therapeutic options. We report analytic validation of a BRAF/NRAS next-generation sequencing (NGS) panel. **Methods:** A custom NGS panel (employing Illumina TruSeq amplification) was used to detect hotspot mutations in BRAF and NRAS. A custom bioinformatics pipeline was used for alignment (BWA, Picard, and Abra), variant calling and annotation (SAMtools, FreeBayes, and Wheeljack). Sequencing was performed on DNA isolated from 53 formalin-fixed paraffin embedded (FFPE) primary CM samples. Two normal controls, NA12877 and NA12878, were used. Confirmatory sequencing on all 55 samples was performed with the Sanger method. Dilution studies were performed to determine the limit of detection (LOD). Intra-assay concordance was analyzed by preparing triplicate libraries for 3 CM samples with 3 different variants and sequencing in the same assay. Inter-assay concordance was evaluated by preparing libraries for and sequencing 8 samples on 3 non-consecutive days. **Results:** Of the 53 CM specimens that were sequenced by the CM NGS panel, 30% were BRAF V600E, 13% BRAF V600K, 9% NRAS Q61R, 8% NRAS Q61K, 4% BRAF L597S, 4% NRAS Q61R, and 2% each of NRAS G12S, NRAS G12R, NRAS Q61L, BRAF D594N, and NRAS T501I; 23% had no detectable mutations in BRAF or NRAS. Results were 100% concordant with Sanger sequencing. Minimal dilutions were made using 23 detected variants and the average detectable VAF was 4.06%. Intra-assay concordance was 100%, with a mean standard deviation (SD) of the observed VAFs of 1.35%. Inter-assay concordance was 100%; with a mean SD of the observed VAFs was 1.01%. Across 6 sequencing runs (7-32 samples in each run) 97-98% of the amplicons had at least 500x coverage. **Conclusion:** A 2-gene NGS panel accurately identifies mutations in BRAF and NRAS in CM FFPE tissue, as confirmed by traditional Sanger sequencing. Results showed a high degree of reproducibility in intra- and inter-assay experiments, and sufficient

coverage was achieved across multiple runs. This test will be useful to help guide clinical decision-making regarding therapy choices for CM patients.

#### ST079. Validation of Cobas HR-HPV Genotyping Assay Head and Neck Squamous Cell Carcinoma FFPE Specimens

W. Zhou, B. Witt, L. Rowe, J. Stanchfield, B. Chadwick, G. Deftereos  
ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT.

**Introduction:** High-risk Papillomaviruses (HR-HPVs) cause a subset of head and neck squamous cell carcinomas (HNSCCs), with better prognosis and higher sensitivity to chemoradiotherapy. Testing for HPV status is standard of care in HNSCC. DNA *in situ* hybridization (ISH) and p16 immunohistochemistry (IHC), currently used for HPV detection are operator-dependent. In addition, ISH has a relatively low sensitivity, and p16 IHC is not 100% specific for HR-HPV infection. The Roche cobas HPV genotyping assay is an automated platform detecting 14 HR-HPV types, with ability to genotype HPV16 and 18. **Methods:** In this study, we examined the performance of the cobas HPV genotyping assay in formalin-fixed, paraffin-embedded (FFPE) samples of HNSCC. Specimens were selected after review of HR-HPV ISH and/or p16 IHC results from patients with HNSCC and a few SCCs of other anatomic sites. HPV status was confirmed using the Linear Array (LA) HPV Genotyping test (Roche). Sections were cut from each FFPE tissue blocks, and either processed as macrodissected slides or as scrolls. Tissues were de-paraffinized, pre-digested, and suspended in PreservCyt (Hologic). Specimens were then processed on the cobas system for DNA extraction, amplification and detection. Known counts of HPV-positive HeLa (HPV18), SiHa (HPV16) and Ca Ski (HPV16) cells were used for limit of detection (LOD) determination. **Results:** A total of 32 samples were tested (23 HNSCCs, 5 SCCs from other sites and 4 cell lines). All HPV16-positive (N=18) samples were found to be HPV16-positive by cobas. Both HPV18-positive (N=2) samples tested HPV18-positive by cobas. The HPV58 and 68 samples (N=2) tested as "other HR-HPV"-positive, while HPV6 and 11 (N=3) samples tested as negative by cobas. All HPV-negative samples (N=7) tested negative by cobas. Interestingly, two HR-HPV ISH and one p16 IHC positive samples tested as negative by both LA and cobas. LODs for HPV16 and 18 were established at 160-320 and 320-1600 copies, respectively. **Conclusions:** The cobas assay showed 100% sensitivity and specificity for HR-HPV detection, when using LA as reference, and demonstrated higher specificity than HPV ISH and p16 IHC. Our data suggest that cobas HR-HPV genotyping is a viable option for detection of HR-HPV in FFPE samples from HN and other anatomic sites and is being validated for clinical use.

#### ST080. Clinical Grade Semi-automated Platform to Annotate Somatic Variants in Solid Tumors per AMP Guidelines

D. Weeraratne, S. Pei, A. Pantazi, J. Jiang, R. Sun, M. Lvova, J. Ring, S. Lyle, M. Russell

KEW, Inc., Cambridge, MA.

**Introduction:** Rapid advances in next generation sequencing (NGS) technology have resulted in massive amounts of clinical informatics data facilitating precision oncology and improved cancer patient care. However, efficiently interpreting the sequencing results and matching variants with targeted therapy and clinical trials remains laborious and time consuming. Manual interpretation of variants is onerous, unreproducible, and non-scalable. At the same time, full automation of clinical interpretation without human intervention is still at a nascent stage and has high error rates. Additionally, prior to the recommendations from AMP in 2017, clinical interpretation and reporting of somatic variants in cancer were largely unstandardized between different clinical labs. Herein, we describe the development of a semi-automated platform to annotate and interpret sequencing data from cancer patients consistent with the AMP guidelines. **Methods:** To facilitate accurate and efficient variant interpretation and clinical reporting we developed and validated GENEKEEPER (KEW, Inc.; Cambridge, MA), a cloud-based knowledgebase and reporting tool. The underlying database architecture seamlessly integrates patient sequence data with numerous resources including, the internal knowledgebase, trial registries, professional guidelines, population databases, sequence repositories, and



variant/gene-specific resources. We sequenced 2,400 solid tumor samples using CANCERPLEX in a CLIA-certified/CAP-accredited laboratory (KEW, Inc., Cambridge, MA). CANCERPLEX is a comprehensive large gene panel comprising 435 cancer associated genes. We analyzed the sequencing results in GENEKEEPER and classified variants based on evidence-based categorization conforming to AMP guidelines. **Results:** The flexible architecture of GENEKEEPER enabled facile implementation of AMP guidelines to enhance standardization and accuracy of variant interpretation. 97% of colorectal cancer specimens (n=580) had variants of strong clinical significance (tier I) or potential clinical significance (tier II). 95% of lung adenocarcinoma specimens (n=402) had tier I or tier 2 variants. Neuroendocrine neoplasm in our cohort (n=35) were the least clinically actionable with only 70% tumors having tier I or II variants. Overall, 93% of the tumors we analyzed had variants with strong or potential clinical significance. **Conclusion:** Standardization of variant interpretation and reporting help streamline and improve uniformity in clinical decision making. Semi-automated analytics platforms such as GENEKEEPER promote precision and efficacy to guide informed treatment decisions. GENEKEEPER significantly reduces the laboriousness of manual curation and will empower clinical laboratories to exponentially scale-up NGS operations.

#### ST081. MSI Status in Primary Pancreatic Carcinoma: A Pilot Study of a New England Cohort

A.M. Strait, L.J. Cook, S.M. Palisoul, B. Ren, G.J. Tsongalis, X. Liu  
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

**Introduction:** Despite extensive research in pathogenesis and therapy, pancreatic carcinoma continues to carry a poor prognosis and is projected to soon become the second leading cause of cancer-related mortality in the US. Although rarely identified in pancreatic cancers, much excitement has been generated in the realm of mismatch repair deficient (MMR-D) tumors, as studies in the field of immune checkpoint inhibition (e.g. PDL-1 inhibitor therapy) continue to demonstrate profound and promising results. Testing for microsatellite instability (MSI) as the hypermutable phenotype of MMR-D therefore remains an intriguing area of research in pancreatic cancer, despite prevalence of approximately 0.5-2% to date. As a pilot study, we set out to determine the prevalence of MSI in pancreatic carcinomas encountered at our institution. **Methods:** All archived resection cases of primary pancreatic carcinoma at our institution from 2014-2017 were reviewed, including clinical history, pathologic reports, and histology of specimens. In order to obtain an adequate amount of tumor DNA for MSI analysis, all untreated carcinoma and carcinomas post chemoradiation therapy exhibiting moderate or poor treatment response (e.g. tumor regression score 2 or 3 of modified Ryan scheme) were selected. From a total of 95 cases, 50 consecutive cases met inclusion criteria. For each case, tumor and normal tissue was dissected after deparaffinization from four unstained slides. Extraction of genomic DNA was accomplished using QIAamp DNA FFPE Tissue Kit and the automated QIAcube workstation. DNA isolations were quantified using Qubit. Paired tissues were tested for microsatellite instability using the MSI Analysis System v1.2 (ProMega) which includes fluorescently labeled primers for co-amplification of 7 markers (5 mononucleotide and 2 pentanucleotide). Tumors showing instability at two or more markers were defined as MSI-High (MSI-H), and those with instability at one repeat or showing no instability were defined as MSI-Low (MSI-L) or microsatellite stable (MSS), respectively. **Results:** Adequate genomic material was extracted from all 50 specimens (e.g. >10 ng/mL). The results of MSI testing revealed high microsatellite instability (MSI-H) in 1 case of ductal adenocarcinoma, likely arising from an intraductal papillary neoplasm. **Conclusions:** While MSI-H pancreatic carcinomas are rarely identified in the existing literature, there is still interest in this area considering the potentially encouraging therapeutic outcomes with use of PDL-1 inhibitors. We identified a single case of MSI-H ductal adenocarcinoma in our cohort of 50 specimens (e.g. 2% prevalence), which is concordant with the current literature.

#### ST082. Clinical Implications of "Indeterminate" UroVysion Fluorescence *in situ* Hybridization Results: An Institutional Retrospective Study of Over 1,200 Patients

J. Xu, E. Vail, D. Engman, J. Lopategui

Cedars-Sinai Medical Center, West Hollywood, CA.

**Introduction:** UroVysion Fluorescence *in situ* Hybridization (FISH) test is more sensitive than urine cytology in screening and monitoring of urothelial carcinoma. Currently, UroVysion is reported as either abnormal or negative. However, cases with 1 to 3 abnormal cells which do not meet the threshold for positivity may be better classified as "indeterminate". The aim of this study is to determine the incidence and clinical significance of these indeterminate UroVysion results. **Methods:** A total of 1,907 urine specimens from 1,213 patients were analyzed by UroVysion FISH over a four-year period. In total, 223 (11.7%) cases were abnormal (4 or more abnormal cells), 1,375 (72.1%) were negative (no abnormal cells), 178 (9.3%) were indeterminate (1 to 3 abnormal cells), and 131 (6.9%) had insufficient cells for analysis. Follow-up of the first-time UroVysion cases, including urine cytology, repeat UroVysion testing or bladder biopsy performed within 12 months of the initial diagnosis of the result was obtained from pathology reports. **Results:** Of 106 patients with initial indeterminate UroVysion results, 61 had follow-up tests and 14 of them (22.9%) were found to have urologic malignancies by biopsy/cytology studies. Of 896 patients with initial normal UroVysion results, 354 had follow-up tests, and 48 (13.6%) patients were found to have urologic malignancies. There was no statistically significant difference of subsequent malignancy rate between these two groups ( $p=0.081$ ). However, for patients without previous history of urinary tract neoplasm, the subsequent malignancy rate in the group with indeterminate UroVysion results (7 of 19, 36.8%) was significantly higher than the group with normal UroVysion results (16 of 121, 13.2%) ( $p=0.017$ ). **Conclusions:** In patients without previous history of urinary tract neoplasm, over one-third of patients with initial indeterminate UroVysion results and follow-up were found to have urologic malignancies on subsequent testing, which was significantly higher than the group with normal UroVysion results. Based on these results, an indeterminate UroVysion FISH result of 1 to 3 abnormal cells should not be reported as negative and may warrant closer clinical follow-up than a truly negative result. We suggest reporting these cases as "aneuploidy of undetermined significance".

#### ST083. Development and Characterization of EML4-ALK and KIF5B-ALK Gene Fusion NSCLC Cell Line using CRISPR/Cas9 Technology as a Reference Material for use with Next-Generation Sequencing Platforms

Q. Zheng, S. Kondapalli, D. Sengupta, S. Thakker, G. Roma, S. Saddar  
AccuRef Diagnostics, Milpitas, CA.

**Introduction:** Acquired chromosomal alterations such as chromosomal translocations often result into generation of pathogenic gene fusions. More than 100,000 common and rare fusion genes have been identified till date, many of which are potent oncogenic drivers for a wide variety of cancer types. The current gold standard for gene fusion identification is fluorescence *in situ* hybridization (FISH) and immunohistochemistry. However, in the recent years, RNA-Seq technologies have emerged as a powerful molecular approach for identification of gene fusions events because of its ability to provide comprehensive information on multiple gene fusions and high-throughput compatibility. While RNA-Seq assays offer a sensitive and cost-efficient method for simultaneous analysis of multiple gene alterations, the underlying complexity of the technology necessitates inclusion of appropriate controls for assay validation, quality control, and troubleshooting. Through the use of CRISPR/Cas9 genome editing technology, it is possible to generate appropriate control cell lines for identification of gene fusion events. Here, we demonstrate use of CRISPR/Cas9 technology for generation of genome edited cell lines displaying EML4-ALK, and KIF5B-ALK gene arrangements, two of the gene fusion events that are being increasingly recognized as drivers for non-small cell lung carcinomas (NSCLC). **Methods:** Using the HCT116 cancer cell line, single stranded guide RNAs (sgRNAs) were designed and built to cause Cas9 to bind and cut desired intronic regions in the EML4, ALK and/or KIF5B gene targets upon co-transfection. First step was directed to cause the deletion of the ALK gene. In second step, donor was

designed which will cause directed repair creating the fusion of two genes. The single-cell colonies containing the fusion genes were confirmed by sequencing. Fusion gene expression was confirmed by next generation sequencing (NGS) and RNA-Seq. **Results:** Using NGS we confirmed the generation of the *EML4-ALK* and *KIF5B-ALK* gene fusion cell lines. No CRISPR/Cas9 off-target events were detected in the newly formed gene-fusion cell lines when compared to the isogenic pair wild type. **Conclusion:** Using the CRISPR/Cas9 technology, we demonstrate the development and characterization *EML4-ALK* and *KIF5B-ALK* gene fusion. This reference material will enable higher sensitivity and confidence in the NGS bioinformatic analysis when detecting difficult *ALK* gene fusion variants in a clinical workflow. These engineered cell lines are biologically relevant materials and allow for a reproducible and renewable source of mRNA reference material for RNA Seq assay development, quality control, and proficiency panel use.

#### ST084. Evaluating Double-equivocal *HER2* Invasive Breast Cancer Cases and Potential Solutions

R.A. Allen, M.D. Lucas, Y. Zhou

University of Oklahoma Health Sciences Center, Oklahoma City, OK.

**Introduction:** One consequence of changes made from the 2007 ASCP-CAP *HER2* breast guidelines to the 2013 guidelines is an increase in breast *HER2* equivocal interpretations. At our institution, surgical pathologists review invasive breast cancer cases and *HER2* IHC. IHC 2+ cases are sent to our molecular pathology laboratory for *HER2* dual-probe fluorescence *in situ* hybridization (FISH), which is interpreted by a molecular genetic pathologist. Under the 2013 guidelines, breast cases with *HER2/CEP17* ratio <2 and ≥4 and <6 *HER2* signals per cell are "equivocal," which is a clinical dilemma for oncologists. From 2017 to the first quarter of 2018, our laboratory observed an increased *HER2* FISH equivocal rate. We evaluated reasons for this apparent increase in equivocal rates and potential solutions. **Methods:** We queried our molecular laboratory database for previously tested breast *HER2* FISH cases and calculated equivocal rates for individual surgical pathologists. We also reviewed the literature for potential benefits of using alternative FISH probes and ways to reduce equivocal cases. **Results:** From 2016 to May 2018, our laboratory resulted 33 equivocal breast *HER2* FISH cases (equivocal rate=12.9% of 238 total), with per year equivocal rates of 11.1%, 11.9%, and 19.7% in 2016, 2017, and Jan-May 2018. Four surgical pathologists reviewed 200 cases (individual pathologists 69, 45, 45, and 41). Two pathologists (equivocal rates 7.3% and 8.9%) reviewed 74 of 109 (68%) cases in 2017. Two other pathologists (equivocal rates 17.8% and 20%) reviewed 39 of 66 (59%) cases in the first quarter of 2018. Our literature review showed that our overall equivocal rate was consistent with that of other institutions. We also found insufficient evidence to support using alternative probes, which are currently available only through reference laboratories. No clinical trials show clear benefit of *HER2*-targeted therapy in dual-equivocal patients. **Conclusions:** One significant challenge to molecular testing is addressing clinician concerns regarding the validity of what appear to be inconclusive results. There was an apparent increase in equivocal rates within the first quarter of 2018, and the reasons for variability amongst our surgical pathologists is unclear. There may be normal statistical variation or real inter-observer differences (e.g., due to differences in subspecialty training). Fortunately, on May 30, 2018, ASCP-CAP released an update regarding *HER2* testing in breast cancer, which subdivides *HER2* dual-probe ISH cases into 5 groups, including one for the 2013 "equivocal" category. While these guidelines do eliminate "equivocals," our molecular pathology laboratory will need to modify our workflow to meet new requirements such as performing concomitant IHC review for dual-probe ISH cases groups two to four.

#### ST085. Large-scale Hybrid Capture-based RNA Sequencing for Clinical Detection of Gene Fusions and Broad Transcriptomic Assessments of Solid Tumors

V. Balagopal, C. Zhen, S. Kadri, A. Plagov, L.L. Ritterhouse, J.P. Segal  
The University of Chicago, Chicago, IL.

**Introduction:** Gene fusions that result in constitutive activation or overexpression of kinases are important drivers of oncogenic transformation. Clinical detection of kinase fusions is important for cancer

diagnosis, subtype definition, and selecting targeted therapy.

Fluorescence *in situ* hybridization (FISH) and RT-PCR assays can be used to detect fusions, but these technologies are low-throughput and may require prior knowledge of fusion breakpoints. RNA sequencing (RNA-Seq) for high-throughput detection of both characterized and novel fusions with high accuracy and sensitivity is an optimal solution in a clinical setting. Here, we report the development and validation of a hybrid capture-based RNA-Seq panel to detect fusions of clinical relevance. Initial validation is focused on *FGFR* and *NTRK* fusion genes; however, the methodology allows for future extension across the entire panel.

**Methods:** RNA was obtained from formalin-fixed, paraffin-embedded (FFPE) tissue samples followed by RNA-Seq library preparation after ribosomal depletion (Stranded RNA-Seq Library Preparation Kit, KAPA). Libraries were subjected to capture targeting 1213 cancer-related genes followed by sequencing via HiSeq 2500 (Illumina). Bioinformatic pipelines for detection of fusion reads included a combination of in-house fusion detection software utilizing both discordant and split reads and publicly available STAR-fusion software. **Results:** We assembled a set of 23 clinical validation samples bearing fusions in *FGFR1/2/3* and *NTRK1/2/3* and were able to detect all expected fusions via the assay (100% sensitivity). Negligible background signal was detected in 21 negative samples, indicating equally high specificity. Intrarun and interrater replicates demonstrated high reproducibility. Beyond *NTRK* and *FGFR* genes, the assay also demonstrated utility in fusion hunting as evidenced by its ability to identify novel fusions involving *PDGFRB*, *STAT2* and *USP6* in several investigational samples and an atypical *BCR-ABL1* breakpoint (p230) in a case with discrepant FISH/qPCR results. **Conclusions:** We have developed and validated a highly sensitive and specific assay for clinical detection of gene fusions using RNA sequencing and targeted capture. This assay works with low quantity and quality RNA from FFPE samples and is capable of detecting both characterized and novel fusions for a large number of genes in a high-throughput fashion. Additionally, in contrast to other commercial targeted-fusion next-generation sequencing platforms using primer extension, this capture-based methodology allows for collection of additional data including gene expression, variants/allele-specific expression and alternative splicing events across all genes in the panel.

#### ST086. Sanger Sequencing Method for the Detection of Extended *RAS* and *BRAF* with an LOD of 10% VAF

D.R. Pringle<sup>1</sup>, M.S. Lalonde<sup>1</sup>, M. Erali<sup>2</sup>, A.P. Matynia<sup>2</sup>

<sup>1</sup>ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT; <sup>2</sup>University of Utah, Salt Lake City, UT.

**Introduction:** Sanger sequencing, developed in 1977, remains the gold standard for generation of sequence information from genomic DNA. The utility of Sanger sequencing for detection of somatic mutations in oncology samples remains limited compared to other methods. Detection of mutants with variant allele frequencies (VAFs) as low as 10% is clinically important. The inability of Sanger sequencing to detect mutations with low VAF has led to the development of other methodologies for detection of somatic variants in cancer that have varying advantages/disadvantages. New data analysis software has become available which claims to significantly improve the limit of detection (LOD) of Sanger sequencing assays. Using this software, we are developing a clinical assay with an LOD of 10% for detection of extended *RAS* and *BRAF* mutations to comply with the clinical practice guidelines for colorectal cancer testing. **Methods:** De-identified, previously tested, formalin-fixed, paraffin-embedded (FFPE) patient samples and FFPE Horizon standards with known VAFs were utilized. Horizon standards were diluted in patient wild-type samples to yield a known VAF dilution series. Samples were then subjected to PCR, using the BigDye Direct PCR MasterMix, to generate seven amplicons. Amplicons were rSAP treated, then, cycle sequenced using the BigDye Direct xTerminator chemistry on an ABI3730. Resulting sequences were analyzed with Minor Variant Finder (MVF, ThermoFisher). WT patient samples were used as reference sequences. **Results:** In preliminary experiments, all mutants tested were detected with MVF with ≥ 90% specificity and ≥ 95% accuracy. Sequence quality was vital for downstream analysis and our results indicate that kit clean DNA is essential for variant detection. No bias was found in detection of mutants and VAF was reproducible (+/-

~5%) between runs. While over reporting of known VAF was observed, under reporting was not, giving further confidence to our LOD of 10%.

**Conclusions:** This Sanger assay meets clinical guideline recommendations for detection of mutants known to be predictive of the lack of response to anti-EGFR therapy or to have prognostic significance in colorectal cancer patients. While also achievable with next generation sequencing (NGS), the focused scope of this panel makes it a relatively inexpensive and quick alternative for clinically significant low-level somatic variants. The basic methodology is well established, traditional Sanger sequencing and data analysis is straightforward, making this assay a reproducible, technically simple, and quick way to identify these variants in either a large reference laboratory or a small hospital laboratory. This methodology also has the potential to be used in other small gene panels, once again, re-iterating that Sanger sequencing is here to stay.

#### ST087. Enabling Standardized Testing of Liquid Biopsy Assays Detecting EGFR Mutations using Bespoke Reference Materials

S. Deans<sup>1</sup>, N. Iley<sup>1</sup>, J. Fairley<sup>1</sup>, R. Butler<sup>2</sup>, C. Clark<sup>3</sup>, J. Wu<sup>4</sup>, O.

Clement<sup>4</sup>, B. Anekella<sup>4</sup>, D. Brudzewsky<sup>4</sup>

<sup>1</sup>United Kingdom National External Quality Assessment Service for Molecular Genetics, Edinburgh, United Kingdom; <sup>2</sup>All Wales Genetics Laboratory, Cardiff, United Kingdom; <sup>3</sup>NHS Grampian, Aberdeen, United Kingdom; <sup>4</sup>SeraCare Life Sciences, Milford, MA.

**Introduction:** Frequently, patients with non-small cell lung cancer have limited (if any) tissue available to perform *EGFR* mutation testing. This can be overcome by testing plasma samples for the presence of mutations in circulating tumour DNA (ctDNA). The application of such highly sensitive testing methods requires assessment of the quality of the processes. One mechanism to measure the standard of testing is through external quality assessment (EQA). UK NEQAS for Molecular Genetics provided An AstraZeneca EQA for all UK laboratories performing *EGFR* mutation plasma testing using bespoke reference materials with patient-like sample attributes to assess the standard of service. **Methods:** A panel of 10 reference plasma samples (SeraCare) were designed to cover a range and combination of *EGFR* mutations at different allelic frequencies. The samples were provided to participants as 5mL plasma aliquots for routine *EGFR* mutation testing. The samples suitable for all cfDNA extraction and mutation analysis methods were batch controlled so benchmarking could be performed between laboratories. Each sample was supplied with a corresponding clinical case according to which the result was to be interpreted. Details of the variants present in the reference samples, collation of the submitted results, methodologies performed and general feedback comments were provided to the participants. **Results:** Nine laboratories participated and only one of the 90 samples distributed failed to obtain a reportable result. Three testing methods were performed (some in combination); Cobas (the majority), digital droplet PCR and Therascreen. False positive results were reported with sporadic occurrence of a range of mutations regardless of testing method used. The laboratories performing the Therascreen assay reported recurring false negatives for p.(T790M), and a low level rare mutation was detected by Cobas. The investigation of these unexpected results will be discussed. Furthermore, the content of the reports was highly variable with multiple sub-optimal interpretative comments being present. The key issues will be summarised and discussed. **Conclusion:** Participation in EQAs enable laboratories to identify issues with testing methods and reporting formats through comparison with other laboratories performing similar tests. The use of bespoke SeraCare reference materials allows challenging clinical cases with corresponding genotypes to be used. The scalability and stability of the EQA material supports the provision of EQA to many laboratories without comprising the benchmarking aspect of the assessment. Future EQA is required to standardise and improve the testing and reporting of *EGFR* mutation testing in plasma samples.

#### ST088. Novel BCOR and CREBBP Fusion Events in High Grade Infiltrating Glioma

D.J. Pisapia, R. Bareja, D.C. Wilkes, E. Hissong, T.Y. MacDonald, J.A. Croyle, J. Kim, J. Saab, S. Beg, C. O'Reilly, P. Rajappa, M.A. Rubin, O. Elemento, J. Greenfield, A. Sboner, J. Mosquera  
Weill Cornell Medicine, New York, NY.

**Introduction:** *BCOR* (*BCL6* interacting co-repressor) is increasingly recognized as an altered gene in a subset of pediatric tumors of the central nervous system. These include the recently described "high grade neuroepithelial tumor with *BCOR* alteration" (HGNET-*BCOR*), an entity characterized in most cases by an internal tandem duplication involving exon 15. Here, we describe previously unreported *BCOR* fusion events in infiltrating gliomas. **Methods:** We analyzed frozen tumor tissue from a 15-year-old male who presented with an infiltrative mass in the right cerebral hemisphere and who died of disease 24 months following presentation. RNA-seq followed by computational analysis (FusionSeq) nominated a *BCOR-CREBBP* fusion. An additional 180 cases of pediatric and adult primary tumors of the central nervous system (CNS), including 99 adult glioblastomas, 42 lower grade gliomas, and 39 pediatric gliomas were assessed by fluorescence *in situ* hybridization (FISH) for *BCOR*. Publicly available RNA-seq data from an additional 509 adult lower grade gliomas (TCGA) was also screened for the presence of fusion events involving *BCOR* and/or *CREBBP* using STAR-fusion. **Results:** In the index case, the fused *BCOR-CREBBP* transcript comprises exons 1-4 of *BCOR* and exon 31 of *CREBBP*. The fused gene retains the Bcl6 interaction domain of *BCOR* while eliminating the domain that interacts with the polycomb group protein POGZ1. Fusion of *BCOR* was validated by FISH. All 180 of the additional adult and pediatric tumors screened for *BCOR* fusions by FISH were negative. Of the additional 509 primary CNS tumors that were screened for *BCOR* or *CREBBP* fusions using RNA-seq data, one case of IDH-wildtype anaplastic astrocytoma in a 45-year-old was found to harbor a *CREBBP-GOLGA6L2* fusion, and in another case of IDH-mutant anaplastic astrocytoma in a 30-year-old, a *CREBBP-SRRM2* fusion was identified. In a third patient, a 24-year-old female with an IDH-wildtype high grade glioma, two fusions involving *BCOR* were seen, *BCOR-L3MBTL2* and *BCOR-EP300*. Of particular interest, *EP300* is a paralog of *CREBBP* and the breakpoint seen involves a similar region of the gene to that of the index case. **Conclusions:** We describe a novel *BCOR-CREBBP* gene fusion in a pediatric high grade astrocytoma. In an additional 689 primary adult and pediatric CNS tumors assessed by FISH or RNA-seq, we identified additional cases demonstrating a *BCOR* fusion to a paralog of *CREBBP*, namely *EP300* (one case), and *CREBBP* fusions with different partners (two cases). These findings add to the existing literature implicating *BCOR* as having a potential driving role in a small subset of CNS tumors, and demonstrate the need to further characterize the role of *CREBBP* in CNS tumor biology.

#### ST089. Utility of GliSeq Next-generation Sequencing Test for Classification of Ependymomas

A. Wald, W. Ernst, S. Mercurio, M. Grupillo, L.S. dos Santos, S. Roy, M. Nikiforova

University of Pittsburgh Medical Center, Pittsburgh, PA.

**Introduction:** Ependymal tumors have been recently re-classified into nine groups based on anatomical compartment of the CNS and molecular findings [Pajtler *et al.*, Cancer Cell 2015]. The new molecular classification outperformed the current histopathological classification and has demonstrated an improved clinical utility in management of patients with these tumors. We evaluated the use of a targeted next-generation sequencing (NGS) test (GliSeq) for classification of ependymal tumors. **Methods:** GliSeq v1 and v2 NGS tests were used to interrogate molecular alterations in ependymoma samples. GliSeq test sequences targeted regions of 30 CNS tumor-related genes for single-nucleotide variant (SNV) and insertion/deletions (indels), 24 genomic regions for copy number alterations, and detects > 50 gene fusions, including *RELA* and *YAP1* fusions. It requires 20ng DNA and 10ng RNA for library preparation and sequencing and is validated for use in small formalin-fixed, paraffin-embedded (FFPE) stereotactic biopsy samples. **Results:** A total of 21 small biopsies and resected ependymoma tumor specimens, including 5 pediatric and 16 adults were tested by GliSeq NGS test from 2015-2018. Based on anatomic location, 7 ependymomas (EPN) were from the spine (SP), 12 from the posterior fossa (PF), and 2 from the supratentorial (ST) compartment. Out of 7 spine ependymomas, 5 (71%) had molecular alterations allowing the classification into 4 SP-EPN (anaplastic ependymoma, NF2 positive, WHO grade II-III) and one SP-MPE (myxopapillary ependymoma with chromosomal instability, WHO



grade I). The posterior fossa ependymomas showed presence of molecular alterations in 8/12 (67%) of tumors that led to classification into 6 PF-EPN-A (anaplastic ependymoma with balanced genome) and 2 PF-EPN-B (anaplastic ependymoma with chromosomal instability), both WHO grade II-III. Finally, 2 (100%) supratentorial ependymomas were positive for C11orf95/RELA fusion allowing classifying them into ST-EPN-RELA (anaplastic ependymoma RELA fusion positive) subgroup, WHO grade II-III. **Conclusions:** GloSeq targeted NGS test allowed to re-classify the majority (71%) of tested ependymomas into new molecular groups based on detected genomic alterations. The new molecular classification improves diagnostic accuracy and prognostication of pediatric and adult patients with ependymomas.

#### ST090. Role of Genomic Profiling in Staging of Patients with Multifocal Lung Carcinomas

S. Jiwani, J. Zhang, N.V. Guseva, A.A. Stence, U. Swami, A.D. Bossler, D. Ma

University of Iowa Hospitals and Clinics, Iowa City, IA.

**Introduction:** Lung carcinoma is the leading cause of cancer-related death in USA. TNM staging is critical in patient management and prognosis. Traditionally, staging of patients with multifocal lung carcinomas relies on their anatomic locations and histology with an aim to distinguish multiple synchronous primaries from single primary tumor with intrapulmonary metastases; the latter has a more advanced T stage and worse prognosis. This staging strategy is limited by various factors including accessibility and/or preoperative selectivity of tumor nodules to be biopsied, morphologic heterogeneity, as well as intra-observer variability in assessing the dominant morphologic patterns. We assessed the role of genomic profiling in accurate staging and effective management of patients with multifocal lung carcinomas.

**Methods:** Fourteen patients with previous known diagnosis of multifocal lung carcinomas were identified from archival tissue. Individual tumor foci were microdissected and tested separately using an RNA-based next-generation sequencing (NGS)-panel for fusion and hotspot mutation detection (FusionPlex Lung Thyroid Panel (CTL), ArcherDx, Inc) for 36 genes commonly mutated in lung carcinomas. Fusion/mutation-negative cases were further tested with the 50-gene hotspot mutation panel (AmpliSeq Cancer Hotspot Panel v2 (CHPv2), Life Technologies). Tumor nodules with the same molecular aberration(s) were considered the same primary. The impact on staging based on the molecular findings was compared with the morphologic classification. **Results:** The efficiency in determining the clonal relationship in multifocal lung cancer by CTL, CHPv2, and CTL+CHPv2 was 86%, 84%, and 86%, respectively. The concordance between morphologic and genomic profiling was 36% (n=14) and the discordance was 43% (n=14). Genomic profiling was inconclusive in assessing clonality in 3/14 cases either due to insufficient quality or quantity of nucleic acid for testing or no variants were identified in the genes included in these panels. Based on the findings from genomic profiling, 2/14 patients were upstaged and 3/14 patients were down-staged. **Conclusions:** Genomic profiling may serve the dual purpose of identifying variants for targeted therapy as well as a diagnostic tool in accurate staging of patients with multifocal lung cancers.

#### ST091. Reduced Sensitivity of Break-apart FISH for ALK Gene Rearrangements in EML4-ALK Fusion Positive Lung Cancer Samples Detected by NGS

S. Knight, C. Sattler, P. Caffes, K. Pearce, M. Aubry, J. Voss, B. Kipp, K. Geiersbach

Mayo Clinic, Rochester, MN.

**Introduction:** EML4-ALK fusion is the most frequent type of ALK rearrangement in lung cancer, and its detection predicts responsiveness to ALK inhibitory therapies. Fluorescence *in situ* hybridization (FISH) has historically been the gold standard for detecting ALK gene rearrangements in lung cancer. Immunohistochemistry (IHC) with certain commercially available antibody clones has been more recently accepted as an alternative to FISH. Our institution recently launched a next-generation sequencing (NGS) assay that evaluates targeted ALK fusion transcripts in patients with lung cancer. The aim of this study was to evaluate the sensitivity of a commercially available ALK break-apart FISH assay in patient samples with a

known EML4-ALK fusion detected by our clinically validated NGS assay.

**Methods:** Lung adenocarcinoma samples from 10 different patients with EML4-ALK fusion identified by NGS testing were selected for ALK FISH and IHC testing. Residual formalin-fixed, paraffin-embedded (FFPE) tissue slides were hybridized with the Abbott ALK (2p23) dual-color, break-apart rearrangement FISH probe set and those with an additional slide available were also stained with the Cell Signaling Technology D5F3 XP Rabbit monoclonal antibody for IHC. FISH and IHC slide interpretations were blinded to results of the other assays including NGS. **Results:** Six different targeted transcript breakpoints were detected by NGS in the 10 samples including EML4 Intronic 6/ALK Exon 20 (n=4), EML4 Exon 14/ALK Exon 20 (n=1), EML4 Exon 13/ALK Exon 20 (n=1), EML4 Exon 20/ALK Exon 20 (n=1), EML4 Exon 20/ALK Intronic 19 (n=1), EML4 Exon 6/ALK Exon 20 (n=1), and EML4 Exon 18/ALK Exon 20 (n=1). FISH testing was positive in 6 of 10 samples, and negative in 4 samples. Additional slides were available for IHC testing in 8 of 10 samples including 2 of the 4 negative FISH samples, and all 8 were IHC positive. **Conclusions:** Our data suggests that FISH with the standard break-apart assay method has reduced sensitivity for detecting ALK rearrangements in lung cancers harboring an EML4-ALK fusion. Our data also suggests that immunohistochemistry may be more sensitive than FISH for detecting this particular subtype of ALK rearrangement, and that NGS may be a superior confirmatory method for a positive IHC result rather than FISH.

#### ST092. Optimization Studies for the Development of Highly Multiplexed Reference Materials in FFPE Format for Solid Tumor Profiling

B. Anekella<sup>1</sup>, C. Huang<sup>1</sup>, D. Ruminiski Lowe<sup>1</sup>, D. Phikana<sup>1</sup>, R. Vemula<sup>1</sup>, L. Nguyen<sup>1</sup>, R. Garlick<sup>2</sup>

<sup>1</sup>SeraCare Life Sciences, Gaithersburg, MD; <sup>2</sup>SeraCare Life Sciences, Milford, MA.

**Introduction:** Next-generation sequencing (NGS) has revolutionized how IVD developers, laboratories, and clinicians are diagnosing, treating, and monitoring disease. NGS assays must be proven robust, accurate, and consistent through validation and verification studies. Sourcing individual formalin-fixed, paraffin-embedded (FFPE) samples (remnant patient specimens or cell line derived) for each of the somatic mutation of interest is expensive and time consuming. Materials with copy number variations (CNV) quantitatively characterized in a stable background have not been available. Therefore, highly multiplexed, engineered reference materials in FFPE format are needed. These FFPE reference materials must be consistently manufactured and must incorporate a wide variety of somatic variations including CNVs in addition to single-nucleotide variants (SNVs), insertion/deletion (indel), and structural variants.

**Methods:** Methods were optimized for embedding engineered cell line pellets in FFPE to minimize variability between and within blocks. Nine different FFPE blocks were made on three independent days. DNA was extracted from FFPE curls using Qiagen QIAamp DNA FFPE Tissue kit, and the consistency of DNA yield across blocks and within a single block were assessed. Prototype materials were made bearing forty SNVs, small indels and structural rearrangements targeted to 20% variant allele frequency (VAF) and were tested by both digital PCR and NGS on the Ion Ampliseq Cancer Hotspot Panel v2 and Illumina TruSight Tumor 15 Assay. Prototypes also incorporated large, whole gene constructs to amplify the copy number of driver genes such as MET, MYC and EGFR in the engineered cells. **Results:** DNA yield across three manufacturing runs (9 different FFPE blocks) varied by less than 12% (average of 178 ng per curl, standard deviation of 20 ng). One FFPE block was completely sectioned and seven non-consecutive curls throughout the block showed intra-block variability was very similar to variability between blocks. Average VAF across all assayed variants was 19.79% by digital PCR, 23.7% by Illumina TruSight Tumor 15 assay, and 20.7% by Ion Ampliseq Cancer Hotspot Panel v2. All evaluated variants in the reference material were positively detected. New prototypes were generated with MYC copy number increased to greater than 4 total copies per cell. **Conclusions:** This study demonstrated that highly multiplexed reference materials in FFPE can be consistently manufactured such that there is both uniformity of curls within an FFPE block and among different FFPE blocks. Forty somatic variants were accurately targeted to the desired

VAF and complex variant types such as CNVs could also be included. These advances make validation and verification of new assays as well as ongoing QC monitoring easier and more cost effective for NGS users.

#### ST093. NEBNext Direct Custom Ready Panels Overcome Challenges Associated with Targeted Re-sequencing

A.J. Barry<sup>1</sup>, K.M. Patel<sup>2</sup>, A.B. Emerman<sup>2</sup>, S. Adams<sup>2</sup>, S.J. Bowman<sup>2</sup>, E. Maucelli<sup>2</sup>, J. Dunn<sup>2</sup>, C. Elfe<sup>2</sup>, S. Corbett<sup>2</sup>, B. Desmond<sup>2</sup>, K. Krishnan<sup>1</sup>, E. Dimalanta<sup>1</sup>, F. Stewart<sup>1</sup>, T.B. Davis<sup>1</sup>, C.L. Hendrickson<sup>2</sup>

<sup>1</sup>New England Biolabs, Ipswich, MA; <sup>2</sup>Directed Genomics, Ipswich, MA.

**Introduction:** Application of targeted gene panels for clinical research is challenged by the wide variation in gene constituents specific to a given study. While focused gene panels efficiently provide the necessary depth of coverage for low frequency variant detection, the high costs and design challenges associated with panel design present challenges. Furthermore, inherent issues with amplification-based enrichment include low uniformity of target coverage, and challenges when modifying content of a given panel. NEBNext Direct Custom Ready Panels employ a novel, hybridization-based approach to selectively enrich nucleic acid targets ranging from a single gene to several hundred genes, without sacrificing specificity. The approach rapidly hybridizes both strands of genomic DNA with biotinylated probes prior to streptavidin bead capture, enzymatic removal of off-target sequence, and conversion of captured molecules into sequencer-ready libraries. This results in a unique read coverage profile that results in uniform coverage across a given target. Unlike alternative hybridization methods, the approach does not necessitate upfront library preparation, and instead converts the captured molecules into dual-indexed illumina sequencer compatible libraries containing an 8 basepair sample ID and a 12bp Unique Molecule Index (UMI). The result is a 1-day protocol that enables the preparation of sequence-ready libraries from purified genomic DNA specific to the gene content included in the panel. We have designed and optimized baits specific to the full exonic content of >800 genes associated with cancer, neurological disorders, autism, cardiovascular disease, and other conditions. These are designed, balanced, and pooled on a per gene basis, and can be combined into NEBNext Direct Custom Ready Panels, allowing rapid turnaround of specific custom gene subsets. **Methods:** NEBNext Direct Custom Ready panels were developed, ranging from 1 to 100 genes, and used to enrich specific targets from control DNA samples with known truth variants. These panels were sequenced, and compared to results obtained using commercially available, amplification-biased enrichment techniques. **Results:** We were able to obtain high-quality sequencing data across the range of panels tested, and metrics were compared based on the specificity of enrichment, uniformity of target coverage, and ability to automatically call nucleic acid variants across a range of frequencies. **Conclusions:** We present here the ability to rapidly deploy custom gene panels across a variety of panel sizes and content, while maintaining high specificity, uniformity of coverage across target content, and sensitivity to detect nucleic acid variants from clinically relevant samples.

#### ST094. Loss of Heterozygosity in Uterine Serous Carcinoma: Prognostic and Therapeutic Implications

E. Abdulfatah<sup>1</sup>, A. Loehr<sup>2</sup>, A. Simmons<sup>2</sup>, S. Bandyopadhyay<sup>3</sup>, R. Morris<sup>4</sup>, R. Ali-Fehmi<sup>3</sup>

<sup>1</sup>Wayne State University, Grosse Pointe Farms, MI; <sup>2</sup>Clovis Oncology, Boulder, CO; <sup>3</sup>Wayne State University, Detroit, MI; <sup>4</sup>Karmanos Cancer Institute, Detroit, MI.

**Introduction:** While uterine serous carcinomas (USCs) are morphologically and immunophenotypically indistinguishable from their ovarian counterparts, questions remain on whether these tumors share similar molecular profile and could benefit from similar targeted therapies. Somatic and germline defects in the homologous recombination (HR) pathway genes (*BRCA1*, *BRCA2* and others) have been implicated in ovarian cancer predisposition and are associated with high sensitivity to platinum-based and targeted therapies (PARP inhibitors). HR deficiency impairs normal DNA repair resulting in loss or duplication of chromosomal regions, termed genomic loss of heterozygosity (LOH). Whether this phenomenon applies to USCs is yet to be determined. In this study, we analyzed genome-wide LOH in USCs and correlated its extent with

clinicopathological parameters and outcomes. **Methods:** Sixty-nine platinum treated USC patients diagnosed between 1998 and 2015 were included. DNA was isolated from formalin-fixed, paraffin-embedded (FFPE) and LOH analysis was performed querying over 220,000 SNPs using the Affymetrix OncoScan. Selecting the top quartile of LOH distribution as a cutoff point to separate patients into LOH "high" and LOH "low" groups, data was analyzed using Kaplan Meier survival analysis.

**Results:** Patient's ages ranged from 45 - 89 (median 68) years. Stage distribution included: 11.5% I, 1.5% II, 62% III and 25% IV. Lymphadenectomy was performed in 74% and omentectomy in 62% of patients. 80% of patients received adjuvant radiotherapy. Median LOH was 1.4% (range 0-30.1%) and the top quartile threshold was 9.9%. 52 tumors showed LOH lower than the top quartile. In the top quartile of LOH, tumors were more common in African Americans (83% vs 56%), advanced stage (100% vs 82%) and more commonly associated with outer half myometrial invasion (MI) (76.5% vs 47%) and lymphovascular invasion (LVI) (88% vs 65%) when compared to tumors with LOH lower than the top quartile. In general, LOH did not significantly correlate with age ( $p=0.41$ ), race ( $p=0.43$ ), tumor size ( $p=0.72$ ), stage ( $p=0.36$ ), MI ( $p=0.52$ ), LVI ( $p=0.40$ ), lymph node metastasis ( $p=0.43$ ) or recurrence ( $p=0.34$ ). Kaplan-Meier analysis showed no survival benefit nor prolonged disease-free interval for platinum treated patients with LOH in the top quartile compared to LOH lower than that. **Conclusion:** In our cohort of USCs, median LOH was 1.4%, with 25% of tumors showing genomic LOH higher than 9.9%. In contrast to their ovarian counterparts, a high degree of genomic LOH does not correlate with survival benefit for platinum treated USC patients, suggesting that these tumors may have distinct biology and genomic profile.

#### ST095. Identification of Rare Clinically Actionable Variants in *KRAS*, *EGFR*, and *BRAF* Using a Comprehensive Gene Panel

D. Weeraratne, S. Lyle, M. Russell KEW, Inc., Cambridge, MA.

**Introduction:** While commercially available hotspot testing detects over 98% of the *KRAS*, *EGFR*, and *BRAF* oncogenic variants in solid tumors, rare oncogenic variants in these genes that are typically not detected in hotspot assays, can significantly impact treatment decisions. In this work, we highlight the utility of comprehensive gene panel testing in identifying low frequency mutations in *KRAS*, *EGFR*, and *BRAF* that impacted the patients' treatment course. **Methods:** 580 colorectal cancer (CRC), 402 lung adenocarcinoma, and 92 melanoma specimens were analyzed using CANCERPLEX in a CLIA-certified/CAP-accredited laboratory (KEW, Inc., Cambridge, MA). CANCERPLEX is a comprehensive large gene panel comprising 435 cancer associated genes. A rare mutation was defined as a variant with a frequency > 0.02% in COSMIC. Variants with clinical actionability were defined as activating mutations with inferred sensitivity or resistance to FDA approved therapy. The analytical sensitivity and specificity for single nucleotide polymorphisms (SNPs) in CANCERPLEX was 99.2% and > 99.9%, respectively. **Results:** We detected low frequency clinically actionable mutations in *KRAS* beyond the common codon 12, 13, 61, 146 mutations, in *EGFR* beyond the common codon 719, 858, 861 and exon 19 deletions, and in *BRAF* beyond codon 600 mutations. *KRAS* Q22K, L19F, and G60D were identified as rare oncogenic variants in CRC. All three variants have been reported to have transforming potential *in vitro* and *in vivo* and trigger activation of downstream oncogenic pathways. As activating mutations in *KRAS* predict lack of response to EGFR antibody therapies, cetuximab and panitumumab were reported as contraindications in all three cases. *EGFR* G724S, S645C, and G465E were identified in lung adenocarcinoma as rare clinically relevant variants that predict resistance to FDA approved kinase inhibitors. In our analysis we reported *EGFR* R776H as predicting sensitivity to TKIs. Finally, CANCERPLEX assay detected *BRAF* K483E, K464V, L485F in NSCLC and CRC as actionable mutations that predict response to MEK inhibitors, but not to RAS inhibitors. Of note, in melanoma all *BRAF* mutations detected were the canonical V600 mutations. **Conclusions:** Low frequency, yet clinically relevant mutations in *KRAS*, *EGFR*, and *BRAF* are overlooked in many hotspot assays currently used for these genes. In this study we demonstrate that a comprehensive gene panel may detect clinically consequential rare mutations that may define patients' treatment and clinical course.

# ST096. A Novel and Accurate Real-time PCR Approach for Simultaneous Detection of Multiple Driver Gene Mutations in Non-small Cell Lung Cancer

G. Zhu, Y. Gao, F. Jiang, G. Huang, Q. Song  
Amoy Diagnostics Co., Ltd., Xiamen, China.

**Introduction:** Patients with non-small cell lung cancer (NSCLC) often harbor driver mutations in multiple oncogenes, including *EGFR*, *ALK*, *ROS1*, *BRAF*, *HER2*, *RET*, etc. The presence of somatic mutations can influence the choice of and the response to targeted therapies. Therefore, identifying mutations in oncogenes and tailoring therapy accordingly are widely accepted in clinical cancer management. Faster detection of driver mutations enables quicker initiation of proper therapy. A fast and sensitive real-time PCR based assay has been developed for detection of mutations in 9 driver genes in NSCLC at a time from a single tissue specimen. Clinical validation was conducted in the present study. **Methods:** AmoyDx Multi-Gene Mutations Detection Kit (ADx Multi-Gene assay) is a one-shot test based on real-time PCR technology for simultaneous detection of most common hotspot mutations/fusions in 9 genes associated with lung cancer. The assay was developed by combining DNA-based mutation detection (for *EGFR*, *KRAS*, *BRAF*, *NRAS*, *HER2*, and *PIK3CA* genes) and mRNA-based fusion detection (for *ALK*, *ROS1* and *RET* genes). A cohort of 1,015 formalin-fixed, paraffin-embedded (FFPE) tissue samples collected from Chinese patients with NSCLC were included in this study. DNA and RNA were isolated and tested for variants by the above assay. The qPCR assays for individual driver genes or next-generation sequencing (NGS) method were used as reference to confirm the mutation status. The concordance of variants determined with the ADx Multi-Gene assay was assessed compared to the reference. **Results:** In the cohort of NSCLC samples, 63.74% (647/1,015) were detected to show mutations, 58 of which were found with co-existing mutations in two or three driver genes. The overall concordance rate of mutations determined with ADx Multi-Gene assay compared with the reference was 96.45%. There was a 1-day turnaround time for the test. **Conclusion:** The ADx Multi-Gene assay provides accurate and efficient detection of mutations in multiple tumor driver genes simultaneously with high concordance rate of 96.45% compared to the reference assay.

# ST097. Clinicopathological and Molecular Characterization of *KIT* and *PDGFRA* Mutations in Advanced Gastrointestinal Stromal Tumors

A. Alsuwaidan, U. Verma, P. Gopal, H. Hwang, M. Wachsmann, D. Oliver  
University of Texas Southwestern Medical Center, Dallas, TX.

**Introduction:** Approximately 85% of gastrointestinal stromal tumors (GISTs) have mutations in the *KIT* proto-oncogene, leading to constitutive activation, while a subset of GISTs instead have activating mutations in the platelet-derived growth factor receptor- $\alpha$  (*PDGFRA*) gene. Both *KIT* and *PDGFRA* are tyrosine kinase receptors that can be targeted by tyrosine kinase inhibitors (TKIs). The clinical response to a TKI, such as imatinib, is associated with the specific mutation identified in *KIT* or *PDGFRA*. This study is designed to correlate specific *KIT* or *PDGFRA* mutations with clinicopathologic features of GISTs, including their effect on targeted therapy choice. **Methods:** After review of the 112 cancer samples (different anatomic sites) run by next generation sequencing (NGS) in our lab since 2016, three clinical GIST cases were selected for this study. Following histologic confirmation, slides were microdissected to enrich for tumor cells and the genomic DNA was extracted using QIAamp DNA FFPE Tissue Kit columns (Qiagen). Next-generation sequencing used Ampliseq Cancer Hotspot Panel (CHPv2) chemistry (ThermoFisher) and an Ion OneTouch System v2. Libraries were sequenced on an Ion Torrent PGM and analyzed using a bioinformatics pipeline that combined Torrent Variant Caller v5.4, Integrated Genomic Viewer v2.3, and Genetict Assistant v1.1.8.1 software. Patient electronic medical records were reviewed for clinical data. **Results:** Three cases of advanced GIST (males aged 56, 64, and 72) were analyzed. Two were rectal tumors with spindle morphology and one was gastric with epithelioid histology. The first case presented with locally advanced disease and was found to have a deletion in exon 11 of *KIT*. The second sample presented with multifocal gastric masses and contained a complex *PDGFRA* insertion/deletion (indel). Clinically, both

cases are well controlled with imatinib following surgical resection. The third case had metastases and two mutations in *KIT*: an exon 11 deletion and an exon 17 single nucleotide resistance variant (Asn822Lys). This tumor had shown initial response to imatinib but eventually progressed despite surgical resections, radiation therapy, and multiple TKIs (imatinib, sunitinib, nilotinib, regorafenib). **Conclusions:** Our data show that assessment of *KIT* mutations in GIST patients treated for locally advanced or metastatic disease can provide relevant prognostic and predictive information. We demonstrate that the same tumor may harbor sensitive and resistant mutations either simultaneously or as an acquired mutation. GISTs with *PDGFRA* mutations can also show a response to TKI therapy. Our study helps to demonstrate the clinical significance of the specific *KIT* or *PDGFRA* somatic mutations detected in these tumors using next generation sequencing technology.

# ST098. Prediction of Tumor Mutation Burden in Lung Adenocarcinoma using a 130 Gene Targeted Sequencing Panel Covering 0.23 Megabases

R.P. Joshi, S. Yang, H. Costa, H. Stehr  
Stanford University, Palo Alto CA.

**Introduction:** Tumor mutation burden (TMB) is a measure of the total number of tumor somatic mutations by whole exome sequencing and is a predictor of response to checkpoint inhibitors in lung adenocarcinoma and other cancers. Here, we investigated prediction of TMB using our 130 gene targeted sequencing Stanford Actionable Mutation Panel (STAMP) that covers 0.23 megabases and compared these predictions to that of a much larger panel. **Methods:** We used previously published lung adenocarcinoma whole exome sequencing data from The Cancer Genome Atlas (TCGA; n=501), Broad Institute (n=159), and Memorial Sloan-Kettering (n=34) as training, test, and clinical outcome datasets, respectively. We applied a cutoff of 272 whole exome mutations, corresponding to a level that predicts response to nivolumab and ipilimumab combination therapy in a recent clinical trial, to define low and high TMB. We filtered whole exome sequencing data for genes and positions present STAMP (130 genes), or genes present in FoundationOne LDT (F1-LDT; 314 genes), to create *in silico* targeted sequencing data. We created two models: (model 1) a logistic regression model using the number of targeted sequencing mutations as a predictor and (model 2) an L2-penalized logistic regression model that additionally includes the identity of individual mutated genes. We applied Cox regression to associate prediction probability scores with progression free survival in response to pembrolizumab. **Results:** Model 1 demonstrated test set area under the curve, sensitivity, and specificity of 0.84, 0.67, and 0.84 using STAMP data, and 0.96, 0.90, and 0.94 using F1-LDT data. To assess whether inclusion of the identity of individual mutated genes could improve STAMP predictions (model 2), we performed nested cross-validation within the TCGA training set. Model 1 demonstrated a mean accuracy of 0.74 and area under curve of 0.82, while model 2 demonstrated statistically significant increases in mean accuracy of 0.78 and area under the curve of 0.86. Model 2 demonstrated test set area under curve, sensitivity, and specificity of 0.90, 0.82, and 0.83 using STAMP data and 0.95, 0.87, and 0.91 using F1-LDT data. Raw probability scores from model 2 using STAMP data correlated with progression free survival in response to pembrolizumab treatment, with a concordance index of 0.707. **Conclusions:** Despite the STAMP being about one-third the size of F1-LDT, only a minor reduction in classification performance is observed when gene identities are included in addition to number of genes. In particular, the sensitivity of our prediction model was increased by inclusion of gene identities. Future directions include validation of this model using paired whole exome and targeted sequencing.

# ST099. An Analysis of the Level of Supporting Evidence used to Guide Treatment Decisions for Off-label Therapy in Cancer Precision Medicine

C.M. Statz, S.E. Patterson, T. Yin, J. Rueter, S.M. Mockus  
The Jackson Laboratory for Genomic Medicine, Farmington, CT.

**Introduction:** When standard of care has been exhausted, current practice in oncology includes seeking alternative options such as off-label therapy, especially in the context of tumor genomic profiling. To assist with



treatment decisions, genomic tumor boards are increasingly being utilized to guide treatment. Tumor board decisions are often made in conjunction with the National Comprehensive Cancer Network (NCCN) guidelines, however, it has recently been suggested that the level of evidence supporting the recommendations set forth by the NCCN is generally weak (Wagner *et al.*, 2018). Here, we sought to assess the decision process utilized within the genomic tumor board setting to recommend off-label treatment. **Methods:** The Maine Cancer Genomics Initiative, a collaboration between The Jackson Laboratory and all Maine oncology practices enables access to cancer precision medicine in the state of Maine. This includes a genomic tumor board (GTB) comprised of clinical experts from major US-based academic medical centers to help guide therapy based on genomic tumor profiling. From August 2017 to May 2018, 46 cases for advanced solid tumor patients who underwent genomic tumor profiling via ActionSeq were discussed. Data collected included tumor origin, actionable variants, variants of unknown significance, therapy recommendations, and supporting evidence. **Results:** Twenty-eight percent (13/46) of the cases reviewed by the GTB included a recommendation for off-label therapy. Tumor types included 6 colorectal, 1 lung, 2 breast, 1 cholangiocarcinoma, 1 hepatocellular carcinoma, 1 prostate, and 1 uterine. The average number of actionable variants was 2.3. 38% (5/13) of the cases included GTB recommendations corresponding to NCCN guidelines. Of those 5, 20% (1/5) of the recommendations were supported by phase II abstract data, 60% (3/5) by phase II published data, and 20% (1/5) by phase III published data. Evidence supporting GTB recommendations for the 8 cases not corresponding to NCCN guidelines comprised: an abstract with phase I data, a personal communication, and publications with preclinical (2), phase I (1), phase I/IIb (1), phase II (1), and phase III (2) data. **Conclusions:** The decision to use off-label therapy in oncology often utilizes guideline recommendations by compendiums such as the NCCN, and genomic tumor boards can provide additional guidance in cases with genomic profiling. Analysis of the decision process utilized by the GTB in this study reveals biomarker-linked therapeutic recommendations include those from the NCCN. The majority of the data comes from phase II (60%) and phase III (20%) studies and may not support the recent claim that the level of evidence underlying NCCN guidelines is low; however, due to the small sample size further analysis is warranted.

#### ST100. Bigger Nets Catch More Fish: Expanded Fusion Analysis Identifies Potential Novel Targets in Pediatric Brain Tumors

N. Willard, A. Gilani, B. Kleinschmidt-DeMasters, M. Ewalt  
University of Colorado, Aurora, CO.

**Introduction:** Since January 2017, our molecular laboratory has assessed 47 low and high grade primary pediatric brain tumors for both routine as well as novel fusions. We now retrospectively assess our results. **Methods:** BRAF mutations were assessed by the Qiagen BRAF RGQ PCR kit or Sanger sequencing. Fusion analysis was performed with the Archer FUSIONPlex Solid Tumor Kit. We reviewed all molecular testing performed in primary pediatric brain tumors at the Colorado Molecular Correlates Laboratory from January 2017 to May 2018. **Results:** Forty-seven primary pediatric brain tumor specimens were genetically analyzed. Of these, 15/47 (31.9%) harbored BRAF alterations including p.V600E mutation (10/47) and BRAF-KIAA1549 fusion (5/47). Additionally, 3/47 (6.4%) cases were better classified by WHO 2016 criteria by identification of fusions, namely two angiocentric gliomas (AGs) with near-archetypal MYK-QK1 fusion and one supratentorial ependymoma with a RELA fusion. Of particular interest, 6/47 (12.8%) cases had unexpected and potentially actionable fusions including 3 patients with FGFR1-TACC1 fusions: a 16 year female with a tectal plate pilocytic astrocytoma (PA), a 1 year male with a spinal cord PA, and a 9 year male with an extraventricular neurocytoma. Other potentially actionable fusions included 2 patients with unusual glioblastomas (GBMs): a 4 month old male with congenital GBM (MZT2B-ALK fusion) and a 3 year old female with an epithelioid GBM (ETV6-NTRK3 fusion); and a 6 year male with a thalamic PA (GOPC-ROS1 fusion in the absence of a BRAF fusion). **Conclusions:** While testing for BRAF alterations is routine in many laboratories for PAs and other glioneuronal tumors, other potentially actionable alterations can be detected by performing a more comprehensive genomic analysis. The results of this testing may identify

patients for inclusion in clinical trials for targeted therapy. In our cohort we identified 3/47 (6.4%) patients with such fusions involving ROS1, ALK, or NTRK 1/2/3 as well as 3/47 (6.4%) of patients with potentially actionable FGFR1-TACC1 fusions. These findings suggest that a more comprehensive approach to genomic profiling in pediatric brain tumors may be useful to identify patients eligible for targeted agents in addition to its diagnostic and prognostic utility.

#### ST101. Association of Microsatellite Instability and Tumor Mutation Burden

J. Au-Young<sup>1</sup>, J. Zheng<sup>1</sup>, E. Schreiber<sup>1</sup>, W. Tom<sup>1</sup>, J. Huang<sup>1</sup>, R. Chaudhary<sup>1</sup>, V. Mittal<sup>2</sup>, D. Cyanam<sup>2</sup>, E. Wong-Ho<sup>1</sup>, R. Bennett<sup>3</sup>, F. Hyland<sup>1</sup>, S. Sadi<sup>2</sup>

<sup>1</sup>Thermo Fisher Scientific, South San Francisco, CA; <sup>2</sup>Thermo Fisher Scientific, Ann Arbor, MI; <sup>3</sup>Thermo Fisher Scientific, Carlsbad, CA.

**Introduction:** The checkpoint inhibitor pembrolizumab was recently approved for treatment of any unresectable or metastatic solid tumor with microsatellite instability (MSI) or mismatch repair (MMR) deficiency. However, MSI or MMR testing is not yet part of the routine clinical workup for all tumors. In certain cancer types, tumors with MSI often have high Tumor Mutation Burden (TMB). Herein, we report the results of testing for MSI, TMB, and for mutations in MMR genes and other biomarkers to understand their associations in multiple cancer types. **Methods:** Forty-five matched formalin-fixed, paraffin-embedded (FFPE) tumor / normal pairs from CRC, NSCLC and endometrial and gastric cancer were tested for MSI and TMB. MSI analysis was performed by capillary electrophoresis fragment analysis using a panel of 8 genomic mono- and dinucleotide loci with an Applied Biosystems 3730 DNA analyzer. TMB was measured on two platforms: 1) The OncoPrint Tumor Mutation Load Assay, a targeted next generation sequencing (NGS) panel with 1.2Mb of exonic coverage, on the Ion GeneStudio S5XL using 20ng of tumor DNA. TMB results, along with mutations in MMR and other genes are reported with Ion Reporter software and 2) Whole Exome Sequencing (WES) targeting 50Mb using 100ng of tumor and normal DNA on a HiSeq X instrument. **Results:** Preliminary results obtained for a cohort of CRC FFPE matched tumor/normal pairs demonstrated that two-thirds of the samples had high MSI (MSI-H). TMB values had a positive correlation with the MSI results. For MSI-H samples, TMB ranged from 11-49 mutations/Mb for nonsynonymous somatic mutations in the exonic region using the OncoPrint TML Assay. Although MSI-H status and TMB values correlated, some of the MSI-H samples had low-to-medium TMB values. TMB values in the MSS samples were < 10 mutations/Mb. We also characterized nonsense/frameshift mutations in the MMR genes as well in additional cancer biomarker genes. Preliminary results indicate similar patterns in endometrial cancer. Further results of a larger cohort in NSCLC, gastric and endometrial cancers in addition to CRC will be reported. **Conclusions:** We further characterized the positive relationship between MSI and TMB in CRC and endometrial cancer and identified additional biomarkers in MMR genes and other cancer genes that may be informative. Multi-dimensional biomarker testing will help to elucidate the optimal approach in different cancer types.

#### ST102. Histomorphometric Features of Nuclei Architecture and Morphology in Digitized H&E Images Correlate with Mutations in EGFR and KRAS in Early-stage Non-small Cell Lung Cancer

P.D. Velu<sup>1</sup>, C. Lu<sup>2</sup>, K. Bera<sup>2</sup>, D.B. Roth<sup>1</sup>, J.J. Morrisette<sup>1</sup>, M.D. Feldman<sup>3</sup>, A. Madabhushi<sup>2</sup>

<sup>1</sup>University of Pennsylvania Health System, Philadelphia, PA; <sup>2</sup>Case Western Reserve University, Cleveland, OH; <sup>3</sup>University of Pennsylvania, Philadelphia, PA.

**Introduction:** Machine learning approaches can help bridge the data gap between molecular and anatomic pathology to achieve better understanding of genotype-phenotype relationships. More practically, visual screening of lung tumors at histologic diagnosis for molecular subtype may facilitate rush genetic testing of cases with mutations that can be treated with targeted therapies. Here we use computational approaches to explore whether visual features of nuclei from digitized lung cancer pathology images are correlated with pathogenic mutations in EGFR and KRAS found in non-small cell lung cancer (NSCLC).

**Methods:** Hematoxylin and eosin-stained (H&E) histologic guide slides for

samples submitted for targeted next-generation sequencing (NGS) on the institutional solid tumor panel (Illumina MiSeq platform) were digitally scanned at 40x resolution (Philips Intellisite). Custom scripts for pre-processing, nuclei segmentation, and nuclei feature extraction were written in Matlab R2017a. Cases that were determined by NGS to be *EGFR*+/*KRAS*- (n=50), *EGFR*+/*KRAS*+ (n=50), and *EGFR*-/*KRAS*- (n=50) were used for classifications. Median allele frequencies of *EGFR* and *KRAS* mutations were 21% (range: 2-80%) and 19% (range: 2-98%) respectively. Representative regions of tumor were annotated for each case by a pathologist and tumor nuclei were segmented using a watershed approach. A total of 1826 shape, Haralick texture, and global graph features as well as local cell cluster graph, cell run-length, nuclei orientation disorder, and diversity of cell morphology features were extracted. 100 iterations of four-fold cross validation were performed using a random forest classifier that used the top nine features as determined through minimal redundancy maximal relevance feature selection. Classification performance was measured using area under the receiver operator curve (AUC). **Results:** Global and local architectural features were the most successful at discriminating between molecular subtypes across all classifications. While the diversity of cell morphology within local tumor cell clusters also highly contributed to discrimination in classifications involving *EGFR*, general tumor nuclei shape was more important for discriminating between *EGFR*+/*KRAS*+ and *EGFR*-/*KRAS*-. The AUCs from using the top features from the best performing feature type were 0.78 for *EGFR*+/*KRAS*- vs. *EGFR*-/*KRAS*-; 0.87 for *EGFR*-/*KRAS*+ vs. *EGFR*-/*KRAS*-; and 0.71 for *EGFR*+/*KRAS*- vs. *EGFR*-/*KRAS*+. **Conclusions:** Histomorphometric features of nuclear architecture and morphology were found to be associated with *EGFR* and *KRAS* mutations in NSCLC. Further validation needs to be performed using multi-site data. To improve model performance, ongoing work includes additional feature analysis to find more robust signals.

#### ST103. Clinical Implementation of Targeted RNA Sequencing for Detecting Fusions in Solid Tumors

J. Reuther<sup>1</sup>, M.P. David<sup>1</sup>, I. Gandhi<sup>1</sup>, H. Voicu<sup>1</sup>, V. Kumar<sup>1</sup>, C.R. Perez<sup>2</sup>, C.R. Webb<sup>2</sup>, D.W. Parsons<sup>1</sup>, D. Lopez-Terrada<sup>1</sup>, K.E. Fisher<sup>1</sup>, A. Roy<sup>1</sup>

<sup>1</sup>Baylor College of Medicine, Houston, TX; <sup>2</sup>Texas Children's Hospital, Houston, TX.

**Introduction:** Gene fusion detection in cancers has traditionally relied on cytogenetics, fluorescence *in situ* hybridization (FISH) and/or RT-PCR. We describe the clinical implementation of a custom targeted next-generation sequencing (NGS)-based RNA-seq assay as a scalable, high-throughput alternative for detecting clinically relevant fusions and exon skipping events at single nucleotide resolution in formalin-fixed paraffin-embedded (FFPE) solid tumor specimens. **Methods:** Assay optimization and validation was performed to detect 73 oncogenic fusions from 59 archival specimens and 1 engineered reference specimen. Pre-analytic RNA integrity was assessed with an optimized quantitative PCR-based assay. Dual-indexed NGS libraries with molecular barcoding were prepared with 100ng total RNA using anchored multiplex PCR-based chemistry (ArcherDx Inc.) targeting 485 exons in 81 genes recurrently involved in fusions in solid tumors. Primer efficiency was determined using the Universal Human Reference RNA (UHRR). Subset of clinical specimens underwent integrated sequencing with a targeted hybridization capture DNA panel featuring the coding region of 124 genes. DNA and RNA libraries were multiplexed and sequenced on an Illumina MiSeq (2x150bp) generating a mean of 2,935,447 total reads per sample. Data analysis for deduplication and variant/fusion detection was performed on a custom DNA pipeline and a virtual-machine hosted RNA pipeline (Archer Analysis). **Results:** At a  $\geq 40$  tumor% cutoff, the assay detected 73/73 (100%) fusions with  $\geq 3$  split reads (range 5 – 8,209) and 2/2 exon skipping events in *MET* and *EGFR*, exhibiting 100% sensitivity and 97.22% specificity. Analysis of the UHRR specimen and 17 formalin-fixed, paraffin-embedded (FFPE) specimens validated the performance of 92% of primers, confirming their high efficiency in gene expression detection. Clinical implementation on 31 specimens to date has yielded significant findings in 42% (n=13) of cases, clarifying the diagnosis in 5/13 cases, and refining and/or confirming the histological diagnosis in 8/13 cases with confounding differential diagnoses; significantly, unexpected fusions were

identified in 2 cases, including a targetable *ETV6-NTRK3* fusion, and an *EWSR1-ERG* fusion in a FISH-negative Ewing sarcoma. Moreover, integrated DNA and RNA sequencing in a subset of cases detected alterations of potential or established clinical utility in 5/11 (45%) cases, with DNA and RNA sequencing identifying alterations in 3/11 and 2/11 cases, respectively, with all alterations being mutually exclusive.

**Conclusions:** The targeted RNA-seq assay offers robust, sensitive and broad detection of targetable and diagnostic gene fusions using minimal RNA input highlighting the potential utility of unbiased sequencing in capturing the gene fusion landscape in cancers.

#### ST104. Development and Analytical Validation of Colorectal Cancer Specific Next-generation Sequencing Gene Panel for Cell-free DNA (cfDNA) Based Molecular Testing of Disease Progression

S. Zalles<sup>1</sup>, D.Y. Duose<sup>2</sup>, P. Hu<sup>2</sup>, E.S. Kopetz<sup>2</sup>, R. Luthra<sup>2</sup>, S. Mallampati<sup>2</sup>  
<sup>1</sup>University of Texas MD Anderson Cancer Center School of Health Professions, Houston, TX; <sup>2</sup>University of Texas MD Anderson Cancer Center, Houston, TX.

**Introduction:** Approximately 21% of newly diagnosed Colorectal Cancers (CRC) are stage IV disease indicating metastasis to distant organs. Chemotherapy is the standard of care for most stage IV patients and response to therapy is assessed through imaging technologies like MRI and CT scans. Recent studies have demonstrated that imaging methods detect response to treatment later than molecular based methods. Therefore, developing a technique that is minimally invasive, yet highly sensitive at detecting tumor burden would be valuable in disease management of CRC patients. It is well established that circulating cell free DNA (cfDNA) also includes DNA originating from tumor cells, thus providing a surrogate source of genetic material for tumor mutational profiling using Next Generation Sequencing (NGS)-based approaches. Incorporation of unique molecular identifiers (UMIs) has been shown to improve detection sensitivity, and allow identification of low level variants accurately. Most UMI approaches cannot completely eliminate false positive variants below 1%. To overcome this limitation, we developed a custom panel and bioinformatics pipeline that accurately identifies low frequency variants down to 0.3%. **Methods:** We designed an 85 kb colorectal cancer specific NGS panel that covers ~85% of most frequently mutated genes in colorectal cancer. Analytical validation was performed with cfDNA from cultured cell line supernatants, plasma from 30 CRC patients, and 15 healthy donors. We also evaluated response to treatment in 5 patients at 3 time points. Libraries were made with NebNext Ultra II library prep kit (New England Biolabs); the target regions were enriched with custom RNA bait sequences, final libraries from 21 samples were pooled and sequenced on NextSeq (Illumina). Data analysis was performed with a custom bioinformatics pipeline to derive consensus reads from UMIs, model position specific errors in normal cfDNA controls and eliminate sequencing background errors in patient samples. **Results:** The limit of detection of our custom CRC panel is 0.3 % MAF with a 95% sensitivity (95% CI 89%-98%) and 100% specificity (95% CI -100%-100%). We also found that the CRC panel could efficiently track multiple variants in the longitudinally collected cfDNA samples from 5 patients evaluated. **Conclusion:** Our results suggest that by detecting low level mutations present in cfDNA, this panel could be used to monitor and determine a patient's response to treatment.

#### ST105. Inhibitory Effects of Toluidine Blue on RNA Sequencing Library Preparation

K.J. Hampel, N. Sidiropoulos

The University of Vermont Medical Center, Burlington, VT.

**Introduction:** Non-cell block (NCB) cytology specimens are increasingly utilized for genomic analysis placing new emphasis on evaluation of cytopreparatory procedures. Rapid on-site evaluation (ROSE) of fine-needle aspirates (FNA) is an essential procedure to optimize specimen adequacy for genomic analysis. The two most common staining procedures utilized to visualize cells on site are Differential Quik and toluidine blue, a stain with high affinity for nucleic acid. Slides stained with toluidine blue on-site are subsequently submitted for Papanicolaou (Pap) staining. We report our investigation of inhibitory effects of toluidine blue staining used for ROSE on clinical RNA sequencing library preparation.

**Methods:** RNA quality assessment was carried out using ArcherDx

reagents. This is a preliminary step in sequencing library preparation and part of our 30 gene solid tumor panel. Pap staining of smear slides from FNAs, with or without preliminary ROSE with toluidine blue, was carried out using Gill's modified-Papanicolaou staining procedure. **Results:** The onset of RNA sequencing failures of nucleic acid derived from Pap-stained cytology slides was attributed to ROSE procedures by first eliminating failed processes internal to the Genomic Medicine Laboratory. Pap and May-Grünwald Giemsa (MGG) stained slides undergo identical processing in the Genomic Medicine laboratory, but only Pap-stained slides consistently failed analysis. Spiking Pap slide-derived RNA that had failed analysis into extracts that had previously passed RNA sequencing analysis caused the previously passing samples to fail. This indicated the presence of a soluble RNA processing inhibitor in the failed samples. Suspicion fell on toluidine blue among other nuclear staining reagents, such as hematoxylin, used in the procedure. Finally, Pap stained slides that had not been previously evaluated on-site with toluidine blue were shown to consistently pass RNA sequencing analysis. ROSE process changes that prolonged toluidine blue contact with cellular material could be tied to RNA quality failures. **Conclusions:** ROSE is a valuable procedure to mitigate NCB cytology specimen insufficiency for genomic analysis. It is important for Genomic Laboratories to be aware of on-site staining procedures and modification of ROSE processes as they can negatively impact testing. The implementation of processes to ensure communication and pre-emptive validation of cytopathology procedural changes when Toluidine blue is used for ROSE is recommended.

#### ST106. Single-Vial Amplification Based NGS with Rapid Turn-Around-Time for Interrogation of Variants in Tumors with Limited Diagnostic Material

S. Barua<sup>1</sup>, C. Pagan<sup>1</sup>, V. Hodel<sup>1</sup>, M. Ko<sup>1</sup>, E. Petrilli<sup>2</sup>, S. Hsiao<sup>1</sup>, M. Mansukhani<sup>1</sup>, H. Fernandes<sup>1</sup>

<sup>1</sup>Columbia University Medical Center, New York, NY; <sup>2</sup>Pillar Biosciences Inc., Natick, MA.

**Introduction:** Targeted Next Generation Sequencing (NGS) is the primary assay for interrogation of variants in tumors, in several clinical laboratories. Due to limitations in the amount of tumor material available for testing, NGS libraries that use amplification methodologies have been successfully sequenced for patient care. However, the total DNA input, PCR artifacts, compromised DNA quality, increased turn-around-time and ease-of-use, are factors that hamper the universal adoption of NGS assays in routine diagnostics. We evaluated the single tube Stem-Loop Inhibition Mediated Amplification (SLIMamp) technology (Pillar Biosciences) for accuracy and sensitivity in the detection of variants present in solid tumors. **Methods:** Forty previously tested formalin-fixed, paraffin-embedded (FFPE) samples harboring 15 different clinically relevant variants present in 7 genes were included in the evaluation. Variants present were previously detected using either, TruSeq Amplicon Cancer Panel (Illumina) (N=35) or Sanger sequencing (N=5). 3/40 samples were wildtype. Sensitivity studies ranging from 2.5ng – 20 ng input DNA, were performed with 4 samples that harbored differing variants. NGS libraries, using the ONCOResolve Multi-Cancer Panel (Pillar Biosciences) were prepared with DNA input ranging from 2- 94ng. For each run, up to 24 samples were normalized, pooled and run on the MiSeq (Illumina). Data analysis including sequence alignment, variant calling and annotation was performed using FASTQ files, with the Pillar Variant Analysis Toolkit (PiVAT). FASTQ files were also analyzed on NextGENE for comparison. **Results:** All 40 samples were successfully sequenced. Overall, there was 100% concordance between the 38 variants identified using the SLIMamp technology and previously validated technologies. The alterations included missense variants (N=29), insertion/deletions (indels) (N=8) and a splice variant. The mutant allele fraction (MAF) percentage in the samples, ranged from 3% to 80%. Correlation of MAF obtained by the two different methodologies was excellent ( $R^2=0.94$ ). The on target percentage was >99% and average coverage obtained across the samples was 3,731x. Sensitivity studies demonstrated that missense variants and indels with MAF of 3% or more were reliably detected at 2ng input DNA. **Conclusions:** Interrogation of variants in solid tumors using the SLIMamp technology can identify actionable alterations, including missense variants and indels in tumors where the input DNA is as low as 2ng. Assays that use the technology can

detect variants with 3% MAF in samples with low input DNA. The simplicity of library preparation together with a rapid turn-around-time of 3-4 days from sample to answer, allows for viable implementation of SLIMamp technology in clinical laboratories.

#### ST107. Clinical Validation of MSK-ACCESS: An Ultrasensitive Next-generation Sequencing Assay for Liquid Biopsies in the Clinic

A. Brannon, G. Jayakumar, J. Patel, I. Johnson, M. Diosdado, M. Hasan, D. Perera, F. Meng, X. Jing, J. Reichel, Y. Hu, A. Syed, B. Li, A. Zehir, B. Houck-Loomis, M. Ladanyi, M. Arcila, R. Benayed  
Memorial Sloan Kettering Cancer Center, New York, NY.

**Introduction:** Knowledge of the genomic alterations underlying the tumor biology at diagnosis, recurrence, and disease progression is important for proper diagnosis and making suitable treatment decisions. However, tumor biopsies cannot always be obtained for many reasons. Tumor DNA alterations can be detected in the plasma of cancer patients, sometimes called "liquid biopsy", providing the necessary clinical information in a less invasive manner while also overcoming the temporal and spatial heterogeneity inherent to standard tumor biopsies. Liquid biopsy can also potentially assess minimal residual disease and identify recurrence earlier than classic radiographic or pathologic means. Here, we describe the design, development and validation of MSK-ACCESS (Analysis of Circulating cfDNA for the Evaluation of Somatic Status), a laboratory-developed, ultrasensitive assay to profile mutations, copy number alterations, and fusion events in clinically actionable genes. **Methods:** Plasma cfDNA for tumor analysis and buffy-coat DNA for germline comparison were extracted from whole blood collected in cell-stabilizing tubes (STRECK BCT cell-free DNA tube). Unique molecular identifiers (UMIs) and dual index barcodes were introduced during DNA library construction. Libraries were captured with a 200kb bait panel designed to interrogate frequently mutated sites in 129 genes, copy number alterations, structural variants, and MSI status. Altogether, these target regions were chosen because they collectively contained at least 1 mutation in 84% of >20,000 tumors, 94% of breast tumors, and 96% of NSCLC tumors analyzed with our institutional clinical sequencing assay MSK-IMPACT. Sequencing reads were first aligned to human genome (hg19) after UMI clipping (primary alignment), then reads originating from the same DNA fragments were collapsed into consensus read sequences using either an in-house developed algorithm or *fgbio* (Fulcrum Genomics). These consensus reads were aligned to the human genome followed by variant calling and genotyping. **Results:** We find that MSK-ACCESS can detect variants down to 0.1% allele fraction based on dilution experiments. A series of plasma samples from healthy individuals were used to create a background error profile for each locus. Further, we analyzed plasma samples from more than 60 patients with MSK-ACCESS and identified variants known to be present in the plasma based upon commercial cfDNA assays performed on the same plasma collection. Intra- and inter-assay reproducibility studies show variants are detected reliably. **Conclusions:** MSK-ACCESS successfully identifies clinically actionable mutations in cancer patients using less invasive techniques, reducing the need for biopsies or rebiopsies.

#### ST108. Suboptimal Somatic Mutation Detection for EGFR by the OncoScan CNV Plus Assay

S.C. Smith, M.S. Farooqi, J.M. Joyce, T.M. Bendorf, K.J. Detherage, K.D. Harris, A.D. Skipp, L.D. Cooley  
Children's Mercy Hospital, Kansas City, MO.

**Introduction:** The OncoScan CNV Plus Assay (OS+) (Thermo Fisher Scientific (TFS)) is a copy number microarray-based test optimized for formalin-fixed paraffin-embedded (FFPE) specimens. It has been clinically validated and used by our laboratory since July, 2016. OS+ can also detect 74 hotspot somatic mutations (SMs)—both single nucleotide variants (SNVs) and insertions/deletions (indels)—in 9 genes (BRAF, EGFR, IDH1, IDH2, KRAS, NRAS, PIK3CA, PTEN, and TP53) via molecular inversion probes. This capability was not examined in our initial validation. Here, we do so using a cohort of pediatric (ped) tumor samples. **Methods:** We reviewed SM data generated by OS+ for all brain tumors (BT) run between 7/12/16 and 5/1/18 (n=46). DNA was isolated using a Maxwell RSC instrument and FFPE kit (Promega). The OS+ assay was performed per TFS's instructions; samples were processed



using Chromosome Analysis Suite (ChAS) software versions 3.0 and 3.3 (TFS). A subset of samples were verified via bi-directional Sanger sequencing (SS) (BigDye Terminator v3.1 Kit, TFS) using lab-developed primers and Sequencher (v.5.4.6, GeneCodes) analysis. **Results:** Of 46 cases, 15 were negative (neg) and 31 positive (pos). OS+ called 71 SMs: 28 high-confidence calls (HCc) and 43 low-confidence calls (LCc), involving *BRAF* (3 HC, 2 LC), *EGFR* (11 HC, 18 LC), *IDH1* (3 HC, 4 LC), *IDH2* (1 LC), *KRAS* (5 LC), *NRAS* (2 LC), *PTEN* (5 LC), and *TP53* (11 HC, 6 LC). We performed SS for 54 / 71 calls (27 HC, 27 LC) and for 21 hotspots in the 15 OS+ neg cases. The latter and all 27 LCc by OS+ were neg by SS. All HCc (n=16) not in *EGFR* were pos by SS. For *EGFR*, only 1 / 11 HCc was pos by SS; 10 / 11 HCc were neg and represented a range of SNVs and indels. TFS recommended a software update (ChAS v3.3) and data reprocessing. Subsequently, OS+ gave only HCc and neg calls; now, 26 cases were neg and 21 pos; OS+ called 26 SMs: 25 of the original (o) 28 HCc were still called (3 oHCc (all in *EGFR*) were now neg); 1 oLCc (in *TP53*) was now a HCc; 42 oLCc were now neg. For *EGFR*, 7 / 10 discordant oHCc were still called HC by ChAS v3.3; these HCc were seen regardless of *EGFR* amplification status. **Conclusions:** These discordant *EGFR* SMs are very likely false pos calls by OS+. The St. Jude PeCan dataset found *EGFR* SMs in only 7 / 769 ped BT; none of which were in exons 18, 19, or 21; however, SMs in these exons were called by OS+ in our dataset. One *EGFR* exon 20 insertion (p.D770>ASVD) was observed by PeCan in ped BT; interestingly, this same SM was the only concordant *EGFR* SM between OS+ and SS above. While SM detection by OS+ appears accurate for certain genes, *EGFR* calls should be treated with caution. Review of data from additional tumor samples is ongoing to examine genes not yet evaluated by this study (e.g. *PTEN*).

#### ST109. Clinical Experience of a Next-generation Sequencing Assay that Evaluates Common Somatic Mutations and Rearrangements in Patients with Lung Cancer

S. Knight, J. Voss, S. Kerr, S. Sankaranarayanan, B. Kipp  
Mayo Clinic, Rochester, MN.

**Introduction:** Next-generation sequencing (NGS) has been shown to be an accurate, cost-effective method to identify alterations across numerous genes from a variety of tumor types. Our institution recently launched a clinically validated NGS assay that assesses for common somatic mutations and gene fusion transcripts involving 11 lung cancer associated genes. The aim of this study was to evaluate the performance characteristics of this panel in routine clinical practice. **Methods:** We analyzed the pathogenic mutation and fusion results of samples reported with our lung cancer NGS assay between January 1, 2017 and April 30, 2018 (484 days). This assay utilizes total nucleic acid (TNA) extracted from formalin-fixed, paraffin-embedded (FFPE) tissue or cytology slides to reduce the amount of tumor cells needed in a single extraction. A custom designed primer pool was used to assess DNA for somatic mutations in hotspot regions of *EGFR*, *BRAF*, *KRAS*, *HRAS*, *NRAS*, *ALK*, *ERBB2*, and *MET* genes. The Ion AmpliSeq RNA Fusion Lung Cancer Research Panel primer pool was used to assess RNA for rearrangements (gene fusions) involving *ALK*, *ROS1*, *RET*, and *NTRK1* genes. Samples were sequenced on an Illumina MiSeq or HiSeq 2500 instrument and raw data was processed through automated custom bioinformatics pipelines.

**Results:** Fourteen hundred and seventy-two samples were included in this review. The majority of submitted samples were FFPE tumor tissue (86%) and the remaining 211 samples were cytology slides. Complete assay failure rate was 1.3% with 19 samples (15 FFPE, 4 cytology) having no mutation or fusion results. There were 27 (1.8%) samples (22 FFPE, 5 cytology) reported with only one component of the assay due to failure of the corresponding component (16 samples with fusion results only and 11 samples with mutation results only). 1,426 samples successfully obtained both mutation and fusion results. 808 (56.7%) samples were reported with a pathogenic mutation and/or a fusion (i.e. positive result), including 94.7% (765/808) reported with a pathogenic mutation only, 5.0% (40/808) with a fusion only, and 0.3% (3/808) with both a pathogenic mutation and a fusion. The remaining 618 (43.3% of 1426) samples were negative for pathogenic mutations and fusions. 810 pathogenic variants (670 SNPs and 140 insertion/deletions or INDELS) involving *KRAS* (n=464), *EGFR* (n=186), *BRAF* (n=59), *MET* (n=46), *ERB*

*B2* (n=24), *NRAS* (n=13), *HRAS* (n=9), *ALK* (n=9) and 41 fusions involving *ALK* (n=27), *ROS1* (n=9), *RET* (n=5), *NTRK1* (n=0) were reported. **Conclusions:** Our clinically implemented lung cancer NGS assay has the ability to detect multiple mutations and fusions in a single multiplex assay, including therapy directing and resistance markers. The assay has a very low failure rate, despite its use on limited specimens such as small FFPE tissue biopsy specimens and residual cytology slides.

#### ST110. Next-generation Sequencing for the Detection of Actionable Genetic Alterations in Advanced Breast Cancer: Should We be Testing and How?

S.E. Abbott<sup>1</sup>, R. Broadus<sup>1</sup>, M.J. Routbort<sup>1</sup>, S. Roy-Chowdhuri<sup>1</sup>, A. Yemelyanova<sup>2</sup>, A. Rashid<sup>1</sup>, R. Luthra<sup>1</sup>, K.P. Patel<sup>1</sup>, H. Chen<sup>1</sup>

<sup>1</sup>The University of Texas MD Anderson Cancer Center, Houston, TX; <sup>2</sup>The University of Alabama at Birmingham, Birmingham, AL.

**Introduction:** Use of generic solid tumor next-generation sequencing (NGS) panels is becoming more routine in clinical practice. Such panels, however, may not have equal relevance across all solid tumor types. Furthermore, there is a need to balance stewardship of resources, often including limited tumor sample, with providing precision oncology care. Thus, our objectives were to 1) determine whether the frequency of potentially actionable genetic alterations identified in advanced breast cancer warrants routine NGS panel testing in this population and 2) if warranted, assess whether a smaller, "hotspot" NGS panel differs significantly from a more comprehensive panel in its ability to identify patients who may benefit from targeted therapy. **Methods:** We included all advanced breast cancer patients who had NGS performed from 2016-2017 (n=142; 50% ER+/HER2-, 39% triple negative, 11% HER2 amplified) using 1 of 2 panels: 1) a 50-gene panel with 38 actionable genes (50-GP; n=59) detecting only mutations and typically requiring 10 ng DNA and no control tissue or 2) a 134-gene panel with 66 actionable genes (134-GP; n=83) detecting both somatic mutations and amplifications, but requiring ≥20 ng DNA and control non-tumor sample. A clinically actionable gene was defined as one that can be therapeutically targeted. *ESR1* was also included due to its prognostic significance. **Results:** Overall, 49% of advanced breast cancer patients in this cohort were found to have at least one alteration in an actionable gene regardless of NGS panel used, a finding which persisted across all breast cancer subtypes (58% in ER+/HER2-, 38% in triple negative, 50% in HER2 amplified). When comparing the capacity of the 50-GP and the 134-GP to identify patients who may benefit from targeted therapy, the 134-GP identified significantly more patients with at least one alteration in a potentially actionable gene among total patients (61% versus 32%, p<0.01), as well as within the ER+/HER2- (73% versus 39%, p<0.01) and triple negative (50% versus 24%, p<0.05) subtypes. Common somatic mutations identified in the 38 actionable genes shared between the 2 panels were *PIK3CA* (25%), *ERBB2* (4%), and *PTEN* (4%). Frequent alterations in actionable genes unique to the 134-GP included *ESR1* (14%) and *NF1* (6%) mutations and *CCND1* amplification (11%). **Conclusions:** Alterations in actionable genes occur with sufficient frequency to justify NGS panel testing in all subtypes of advanced breast cancer. The 134-GP, which covers 28 additional actionable genes and detects amplifications, identifies significantly more patients who may benefit from targeted therapies and should be preferentially used. When testing requirements for the 134-GP cannot be met, the 50-GP still provides utility, albeit with less coverage.

#### ST111. Validation of the MSI Analysis System, Version 1.2 (Promega) Using the ABI 3130XL Genetic Analyzer System

J. Sen, C. Chang, A. Walden, K. Curless, L. Cheng  
Indiana University Health, Indianapolis, IN.

**Introduction:** Microsatellite instability (MSI) is a condition that results from impaired DNA mismatch-repair (MMR) activity. MSI is a good marker for determining hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) and the presence of MSI is a marker for a favorable prognosis of cancer treatment. The MSI Analysis System, Version 1.2 (Promega), is a fluorescent PCR-based assay to detect microsatellite instability (MSI) in human cells. It involves comparing allelic profiles of microsatellite markers generated by amplification of DNA from matching normal and tumor samples, which may be mismatch-repair deficient. Alleles that are present

in the tumor sample but not in corresponding normal samples indicate MSI. **Methods:** A total of 71 samples were included in this study. These included 49 in-house samples that were previously tested for MMR protein status by Immunohistochemistry (IHC). Also included were 22 samples, previously tested for MSI, at outside institutions. All 71 samples were amplified using the MSI Analysis System, Version 1.2 (Promega). The PCR products were separated by capillary electrophoresis using an Applied Biosystems 3130xl Genetic Analyzer, and the output data analyzed with ABI GeneMapper software. All samples included both tumor tissue and normal tissue. The MSI Analysis System includes fluorescently labeled primers for co-amplification of seven markers including five mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and two pentanucleotide markers (Penta C and Penta D). The mononucleotide markers are used for MSI determination, and the pentanucleotide markers are used to detect potential sample mixups or contamination (determines that the tumor and normal tissue belong to the same patient). **Results:** Of the 22 cases from outside institutions, the MSI Analysis System, Version 1.2 (Promega) detected eleven cases of MSI-H (high), 1 case of MSI-L (low) and 10 cases of MSS (Microsatellite stable). All 22 cases correlated with the expected results. In addition, of the 49 in-house samples, 46 samples matched with their corresponding IHC result. Two samples showed no loss of staining with IHC but were MSI-H and the third showed loss of staining with IHC but was MSS with the MSI Analysis System. **Conclusions:** Our testing, with the MSI Analysis System, Version 1.2 (Promega) matched for all the expected results with previously tested samples for MSI. The 3 samples where IHC results and MSI PCR did not match were determined not to be true discrepancies – since such correlation between MSI-PCR testing and IHC fits with studies in literature. The MSI analysis protocol, using the MSI Analysis System, Version 1.2 (Promega) has been deemed acceptable based on our validation studies and will be used in clinical testing.

#### ST112. Development of a Plasma-based Method for Microsatellite Instability (MSI) Detection using a Next-generation Sequencing Panel

M.I. Lefterova<sup>1</sup>, A. Artyomenko<sup>1</sup>, M. Sikora<sup>1</sup>, C. Barbacioru<sup>1</sup>, V.M. Raymond<sup>1</sup>, D. Gavino<sup>1</sup>, C. Artieri<sup>1</sup>, E. Helman<sup>1</sup>, D. Chudova<sup>1</sup>, R.B. Lanman<sup>1</sup>, J.I. Odegaard<sup>1</sup>, J.A. Willis<sup>2</sup>, M. Fakih<sup>3</sup>, S. Kopetz<sup>2</sup>, A. Talasz<sup>1</sup>  
<sup>1</sup>Guardant Health, Redwood City, CA; <sup>2</sup>The University of Texas MD Anderson Cancer Center, Houston, TX; <sup>3</sup>City of Hope National Medical Center, Duarte, CA.

**Introduction:** Microsatellite instability (MSI) is a guideline-recommended biomarker with prognostic significance in a variety of tumor types as well as predictive significance for treatment with checkpoint inhibitors. However, published data on MSI testing practices suggest many patients are undergenotyped for MSI despite guideline recommendations. A plasma-based MSI detection method could provide non-invasive, real-time assessment of MSI status, especially when combined with comprehensive genomic profiling of the tumor. However, to be clinically effective, blood-based diagnostics must be highly sensitive and account for tumor shedding. Here, we present an accurate and sensitive cfDNA-based MSI caller using a 73-gene next generation sequencing (NGS) panel.

**Methods:** We developed a method to assess MSI status using targeted sequencing of cfDNA on the Guardant360 clinical platform, which allows broad coverage of simple repeats. For each microsatellite locus, the number of differentially sized repeats in a sample is quantified using a probabilistic log likelihood-based score. This approach is designed to accurately discriminate biological signal derived from a small number of cfDNA fragments of somatic origin from noise arising from technical artifacts. Loci are considered unstable if the likelihood score is statistically greater than a threshold computed from a cohort of normal samples. The scores of individual unstable loci are integrated into an overall MSI score for the sample. The performance of this method was optimized on simulated data and normal samples, and then validated on an independent cohort of 134 (7 MSI-H, 127 MSS) clinical samples from several cancer types for which tissue-based MSI results were available.

**Results:** We simulated MSI high (MSI-H) samples across a range of tumor fractions by combining data from 82 healthy donor samples with *in silico* spike-ins of differentially-sized repeats. This simulation predicted a sensitivity of 94% at 0.2% tumor fraction (limit of detection) at an expected

specificity of 99.9%. When applied prospectively to a test set of 134 advanced cancer cfDNA samples with maximum somatic variant allele fractions ranging from 0.1%-15%, this method demonstrated 98.5% specificity (125/127) and 86% sensitivity (6/7) relative to tissue-based MSI assessment. **Conclusions:** We describe a plasma-based MSI assay that is highly concordant with tissue-derived MSI at tumor fractions down to 0.1%, enabling highly accurate detection of MSI even for samples with low tumor content. Accurate reporting of MSI status performed concurrently with comprehensive genomic profiling allows potential access to immunotherapies for patients whose tumor types are not routinely tested for MSI.

#### ST113. Concomitant PD-L1 Expression and Driver Oncogenes in Non-small Cell Lung Cancer

N. McNeill<sup>1,3</sup>, J.J. Horton<sup>1,3</sup>, T.A. Freitas<sup>1</sup>, M. Shiller<sup>2</sup>  
<sup>1</sup>Med fusion, Lewisville, TX; <sup>2</sup>Pathologists Bio-Medical Laboratories, Dallas, TX; <sup>3</sup>Quest Diagnostics, Lewisville, TX.

**Introduction:** Immune checkpoint inhibitors (I-O) have offered an effective therapeutic option for patients with non-small cell lung carcinoma (NSCLC). Similarly, NSCLC with identified oncogenic drivers also have efficacious therapeutic options. PD-L1 by immunohistochemistry (IHC) is a method to assess the presence of PD-L1 expression to establish I-O therapy candidacy. However, unlike most oncogenic drivers in NSCLC, its expression is not mutually exclusive with the presence of other genetic biomarkers and the reported frequency of overlap in routine testing has been inadequate. **Methods:** A one-year retrospective analysis of 332 NSCLC cases tested in our CLIA-certified laboratory was carried out to investigate the presence of concomitant PD-L1 expression and oncogenic drivers. Gene mutations were detected from formalin-fixed, paraffin-embedded (FFPE) tumor specimens by next generation sequencing using the Ion Ampliseq Cancer Hotspot Panel V2 with the Ion PGM sequencing platform. Gene rearrangements/amplifications were detected from interphase nuclei of lung carcinoma cells derived from FFPE tumor tissue by multiplex fluorescence *in situ* hybridization. PD-L1 expression was assessed by IHC analysis with the 22C3 antibody using the tumor proportion. **Results:** 62.05% of NSCLC patients tested for PD-L1 exhibited PD-L1 overexpression, 24.7% high and 37.35% low. The most frequent oncogenic driver with high PD-L1 expression (n=82) involved KRAS (29.27%) followed by MET amplification (8.54%), and alterations in BRAF (7.32%), EGFR (6.10%) and PIK3CA (3.66%). In patients with low PD-L1 expression (n=124), the most frequent genetic abnormality again involved KRAS (20.16%), followed by alterations in EGFR (10.48%), MET amplification (7.26%), PTEN (6.45%), BRAF (5.65%), PIK3CA (5.65%), ALK translocation (2.42%), TP53 (2.42%), and HER2 (1.61%). In patients with no PD-L1 expression (n=126), mutations in EGFR (23.02%) and KRAS (23.02%) were the most common followed by alterations in PIK3CA (6.35%), PTEN (4.76%), MET amplification (3.97%), HER2 (1.59%), and NRAS (1.59%). **Conclusion:** PD-L1 expression occurs concomitantly with oncogene drivers in 51.22% of high and 54.84% of low NSCLC cases. KRAS mutation occurred most frequently in PD-L1 expressing patients, particularly in high expression at ~30%. EGFR mutations were present at a higher frequency in low compared to high expression. FDA-approved therapies assume EGFR and ALK-negative status prior to prescribing I-O therapy. Turnaround times for these biomarkers vary, impacting therapeutic decisions which are often unchanged when therapy is initiated prior to release of all results. More data is needed to understand the sequence of therapy delivery based on biomarker findings, ultimately optimizing tissue stewardship in limited specimens.

#### ST114. HOXB13 IHC Expression and Mutational Profile in Ductal Adenocarcinoma of the Prostate

S. Zomorodian<sup>1</sup>, F. Saeed<sup>1</sup>, M. Zhong<sup>1</sup>, J.T. Fallon<sup>1</sup>, J. Zhuge<sup>1</sup>, J.I. Epstein<sup>2</sup>, F. Ye<sup>1</sup>

<sup>1</sup>Westchester Medical Center, Valhalla, NY; <sup>2</sup>The Johns Hopkins Medical Institutions, Baltimore, MD.

**Introduction:** HOXB13 encodes for a transcription factor involved in prostate development and expressed at a high level of in normal prostate and most prostatic adenocarcinomas by IHC (immunohistochemistry). Previous studies have implicated the germline HOXB13 single nucleotide variant (SNV) G84E in roughly 5% of

familial prostatic adenocarcinomas. However, ductal prostatic carcinomas have not been studied with regards to this gene. This study originally sought to examine HOXB13 expression in prostatic ductal cancer by IHC. The IHC results observed led to subsequent sequencing of the gene in order to deduce a potential role for HOXB13 mutations in carcinogenesis of prostatic ductal adenocarcinoma. **Methods:** Thirteen cases of ductal adenocarcinoma containing separate areas of acinar cancer and 89 cases of pure acinar carcinoma were subjected to HOXB13 immunostaining. 12 of the ductal carcinoma cases were macrodissected to isolate tumor for bidirectional Sanger sequencing. Genome databases were utilized to analyze the data. **Results:** All benign prostatic luminal cells were positive for HOXB13. 81/89 (91%) cases of acinar carcinoma were positive for HOXB13. However, only 4/13 (31%) ductal prostate adenocarcinomas were positive for HOXB13. The acinar carcinoma component associated with the ductal carcinoma was positive in 6/13 (46%) cases. Subsequent Sanger sequencing revealed 9 mutations in HOXB13 in 5 out of 12 samples. All mutations observed were SNVs. Only two distinct mutations were shared amongst different cases, one of them shared amongst two cases (c.513T>C, p.Ser171), the other shared amongst three different cases (c.366C>T, p.Ser122). However, these mutations were synonymous and not located at the splice site. Three non-synonymous missense mutations were identified by Sanger sequencing. One was the aforementioned Gly84Glu, categorized as pathogenic in COSMIC. Two were not identified in COSMIC (c.224C>T, p.Pro75Leu and c.824T>C, p.Leu275Pro), but designated as being of uncertain significance in Varsome due to the absence of their alleles in gnomAD. **Conclusions:** Compared to usual acinar carcinoma, ductal carcinomas showed more frequent loss of HOXB13 expression. This suggested the possibility that down regulation of HOXB13 is involved in prostatic ductal carcinogenesis. However, this study did not find a candidate mutation that could account for the lack of expression of HOXB13. Sequencing of the promoter region of HOXB13 might yield answers in this regard. The G84E mutation has not been reported to date for ductal prostate adenocarcinoma. Future high-power studies on ductal adenocarcinomas are warranted to identify the frequency of this mutation in this cancer subvariant.

#### ST115. Novel Solvent-Free Deparaffinization Method for FFPE Sample Prep Enabling a More Convenient Workflow

A. Cheng, N. Hernandez, X. Fang  
Thermo Fisher Scientific, Austin, TX.

**Introduction:** Formalin-fixed paraffin embedded (FFPE) is the most common preservation method to archive solid tissue samples. Traditionally, FFPE samples have to be deparaffinized prior to proteinase digestion and nucleic acid extractions. The gold-standard for deparaffinizations involves a toxic and flammable organic solvent, xylene. In addition to the health hazards and burdensome of chemical waste disposal, it involves multiple rounds of washes with xylene and ethanol, which could lead to tissue loss. Alternative methods using less toxic solvents exist, but the protocol is still tedious. Here, we present a novel solvent-free method to deparaffinize FFPE samples using the Autolys M spin tubes. These tubes eliminate the need for organic solvents, alcohols, and manual washing steps with only one pipetting step needed. Hands-on time is decreased resulting in more convenience and most importantly, tissue loss is minimized. These tubes can work upfront different extraction methods but it has been validated with the MagMAX FFPE Ultra kit and the KingFisher Purification Systems. **Methods:** Different deparaffinization methods (Autolys M tubes versus xylene versus an organic separation method) were compared in lung, colon and breast FFPE cancer tissues. For the Autolys M tube method, the Autolys M TubeLifter was used to process the tubes. After the different deparaffinization methods, the samples were treated together under the same extraction conditions and sequential DNA and RNA from the same sample were isolated using the MagMAX FFPE Ultra kit automated on the KingFisher Flex Purification instrument. DNA and RNA concentrations were measured with the Qubit and the nucleic acids were functionally tested by real-time PCR on the QuantStudio 12K Flex system and next-generation targeted sequencing on the Ion Torrent sequencing platform. **Results:** Overall, yield and functionality of the nucleic acids were comparable or even slightly better with the Autolys tube method as assessed by Qubit, real-time PCR and high sequencing parameters. DNA sequencing metrics include >97%

uniformity, >112bp mean read length, >97% on target and comparable variant calling frequencies among the methods. RNA sequencing metrics include high mean read lengths >132bp and >99% targets detected. RNA gene expression correlation plots comparing the different methods also provided an  $R^2 > 0.97$ . **Conclusions:** The Autolys M tubes provides a convenient FFPE deparaffinization workflow by decreasing hands on time and minimizing tissue loss while eliminating the use of hazardous chemicals. These tubes in combination with our MagMAX FFPE Ultra chemistry and our KingFisher instrumentation increases user's FFPE sample processing throughput.

#### ST116. Optimization of ctDNA Quantification Methods for Longitudinal Disease Monitoring in Lung Adenocarcinoma

X. Max<sup>1</sup>, C. Ju<sup>1</sup>, A. Balasubramanyam<sup>1</sup>, L. Xi<sup>1</sup>, S. Young<sup>1</sup>, D. Klass<sup>1</sup>, M. Thomas<sup>2</sup>, F. Lasitschka<sup>2</sup>, M. Meister<sup>2</sup>, M. Schneider<sup>2</sup>, F. Herth<sup>2</sup>, T. Muley<sup>2</sup>, B. Wehn<sup>3</sup>, J. Palma<sup>1</sup>  
<sup>1</sup>Roche Sequencing Solutions, Inc., Pleasanton, CA; <sup>2</sup>Thoraxklinik at Heidelberg University Hospital, Heidelberg, Germany; <sup>3</sup>Roche Diagnostics GmbH, Penzberg, Germany.

**Introduction:** Longitudinal monitoring of solid tumors by quantifying circulating tumor DNA (ctDNA) is an emerging method to help assess early molecular responses. To quantify ctDNA, allele fraction (AF) of the somatic variants in plasma has been commonly used. We hypothesized mutant AF may misrepresent the level of ctDNA under certain circumstances, and by combining mutant AF with other ctDNA measurement metrics we could improve the existing ctDNA quantification methods. **Methods:** We employed a 197-gene next generation sequencing (NGS) assay, the AVENIO ctDNA Surveillance Kit, which allowed us to measure changes in the AF as well as in the mutant molecules per milliliter-of-plasma (MMPM), the latter of which quantifies ctDNA at the variant level by taking into consideration the plasma volume and extracted DNA mass from each sample. Using mutant AF or MMPM measurement methods separately, we evaluated the association between change in ctDNA levels and survival of advanced lung adenocarcinoma subjects treated with first-line chemo or chemoradiation therapies. We identified subjects that have been categorized differently, i.e. with or without early molecular response, based on the quantification methods used. We then evaluated several combination methods where both AF and MMPM-based measurements were used in algorithms to improve the association between change in ctDNA levels and survival benefit. From the prospective, observational German Lung Cancer Multi-Marker Study, the algorithms were tested in a training cohort (N=36) of UICC-stage IV adenocarcinoma subjects. The final method was validated in a separate independent cohort (N=28). **Results:** We found that when used alone, AF- and MMPM-based methods agree with each other in most of the subjects (21/36 and 23/28 for training and validation, respectively). However, when extracted DNA mass / mL of plasma was greater than 100 ng, MMPM-based method was better in predicting a molecular response. Applying this combination algorithm, the difference in the survival benefit between with or without early molecular responder was widened. In the training cohort, difference in median progression-free survival was 3.1 using the combination algorithm vs. 2.3 mo with AF alone (log-rank P=0.0067 vs. 0.0215 for combination vs. AF alone, respectively), and difference in median overall survival was 7.1 vs. 5.3 mo (log-rank P=0.0085 vs. 0.0352 for combination vs. AF alone, respectively). We saw similar improvements in the validation cohort. **Conclusion:** Taken together, different methods of quantifying ctDNA may each have their own strengths and limitations. We have found that a combination algorithm employing both AF and MMPM improved assessment of early molecular response in a cohort of advanced lung adenocarcinoma.

#### ST117. Clinically Validated Fusion Transcript Panel Identifies TERT Fusions

C.R. Orr, R. Sussman, J. Morrisette, J.N. Rosenbaum  
PennMedicine, Philadelphia, PA.

**Introduction:** One mechanism by which tumor cells escape senescence to transform and self-renew is via the activation of Telomerase Reverse Transcriptase (TERT). The best understood variants driving TERT over-expression are promoter mutations, which have been well described in a



variety of neoplasms. Many tumors with reactivated telomerase activity lack TERT promoter mutations, suggesting TERT activation can occur through alternative mechanisms. TERT fusions have been described as an alternate mechanism of TERT activation, but until recently have been difficult to detect in a clinical setting due to limited technologies. Here, we describe several TERT fusions identified through our clinically validated Next-Generation Sequencing (NGS)-based Fusion Transcript Panel (FTP), suggesting alternate pathways for reactivation of TERT may be more prevalent than the literature has documented. **Methods:** Total nucleic acid (TNA)-based libraries from 416 clinical samples were generated on the FTP (Archer FusionPlex) and sequenced on the Illumina HiSeq platform. Resultant sequencing data was processed on the Archer Analysis 5.0 software and subsequently analyzed by two independent reviewers. **Results:** Over the course of eight months, we detected 51 fusion and oncogenic isoforms from 416 clinical samples (12.3%). Of the 51 positive cases, three cases harbored fusions involving TERT (6%). Two of these fusions were identified in glioblastoma specimens. One was a DPP6-TERT fusion, joining exon 16 of *DPP6* to exon 2 of *TERT*, and the second was a MYO10-TERT fusion joining exon 23 of *MYO10* to exon 2 of *TERT*. These two fusions represent 6% of all the positives found in clinical glioblastoma samples assayed. Finally, a SLC12A7-TERT fusion joining exon 1 of *SLC12A7* to exon 2 of *TERT* was observed in an intrahepatic cholangiocarcinoma. **Conclusion:** Chromosomal rearrangements and the resulting fusion transcripts have proven technically challenging to detect, but the growth of RNA-based NGS platforms allows for the multiplexed detection of these events. These platforms enable the detection of rare or novel fusions that can provide insight for patient treatment or greater understanding of the progression of their cancer. We describe the detection of three TERT fusions, all consistent with the known pathobiology of the neoplasms in which they were detected. We detect these fusions at a frequency that suggests TERT fusions may be more prevalent than previously understood. Much like TERT promoter variants, TERT fusions may prove to be a significant, clinically relevant biomarker.

#### ST118. A Robust End-to-end Next-generation Sequencing Solution for Cancer Genome Profiling of Tumor Tissue Samples

B. Min, L. Feng, C. Choi, L. Qin, M. Pimentel, S. Sealee, L. Li, F. Casey, A. Lovejoy, D. Klass, J. Jin, J. Li  
Roche Sequencing Solutions Inc., Pleasanton, CA.

**Introduction:** Most clinical research studies and clinical trials rely on formalin-fixed, paraffin-embedded (FFPE) tissue samples for biomarker discovery and therapy prediction. While FFPE samples are FFPE samples is often challenging because FFPE-derived nucleic acids are of low abundance and damaged in a way that affects sequencing data quality. We have developed an end-to-end, next-generation sequencing (NGS) based solution for tumor tissue profiling, the AVENIO Tumor Tissue Analysis Kits (For Research use only. Not for use in diagnostic procedures). When used with the AVENIO Oncology Analysis Software (RUO), these kits accurately detect single nucleotide variants (SNVs), insertions/deletions (indels), fusions, and copy number variations (CNVs) from FFPE samples. **Methods:** The end-to-end workflow began with a simple, xylene-free FFPE DNA extraction, followed by a qPCR-based DNA quality assessment to determine input into library preparation, and a DNA polishing step to reduce fixation induced sequencing artifacts. Polished DNA was fragmented and used as input into a NGS library preparation workflow, which has been optimized to be robust for samples of widely varying quality. Three different panels designed to meet various cancer genome profiling coverage needs were used during the hybrid capture-based target enrichment step. Analytical verification studies to assess assay robustness, reproducibility, sensitivity, specificity, and concordance to orthogonal methods were executed. **Results:** Our assay achieved robust and reproducible performance with high on-target rates, low error rates, and high sequencing uniformity for the 500+ samples tested. More than 99% of the samples that met minimum input requirements obtained  $\geq 300\times$  coverage in greater than 98% of panel exon regions. Characterization of analytical sensitivity using samples with droplet digital PCR (ddPCR) verified variants demonstrated robust assay performance, with 100% sensitivity for variants at a limit of detection of 5% allelic frequency (AF) for SNVs, indels, fusions, and 5 copies for CNVs.

Specificity for SNVs was 99.999% per base, and specificities for indels, CNVs, and fusions were greater than 98%. We demonstrated high positive percent agreement for the detection of variants in surgical FFPE specimens verified using orthogonal NGS and fluorescence *in situ* hybridization (FISH) assays. **Conclusions:** The AVENIO Tumor Tissue Analysis kits provide a streamlined workflow from DNA extraction to data analysis for reliable detection of SNVs, insertion/deletions (indels), fusions, and CNVs from FFPE samples of varying quality with high sensitivity and specificity, providing clinical researchers a comprehensive end-to-end solution to profile cancer genomes using archival FFPE samples.

#### ST119. Rapid Assessment of Microsatellite Instability Status using the Idylla MSI Test

K. Nafa, P.A. Salazar, I. Rijo, D. Elezovi, M. Ladanyi, M.E. Arcila  
Memorial Sloan-Kettering Cancer Center, New York, NY.

**Introduction:** Microsatellite instability (MSI) is a condition of genetic hypermutability that results from impairment of DNA mismatch repair (MMR). Establishing the MSI/MMR status in cancer has important diagnostic and prognostic implications and is becoming increasingly important to predict response to immune checkpoint inhibitors. Current testing methods are often laborious and difficult to optimize. In this study, we describe the use of a fully automated system to enable ultrarapid assessment of MSI status on formalin-fixed, paraffin-embedded (FFPE) tissue sections without the need for prior DNA extraction or concurrent testing of a normal control. **Methods:** Tumor samples (38 FFPE tissue sections and 14 extracted DNA) with known MSI status were selected for the study. MSI status was assessed by the Idylla MSI Test using an integrated, real-time PCR-based system (Idylla - Biocartis). The test analyzes a set of 7 novel proprietary biomarkers located in the *ACVR2A*, *BTBD7*, *DIDO1*, *MRE11*, *RYR3*, *SEC31A* and *SULF2* genes. Based on these markers, instability of  $\geq 2/7$  markers = MSI-H,  $\leq 1/7$  = MSS (microsatellite stable). Results were compared to previously determined MSI status based on MSIsensor Score from large-panel targeted next generation sequencing (NGS) data generated by MSK-IMPACT, MSI-PCR (Promega) and/or MMR IHC. **Results:** Fifty-two samples (25 MSI-H with MSI-sensor score  $\geq 10\%$ ; 27 MSS score  $\leq 10\%$ ), were analyzed. Concordant results were obtained for 24/25 MSI-H and 26/27 of the MSS cases for an overall concordance of 96% (50/52). The MSI-H discordant case had an MSI-sensor score of 12%, MSI-PCR 3/5 unstable loci and MSH6 was not expressed by MMR IHC. The MSS discordant case had 4/7 unstable markers and an MSIsensor score of 5% (indeterminate for MSI) and was MSS by MSI-PCR assay. The average hands on time for the Idylla test was 4 minutes and the time from set up to report generation was 150 minutes. **Conclusions:** The Idylla MSI test is a simple and fully automated solution for MSI status determination that provides rapid results that are highly concordant with other MSI testing approaches.

#### ST120. Disease-specific Targeted NGS for Diverse Types of Genetic Cancer Biomarkers

A. McUsic<sup>1</sup>, J. RoseFigura<sup>1</sup>, S. Sandhu<sup>1</sup>, K. Nakama<sup>1</sup>, B. Reed<sup>1</sup>, A. Wood<sup>1</sup>, J. Irish<sup>1</sup>, P. Dottino<sup>2</sup>, O. Camacho-Vanegas<sup>2</sup>, L. Kurihara<sup>1</sup>, J. Martignetti<sup>2</sup>, V. Makarov<sup>1</sup>

<sup>1</sup>Swift Biosciences, Ann Arbor, MI; <sup>2</sup>Icahn School of Medicine at Mount Sinai, New York, NY.

**Introduction:** In the rapidly evolving cancer field, focused assays for different biomarkers are increasingly important tools. We have developed targeted next generation sequencing (NGS) panels consisting of disease-specific, predesigned content that allow the flexibility to assess single-nucleotide variants (SNVs), small insertion/deletions (indels), and CNVs in a multiplexed single tube prep. Herein, we demonstrate the use of these panels to assess these variants in validated standards and combine multiple pre-existing panels to evaluate clinical samples from patients with a known disease. **Methods:** Disease-specific targeted NGS panels were developed using multiple resources for reference, including the OncoKB database (Memorial Sloan Kettering Cancer Center). The panels include a pan-cancer panel and panels targeting specific cancers, including lung, colorectal (CRC), Lynch Syndrome, myeloid disease, and breast/ovarian. These panels are based on a technology which generates

overlapping amplicons for exon-level coverage of contiguous regions in a single tube assay. All libraries were prepared from 10 ng DNA and sequenced on an Illumina MiniSeq. CNV standards were acquired from NIST for *ERBB2* amplification and from SeraCare for *EGFR* and *MET* amplification. The program CNVkit (Talevich *et al.*, 2014) was used to assess copy number. Cell-free DNA (cfDNA) and peripheral blood DNA was acquired from healthy donors. Clinical samples from known Lynch Syndrome patients were provided by the Martignetti lab at the Icahn School of Medicine at Mount Sinai. **Results:** The targeted NGS panels demonstrated consistent performance with a wide variety of DNA types, including cfDNA and formalin-fixed, paraffin-embedded (FFPE) DNA. All panels exhibited >90% on target and >90% coverage uniformity. CNV results agreed with DNA standards at copy number ratios >2.9. Six clinical samples, with 2 corresponding tumors, were initially evaluated using the pan-cancer panel. These results were indicative of Lynch syndrome, so a combined CRC/Lynch panel was also run on these samples to further probe the disease state. **Conclusions:** Targeted NGS panels are a powerful tool for assessing the genetic etiology of multiple disease states. We have demonstrated the ability of this technology to identify important disease biomarkers in clinical samples, and to assess CNVs in pre-validated standards. The ability to assess SNV, insertion/deletions (indels), and CNV in a single assay from 10 ng of sample represents an important tool for assessing diseases in the clinic as well as in research and discovery settings.

#### ST121. Novel Synthetic Plasma Liquid Biopsy (ctDNA) Reference Material

Q. Zheng, G. Roma, S. Saddar  
AccuRef Diagnostics, Milpitas, CA.

**Introduction:** Recent advances in Next-Generation Sequencing (NGS) and digital PCR (dPCR) technologies have enabled low allele frequency detection with greater precision, allowing for non-invasive detection of cancer-associated somatic variants from circulating tumor DNA (ctDNA) in plasma (i.e. liquid biopsy). Due to high heterogeneity in cancer cells and the low prevalence of ctDNA in plasma, there is a need for a reproducible source of ctDNA-mimetic reference material for use in assay development, limit-of-detection (LOD) assessment, quality assurance, and proficiency testing. Currently available ctDNA reference standards are developed by either using sonication of cell line DNA or synthetic oligonucleotides. These methods do not mimic naturally-occurring ctDNA fragment properties (e.g. size distribution and DNA blunt ends) nor provide the cell line-based genomic complexity. Here, we demonstrate the development of a novel ctDNA-mimetic reference material based on nucleosomal-digestion of CRISPR/Cas9 engineered cell lines in synthetic plasma to mimic patient circulating tumor DNA. **Methods:** We engineered 8 actionable cancer mutations in four cancer genes (*EGFR*, *PIK3CA*, *NRAS* and *KRAS*) using CRISPR/Cas9 technology. Mutations were confirmed by Sanger sequencing. A nucleosomal digestion protocol was then utilized to fragment the gDNA into lengths that mimic true patient ctDNA. Targeted mutant allele fractions (MAFs) of 0%, 0.1%, 1.0%, and 5.0% were then created by mixing digested gDNA at various ratios with the wild-type cell line, and validated by dPCR and NGS. Fragment size was assessed using a Bioanalyzer and compared to human extracted ctDNA. **Results:** Using our proprietary nucleosomal digestion protocol, we were able to reproducibly achieve fragmentation profiles centered at approximately 166bp. Targeted allele frequencies of 0.1%, 1.0%, and 5.0% were obtained with high accuracy ( $\leq \pm 50\%$ ,  $\pm 30\%$ ,  $\pm 20\%$ ), respectively using dPCR and NGS. The synthetic plasma extraction behaved similarly to extracted ctDNA from human plasma specimens. **Conclusion:** We have shown the feasibility of developing a low allelic frequency, quantitative multigene ctDNA mimetic reference material in synthetic plasma with high accuracy and high reproducibility using CRISPR/Cas9 engineered cell lines. This reference material is ideal for use in the nucleic acid extraction to allow optimization of the NGS or dPCR clinical workflow prior to running clinical specimens. CRISPR/Cas9 engineered cell lines are biologically relevant materials and allow for a reproducible and renewable source of ctDNA reference standards for assay development and quality control for use in clinical diagnostic workflows.

#### ST122. Lung Adenocarcinoma *EGFR*, *KRAS* and *BRAF* Mutational Analysis, Histologic Correlation and Longitudinal Review

M.B. Wachsmann, C. Glass, Y.M. Butt, S. Yan, J. Torrealba, T. Gokaslan, V. Sarode, D. Gerber, J. Dowell, R. Hughes, K. Kernstine, M. Abu-Hijleh, H. Chiu, D. Oliver  
The University of Texas Southwestern Medical Center at Dallas, Dallas, TX.

**Introduction:** Lung cancer is the leading cause of cancer death in the United States with adenocarcinoma the most common subtype of lung cancer in females as well as non-smoking males. Large-scale genomic studies have found specific molecular alterations in lung adenocarcinoma that are associated with prognosis and treatment response. Not all mutations are equal or common, therefore, it is critical to evaluate mutation profiles and their relationship to clinicopathologic parameters in specific populations. We are an NCI-Designated Comprehensive Cancer Center in North Texas and have accumulated several hundred clinical lung adenocarcinoma cases for mutational analysis of *EGFR*, *KRAS* and *BRAF*. Our objective is to characterize molecular alterations in our population with regards to histology as well as other clinical parameters. **Methods:** A total of 679 formalin-fixed paraffin embedded (FFPE) cases were studied and analyzed by one of two methods to query *EGFR* exons 18-21, 452-FFPE cases for *KRAS* codons 12, 13, 61 and 146 as well as 329-FFPE cases for *BRAF* codons 600 and 601. DNA was extracted using Qiagen QIAamp FFPE Tissue kit. Sanger sequencing used BigDye Terminator v3.1 chemistry with lab developed primers. Sequenom MassARRAY mass spec analysis was done using software-designed primers and iPLEX chemistry. Histologic review was performed by a pathologist and morphology was categorized. **Results:** We identified 25% (173/679) mutated *EGFR* cases, 30% (137/452) mutated *KRAS* cases and 4% (13/329) mutated *BRAF* cases. For mutated *EGFR*, 5% (8/173) were in exon 18, 47% (82/173) in exon 19, 8% (13/173) in exon 20 and 40% (69/173) in exon 21. T790M mutations were identified in 16% (28/173), one was confirmed germline. For mutated *KRAS*, 86% (117/137) occurred in codon 12, 4% (6/137) in codon 13 and 10% (14/137) in codon 61. For mutated *BRAF*, 100% (13/13) were V600E. The *EGFR* wild-type tumors had a higher frequency of a solid growth pattern. We had 20 patients with longitudinal testing and follow up for activating *EGFR* mutations. Of these, 90% (18/20) were treated with erlotinib and 44% (8/18) developed a T790M resistance mutation. One of the 18 patients treated with erlotinib that did not develop a T790M did develop a *BRAF* V600E resistance mutation. **Conclusions:** Although *EGFR* and *KRAS* mutations occur at a similar frequency, the nature of the mutations is more complex with regards to *EGFR* and to a much lesser extent with *KRAS* and *BRAF*. *EGFR* mutations most commonly occurred in exons 19 and 20, with the majority of *KRAS* mutations occurring in codon 12. *BRAF* mutations were much less frequent and they occurred exclusively at codon 600. The *EGFR* wild-type tumors seem to have a high frequency of aggressive/poorly differentiated growth pattern that is associated with a poorer prognosis.

#### ST123. Clinical Evaluation of the Pillar Bioscience ONCOREveal Multi-cancer Panel

A. Atkinson, M. Al-Turkmani, F.B. de Abreu, G.J. Tsongalis, J.A. Lefferts  
Dartmouth-Hitchcock Medical Center and the Geisel School of Medicine at Dartmouth, Lebanon, NH.

**Introduction:** Next-generation sequencing (NGS) panels are a powerful tool in guiding patient treatment in clinical oncology and are increasing in usage. Targeted NGS panels that offer streamlined library preparation of highly multiplexed samples are required to reduce both turn-around time and test complexity while also managing increased test volumes. Here, we evaluated the ONCOREveal Multi-Cancer Panel (Pillar Biosciences) in comparison to the Ion Torrent Cancer Hotspot Panel v2 (CHPv2, Thermo Fisher Scientific). **Methods:** Fifty-six samples were included: thirteen lung adenocarcinomas (NSCLC), sixteen colorectal cancers, ten melanomas, five gliomas, and nine from additional cancer types including breast, thyroid, bladder, and gastrointestinal stromal tumors. Library preparation was performed using 10 ng of gDNA per sample for both the CHPv2 and ONCOREveal assays. For the ONCOREveal 6-8 hour workflow, libraries were normalized using Qubit 3.0, pooled and sequenced with an Illumina

NextSeq system. For data analysis, FASTQ files were uploaded to Pillar Biosciences analytical pipeline (PIVAT) for sequence alignment, annotation, and variant classification. Variant calls within the genomic regions covered by both panels were compared. **Results:** A large degree of concordance was observed between the ONCReveal Multi-Cancer Panel and CHPv2 variant calls (94.5%, 120/127 variants). Eight variants called by the ONCReveal panel were not called using the CHPv2, whereas one variant was called by the CHPv2 analysis and not the ONCReveal panel. **Conclusions:** With NGS tumor profiling as an integral component in determining patient treatment, clinical laboratories will need to accommodate high sample volumes together with both variable specimen quantity and quality. The 6-8 hour workflow and sensitivity of the ONCReveal Panel allows laboratories to perform accurate, highly-multiplexed, targeted NGS using benchtop instruments.

#### ST124. Determining Clonal Relatedness Between Tumors Using a Targeted Next-generation Sequencing (NGS) Assay

R.J. Maglantay<sup>1</sup>, I. Ostrovskaya<sup>2</sup>, C. Begg<sup>2</sup>, W.L. Ernst<sup>1</sup>, M. Barbi de Moura<sup>1</sup>, M. Miller<sup>1</sup>, A. Priddy<sup>1</sup>, S.I. Chiosea<sup>1</sup>, S. Dacic<sup>1</sup>, Y.E. Nikiforov<sup>1</sup>, M.N. Nikiforova<sup>1</sup>, S. Roy<sup>1</sup>

<sup>1</sup>University of Pittsburgh Medical Center, Pittsburgh, PA; <sup>2</sup>Memorial Sloan Kettering Cancer Center, New York, NY.

**Introduction:** Synchronous or metachronous tumors can be either independent primaries or metastatic/recurrent. Next generation sequencing (NGS) allows for comprehensive genomic profiling of tumors including known cancer driver mutations. In this study, we aim to determine the utility of Ion AmpliSeq Hotspot V2 panel in assessing clonal relatedness of tumors. **Methods:** We performed mutational profiling of 21 tumor pairs (42 tumors) (lung squamous cell and adenocarcinomas (LUSC, LUAD); head and neck squamous cell carcinomas (HNSC); urothelial carcinomas (BLCA); without normal tissue) using NGS AmpliSeq Hotspot v2 panel (50 cancer-related genes). Histopathologic information, LOH-PCR analysis data if available, and clinical information were reviewed to establish tumor pairs as independent primaries or metastasis/recurrence. Variants with a minimum coverage of 300x, allelic fraction of 3%, and classified as Tier I/II or III (Li *et al.*, 2017) were included. Clonal relatedness was assessed for each pair using the Clonality R-package (Ostrovskaya *et al.*, 2015) based on a statistical test for evaluating evidence for clonality against a null hypothesis that the tumors are independent. A conditional likelihood ratio (LR) statistic is then calculated from the weighted contributions of matched mutations in a pair and the corresponding marginal frequencies derived from the TCGA database. A high LR indicates clonal relatedness and rejection of the null hypothesis. The p-value is calculated using the null distribution generated from the preassigned marginal frequencies. **Results:** Eighty-seven mutations were detected in 13 genes across the 4 tumor types studied. Most frequent were *TP53* (31/42, 74%), *PIK3CA* (11/42, 26%) and *CDKN2A* (6/42, 14%). In the 13-known tumor-metastasis/recurrent pairs, mutations in each pair ranged from 2-9. Cases shared from 1-4 mutations. LR ranged from 3.6-21 (p=0.02 - <0.001). Of the 8 independent tumor pairs (3 HNSC, 2 LUAD, 2 LUSC, 1 BLCA), 3 cases (2 LUAD, 1 LUSC) were excluded due to the absence of mutations in at least 1 of the tumors. No mutations were shared in the remaining 5 pairs. Mutations detected per tumor pair ranged from 2-7. The LR statistic ranged from -3.5x10<sup>-6</sup> to -1x10<sup>-6</sup> (all p=1). One of these tumor pairs (LUSC) with both LOH and NGS data has concordant results (independent primaries). **Conclusions:** Targeted NGS utilizing a 50-gene panel of known cancer-associated genes showed good concordance with traditional criteria and methodologies used for assessing clonal relatedness between tumors. This approach has several advantages, including lower input DNA requirements than LOH analysis, sequencing-based clonality assessment, higher sensitivity, variant prevalence-weighted statistical analysis, and simultaneous availability of therapeutically relevant information.

#### ST125. Rapid EGFR Mutation Testing in Lung Cancer Tissue Samples Using a Fully Automated System and Single-use Cartridge

M. Al-Turkmani, M.A. Suriawinata, C.C. Black, K. Shirai, K.H.

Dragnev, G.J. Tsongalis

Dartmouth-Hitchcock Medical Center and Geisel School of Medicine, Lebanon, NH.

**Introduction:** Mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) gene are observed in approximately 15% of non-small cell lung cancer (NSCLC) patients in the US. In advanced NSCLC, the presence of an EGFR mutation confers a more favorable prognosis and strongly predicts response to EGFR tyrosine kinase inhibitors (TKIs). The Idylla system (Biocartis, Mechelen, Belgium) is a fully integrated, cartridge-based platform that provides automated sample processing (deparaffinization, tissue digestion and DNA extraction) and real-time PCR-based mutation detection with all reagents included in a single-use cartridge. This retrospective study aimed at evaluating the Idylla EGFR Mutation Assay cartridges (Research Use Only) against next-generation sequencing (NGS) using lung cancer tissue samples.

**Methods:** Thirty-four archived formalin-fixed paraffin-embedded (FFPE) tissue samples obtained from patients with adenocarcinoma of the lung were tested on the Idylla system. Among these samples, 21 had at least one mutation in EGFR and the remaining 13 had no EGFR mutation as determined by NGS analysis using the Ion AmpliSeq 50-gene Cancer Hotspot Panel v2 (Thermo Fisher Scientific). Additionally, 6 samples previously determined to have insufficient DNA quantity for NGS analysis (QNS samples) were analyzed on the Idylla system. One 10 µm FFPE tissue section was used for each Idylla run and all cases met the Idylla minimum tumor content requirement (≥ 10%). **Results:** Idylla results were in complete agreement with those obtained by NGS for EGFR mutations targeted by the Idylla. These included L858R (8 samples), G719A (2 samples), G719C (2 samples), S768I (3 samples), and exon 19 deletions (8 samples). NGS identified two additional EGFR mutations that are not targeted by the Idylla in two samples (E709V and V774M). No EGFR mutations were detected by the Idylla in samples determined by NGS as having wild-type EGFR. Five of the 6 QNS samples gave valid results when tested by the Idylla system, indicating enhanced sensitivity and additional potential utility of the system for testing samples that do not meet minimum quantity requirement for NGS analysis. The Idylla system produced results rapidly with a turnaround time of approximately 2.5 hours. **Conclusion:** The fully automated Idylla system offers rapid and reliable testing of clinically actionable mutations in EGFR directly from FFPE tissue sections. Its simplicity and ease of use compared to other available molecular techniques make it suitable for small centers that lack highly trained staff and molecular expertise. Additionally, it can complement NGS and other molecular testing systems at larger diagnostic centers by providing significantly faster turnaround times.

#### ST126. Detection of Disease-defining Fusion Genes in Sarcomas and Related Neoplasms by Utilizing Multiplex Targeted RNA-Seq Assay

Y. Wu<sup>1</sup>, W. Wang<sup>1</sup>, R. Ricciotti<sup>1</sup>, J. Mantilla<sup>1</sup>, B. Hoch<sup>2</sup>, E. Chen<sup>1</sup>, Y.J. Liu<sup>1</sup>

<sup>1</sup>University of Washington, Seattle, WA; <sup>2</sup>University of New Mexico, Albuquerque, NM.

**Introduction:** The detection of disease-defining gene fusions has facilitated and expanded the classification of sarcoma and related neoplasms. Differential diagnosis of similar-appearing lesions often involves a reflex testing paradigm using multiple Fluorescence *in situ* Hybridization (FISH) probes and Immunohistochemistry (IHC) for a definitive diagnosis. FISH and IHC cannot determine breakpoint or fusion partner with limited multiplexing capability. **Methods:** We evaluate the utility of an Anchored Multiplex PCR and next generation sequencing (NGS) assay to target captured RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tumor tissue and simultaneously detect multiple gene fusions associated with sarcomas. Seventy-four tumors were tested by multiplex RNA-seq assay, including 13 subtypes of bone and soft tissue neoplasms with disease-defining gene fusions and previously unclassified sarcomas. Non-neoplastic tissues were also used as negative controls. The morphologic, IHC and cytogenetic features of the tumors were retrospectively reviewed by four pathologists and recorded. **Results:** Our data showed no discrepancy with previous FISH



results of the 41 fusion-positive sarcoma cases representing 10 subtypes. A novel fusion of *EWSR1-PBX3* was identified in the angiomatoid fibrous histiocytoma. One case initially diagnosed as synovial sarcoma was found to harbor a *BCOR-CCNB3* fusion and reclassified accordingly. In addition, we also used the multiplex RNA-seq to better define other 3 subtypes of bone and soft tissue tumors. For undifferentiated round cell sarcomas, 8 of 15 cases showed disease-defining fusions, including *BCOR-CCNB3*, *CIC-DUX4*, *CIC-NUTM2A*, *EWSR-NFATC2*, *NUTM1-MGA*, and *HEY1-NCOA2*. For malignant gastrointestinal neuroectodermal tumors (GNET), 2 of 4 cases showed the characteristic translocations *EWSR1-ATF1* as GNET, which is a rare entity and can be histologically misdiagnosed as varies other types of neoplasms. Our study also better defined the morphologic spectrum and molecular pathogenesis of cellular fibroma of tendon sheath (FTS). Six of 14 FTS showed *USP6* fusion with partner genes *ASPEN*, *COL1A1*, *COL3A1*, *MYH9*, *PKM*, and *RCC1*, supporting FTS as part of the growing family of *USP6* translocated neoplasms. **Conclusions:** This study demonstrated that such targeted RNA gene panel could provide a novel effective methodology to detect gene fusions, aiding in the diagnosis of neoplasms with recurrent gene fusions. Advantages over FISH include the ability to simultaneously test for multiple gene rearrangements as well as detect novel fusion partners that would not be otherwise identified. Targeted RNA gene panel can be routinely utilized in diagnosing sarcomas, especially those with round cell morphology, unusual features or morphology suggestive of a translocation sarcoma.

#### ST127. Detection of Microsatellite Instability in Endometrial Carcinoma Using the Novel Idylla MSI Assay

C.M. Nicka, D.C. Green, G.J. Tsongalis, L.J. Tafe  
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

**Introduction:** Endometrial cancer (EC) is the most common gynecologic malignancy and approximately 30% are due to microsatellite instability (MSI) caused by deficiencies in the DNA mismatch repair (MMR) genes (dMMR). MSI and MMR testing are frequently used to screen EC patients for Lynch syndrome. Additionally, the PD-1 inhibitor pembrolizumab was recently FDA approved for the treatment of MSI-high or dMMR solid tumors. The Biocartis Idylla MSI Research Use Only assay is a cartridge-based assay that does not require a reference normal sample. It is designed to detect seven novel MSI Biomarkers consisting of short homopolymers located in the *ACVR2A*, *BTBD7*, *DIDO1*, *MRE11*, *RYR3*, *SEC31A* and *SULF2* genes using one formalin fixed paraffin embedded (FFPE) tumor section or previously extracted DNA. Mutation in two of these markers is considered MSI-H. **Methods:** Previously extracted DNA of 12 dMMR ECs (with loss of *MLH1* and *PMS2* by immunohistochemistry (IHC) and presence of *MLH1* gene promoter methylation detected by methylation specific PCR) was used in this study. The tumors were screened previously by IHC for deficiency of mismatch repair proteins *MLH1*, *MSH2*, *MSH6* and *PMS2* and *MLH1* promoter methylation when deficient for *MLH1/PMS2*. One microliter (μL) of DNA (QiaAmp, Qiagen) was added to the Idylla MSI cartridges (concentrations ranging from 63.4 to 152.4 ng/μL) in which DNA preparation, PCR and melt curve analysis are performed. **Results:** Nine out of 12 cases (75%) were classified as MSI-high (MSI-H), and 3 out of 12 cases (25%) were classified as microsatellite stable (MSS). None of the cases had invalid results for any of the 7 markers. In the MSI-H samples, the number of MSI gene mutations ranged from 2/7 to 6/7. Of the 7 novel MSI Biomarkers, *MRE11* was the most commonly mutated (8/9). No sample showed mutations at all seven MSI biomarkers. **Conclusions:** The Idylla MSI Assay showed concordance with dMMR in 9/12 EC previously extracted DNA samples. Additional studies are needed to identify reasons for discrepant results. The MSI assay is a benchtop assay with a small footprint that can be performed within 150 minutes from previously extracted DNA or an FFPE tissue section and does not require a normal control reference.

#### ST128. Development of a Pan-Cancer Comprehensive Genomic Profiling System to Detect Actionable Genetic Alterations and Tumor Mutation Burden in Solid Tissue

J. Simmons<sup>1</sup>, S. Jones<sup>1</sup>, S.V. Angiuoli<sup>1</sup>, K.M. Gerding<sup>1</sup>, J. Hernandez<sup>1</sup>, L. Keefe<sup>1</sup>, A. Georidis<sup>1</sup>, J.R. White<sup>2</sup>, E. Kim<sup>1</sup>, M. Sausen<sup>1</sup>

<sup>1</sup>Personal Genome Diagnostics, Baltimore, MD; <sup>2</sup>Resphera Bio, Baltimore, MD.

**Introduction:** Tumor mutation burden (TMB) has emerged as a promising biomarker, with multiple clinical studies correlating high TMB with improved response to immunotherapy. The spectrum of mutation burden varies widely across cancer types, with a considerable range within certain cancer types as well, thereby highlighting the need for an objective, accurate, and robust TMB assay. However, analytical accuracy of TMB across a broad dynamic range with standardized reporting is necessary prior to clinical implementation. Here, we describe the development and analytical validation of a >500-gene, decentralized, comprehensive genomic profiling system to determine TMB, microsatellite instability (MSI), and other clinically relevant somatic variants in a variety of cancers. **Methods:** To determine mutation burden across different cancer types, we developed PGDx elio Tissue Complete, a >500-gene assay for accurate determination of TMB over a wide range when compared to whole exome sequencing analyses. Utilizing the Personal Genome Diagnostics (PGDx) machine learning algorithm, optimized for accurate somatic sequence mutation detection, we assessed the overall performance of our gene panel to determine TMB *in silico* across a wide dynamic range of mutation burden and cancer types, including NSCLC. Overall accuracy and sensitivity was determined using independent patient cohorts and compared to gold standard, tumor and patient matched normal whole exome sequencing. Microsatellite status was also determined using over 70 mononucleotide tracts and compared to microsatellite status as defined by a reference PCR-based approach. Additionally, in the NSCLC cohort, sensitivity and specificity of clinically-relevant genetic alterations were determined using both contrived and orthogonally-validated clinical samples. **Results:** Our results indicate that the >500-gene system achieves high analytical accuracy for determination of TMB when compared to whole exome sequencing, across a variety of cancer types. In addition, microsatellite status was classified with high sensitivity and specificity. **Conclusions:** Our comprehensive decentralized system provides accurate results for both the determination of TMB across a broad range of cancer types and tumor mutation loads, and has the capability to detect microsatellite instability, as well as clinically relevant genetic alterations in specific cancers.

#### ST129. Evaluation of Residual Fluids from Fine Needle Aspirates for Interrogation of Variants Using NGS

S. Sung, A. Saqi, S. Barua, A. Pavis, H. Fernandes  
Columbia University Medical Center, New York, NY.

**Introduction:** Cell blocks and smears prepared from fine needle aspirations (FNAs) are an excellent source of DNA for molecular analyses. Recent studies have shown that DNA extracted from otherwise discarded, residual FNA needle supernatants, is also a viable source of DNA for interrogation of variants using targeted next generation sequencing (NGS). We compared the potential to detect variants in DNA extracted from residual supernatants derived from alcohol-based rinse and formalin. **Methods:** Smears, cell blocks and/or ThinPrep slides were prepared for all FNAs. Cell blocks were formed from specimens placed in formalin and/or Cytolyt, an alcohol-based solution. Following final diagnoses and ancillary testing (immunohistochemistry, fluorescence *in situ* hybridization (FISH) and/or NGS), the residual supernatants from the alcohol-based rinses and formalin, were submitted for molecular testing. Only 3 mL of the supernatant was centrifuged for 10 minutes at 4500g, and DNA was extracted from the pellet using the QIAamp DNA QIAcube kit (Qiagen, GmbH). Recovered DNA ranging from 1 – 10 ng was used for library preparation employing the SLIMamp Lung and Colon Hotspot Panel (Pillar Biosciences). Samples with quantifiable libraries were sequenced on the MiSeq (Illumina). Data analysis including sequence alignment, variant calling and annotation was performed using FASTQ files, with the Pillar Variant Analysis Toolkit (PIVAT). **Results:** Consecutive 15 available preparations obtained from 9 FNAs, including pancreatic adenocarcinoma (N=6), lung adenocarcinoma (N=2) and

metastatic adenocarcinoma (from lung) to the liver (N=1), were selected for analyses. Five (of 9 FNA) samples had supernatants derived from both alcohol- and formalin-based preparations. All 5 cases harbored clinically relevant variants in the alcohol-based specimens. The 5 samples derived from formalin failed to produce sequencable libraries. From the additional 4 FNA samples with only formalin supernatant, NGS was successfully performed on one sample. Collectively, 100% of the supernatants collected from the alcohol specimens were informative versus 11% of the formalin-based supernatants. Four *KRAS* variants and one 15bp *EGFR* exon 19 deletion was identified in the samples that were sequenced. Corresponding cell blocks harbored the same variants. The mutant allelic fraction of the variants identified ranged from 13% to 37% and the average coverage obtained was >2000x. **Conclusions:** Supernatants from FNA samples can be used for interrogation of actionable alterations, including missense variants and insertion/deletions (indels) using NGS with SLiMamp technology. Based on this limited study, alcohol-based rinses are superior to formalin-based solutions for use in NGS assays. In conclusion, supernatants provide an additional source of DNA for NGS.

#### ST130. Analytical Validation of the QuantideX NGS DNA Hotspot 21 Kit, a Diagnostic Next-Generation Sequencing (NGS) System for the Detection of Actionable Mutations in FFPE Tumors

K. Kelnar, M. Church, W. Pickens, J. Kaplan, M. Popowski, A. Jeiranian, M. Fahey, L. Manzano, G.J. Latham, H. Zhu Asuragen, Austin, TX.

**Introduction:** Accurate multi-gene molecular diagnostics are critical for effective precision medicine in cancer. We describe analytical validation studies of the QuantideX NGS DNA Hotspot 21 Kit\* designed for targeted sequencing of cancer-related mutation hotspots in 21 oncogenes (*ABL1*, *AKT1*, *AKT2*, *ALK1*, *BRAF*, *EGFR*, *ERBB2*, *FGFR1*, *FGFR3*, *FLT3*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *KRAS*, *MET*, *NRAS*, *PDGFR*, *PIK3CA* and *RET*) using formalin-fixed, paraffin-embedded (FFPE) tumor DNA. This assay integrates reagents and bioinformatics software to report clinically-actionable variants and aid in the molecular diagnosis of cancer patients to predict sensitivity to a growing number of therapeutic agents.

**Methods:** Analytical validation studies were designed in compliance with CLSI and ISO guidelines. Residual clinical FFPE tumor biopsies and commercially available reference materials were used to assess assay performance. Amplifiable DNA copy number was measured using the included qPCR QC assay to guide inputs for library preparation and inform the variant analysis pipeline using sample-specific data. All samples were sequenced on the MiSeq or MiSeqDx systems (Illumina). Raw FASTQ files were analyzed using companion QuantideX NGS Reporter software to identify single-nucleotide variants (SNVs) and insertion/deletions (indels). **Results:** All mutation calls in evaluated samples and replicates were accurate when the DNA input was 400 to 24,000 functional copies (as low as 1.5 ng DNA). The lower limit of detection of variant allele frequency was 4.85% for SNVs, 4.82% for insertions and 6.16% for deletions. No false-positive calls were made in wild-type samples resulting in a limit of blank of zero. A precision study was performed with three reagent lots, three operators, and three MiSeq. The positive percent agreement was 100% for all variants tested with the lower limit of 95% CI higher than 90%. Accuracy was determined by analyzing 57 clinical FFPE specimens (lung, breast, colorectal cancer, and melanoma) with 71 known mutations confirmed by a comparator method resulting in a PPV of 100% and a NPV of 99.99% (when all base positions were combined).

**Conclusions:** Results from analytical validation show that the QuantideX NGS DNA Hotspot 21 Kit is an accurate IVD system that demonstrates high sensitivity and specificity. The kit can be used in clinical and research laboratories for reliable detection of ≥1600 COSMIC mutations within the 46 targeted cancer hotspot regions. \*CE-IVD for US export only.

#### ST131. Development of Quality Control Materials for Characterization of Comprehensive Next-generation Sequencing Panels Targeting Cancer Hotspots

M. Christian<sup>1</sup>, N. Holihan<sup>2</sup>, G. Bien<sup>3</sup>, S. Dasari<sup>1,3</sup>, K. Norman<sup>1</sup>

<sup>1</sup>ThermoFisher Scientific, Fremont, CA; <sup>2</sup>Health Network Laboratories, Allentown, PA; <sup>3</sup>ThermoFisher Scientific, Carlsbad, CA.

**Introduction:** Targeted next-generation sequencing (NGS) panels can detect hundreds of mutations in key genes using amplification based and hybrid-capture based NGS technologies. Although NGS technology is a powerful tool, optimizing and characterizing test performance on hundreds of variants is extremely challenging, time consuming, and expensive. Samples must be sourced, variants identified and orthogonally confirmed, then quantified and diluted. This effort is then multiplied across dozens of samples, and then samples must be run over many runs and days to assess assay reproducibility, precision, sensitivity, etc. In this study, we developed a novel reference material, experimental design, and analysis pipeline that allows for highly streamlined NGS assay characterization, enabling thorough test characterization across 500+ variants within only 6 runs. **Methods:** The AcroMetrix Hotspot Frequency Ladder was developed based on the highly characterized NIST Genome in a Bottle GM24385 DNA as the background genome. 555 single-nucleotide variants (SNVs), insertion/deletions (indels), and MNVs were multiplexed into one sample, and a frequency ladder was created, targeting 50%, 25%, 15%, 10%, 5%, 2.5% and 0% allelic frequencies. Variant Allele Frequency (VAF) was confirmed using the Bio-Rad Droplet Digital PCR (ddPCR) system. To assess the ability of the frequency ladder to characterize assay performance, six independent replicate libraries were prepared for each of the seven levels. Libraries were run over six runs on the Ion Torrent Ion S5 XL system using OncoPrint Comprehensive Assay v3, allowing for 6 replicates of all variants at decreasing frequencies.

**Results:** Digital PCR VAF was observed to be 49.98%, 26.30%, 15.83%, 10.56%, 5.12% and 2.77% which compares well with target VAF. NGS data also showed good correlation and the observed frequencies were within ±20% of the expected target VAF for 131 variants covered by the assay. The sensitivity was >90% for 50%, 25% and 15% VAF controls. However, the sensitivity dropped to >70% for 5% and 10% VAF controls. Limit of detection was determined for all variants covered by the assay, using six replicates of seven frequency levels across six runs. Replicate data was also used to calculate assay reproducibility, precision, and specificity. **Conclusion:** Carefully designed quality control materials, experimental design and analysis pipeline significantly accelerated the assessment of reproducibility, precision, specificity, and limit of detection of an NGS assay. Exhaustive analytical characterization of the NGS panel was completed across hundreds of variants within 3 days.

#### ST132. BRAF V600E and Beyond in Non-small Cell Lung Cancer and Other Solid Tumors

J. Horton<sup>1,3</sup>, N. McNeill<sup>1,3</sup>, T.A. Freitas<sup>1</sup>, M. Shiller<sup>2</sup>

<sup>1</sup>Med fusion, Lewisville, TX; <sup>2</sup>Pathologists Bio-Medical Laboratories, Dallas, TX; <sup>3</sup>Quest Diagnostics, Lewisville, TX.

**Introduction:** BRAF alterations are described in a wide variety of malignancies, such as melanoma, thyroid carcinoma, colon cancer, and non-small cell lung cancer (NSCLC). Unequivocally the most common and clinically relevant genetic alteration is V600E. However, uniquely, NSCLC is distinguished by a greater percentage of non-V600E alterations, in some sources as high as 50% of the time. **Methods:** Three-year retrospective analysis of 4459 solid tumor cases tested in our CLIA-certified laboratory for the incidence of BRAF mutations in the following tumor types: NSCLC, colorectal, melanoma, and other solid tumors. Two methods of detection were utilized: 1) SNaPshot Multiplex System on a 3730 Genetic Analyzer, 2) next generation sequencing using the Ion AmpliSeq Cancer Hotspot Panel v.2 on the Ion PGM System platform.

**Results:** A BRAF mutation was present in 334/4459 (7.49%) cases. The V600E mutation comprised 212 (63.47%) cases, while 125 (37.42%) had non-V600E mutations. Of 2278 NSCLC cases tested, 80 (3.51%) carried a BRAF mutation; 35 (43.75%) with a V600E and 45 (56.25%) with a non-V600E. The remaining solid tumor cases found 254 (11.64%) BRAF mutations as follows: 177 (69.68%) V600E and 80 (31.5%) non-V600E. In the 1300 colorectal cases, 103 (7.92%) had a BRAF mutation: 91 (88.35%) with a V600E and 14 (13.59%) with a non-V600E mutation. In

the 339 melanoma cases, 140 (41.3%) had a *BRAF* mutation with 78 (55.71%) having a V600E and 63 (45%) having a non-V600E. With respect to codon 600 mutations, 28 (44.4%) were V600K and 8 were V600R (12.7%). Of all cases, 2358 (52.88%) were male and 2100 (47.1%) female, with 207 (61.98%) males and 127 (38.02%) females being positive for *BRAF* mutations. Specifically, 110 (51.89%) males and 102 (48.11%) females for V600E, and 98 (78.4%) males and 27 (21.6%) females for non-V600E. Of the NSCLC cases, 1151 (50.53%) were male and 1126 (49.43%) female. *BRAF* mutations involved 47 (58.75%) males and 33 (41.25%) females, consisting of 13 (37.14%) males and 22 (62.86%) females for V600E and 34 (75.56%) males and 11 (24.4%) females for non-V600E. **Conclusions:** *BRAF* mutations occurred in 7.5% of solid tumor and 3.5% of NSCLC cases. Of the NSCLC cases with *BRAF* mutations, over half were non-V600E. The rate of non-V600E in NSCLC (56.25%) is distinct from other solid tumor types including colorectal (13.59%), melanoma (45%), and other types (27.27%). There was a male predilection for non-V600E mutations in solid tumor cases with 78.4% cases being male. In NSCLC, there was a female predilection for V600E with 62.8% of cases being female, while solid tumor cases had a rate of 48.11% female. Excluding other alterations in codon 600, these unique alterations may represent a therapeutic opportunity or prognostic indicator.

### ST133. Anchored Multiplex PCR Enables Sensitive NGS-based Mutation Detection in the Context of Large Primer Panels

V. Johnson, K.E. Moore, L.M. Griffin, A. Licon, R. Walters  
ArcherDX, Boulder, CO.

**Introduction:** The mutational landscape of a vast array of pediatric and adult cancers is changing at a rapid pace, however targeted next generation sequencing (NGS) assays typically lack the flexibility to adapt to changing mutational landscapes. For example, adding new targets to existing primer panels often requires redesign of the entire panel and expansion of panel size risks reducing the sensitivity of variant detection. Here, we describe a target enrichment method for NGS, Anchored Multiplex PCR (AMP), which enables sensitive detection of variants regardless of panel size while permitting flexibility in panel design.

**Methods:** AMP is a target enrichment strategy that uses unidirectional gene-specific primers and molecular barcoded (MBC) adapters ligated to DNA ends for amplification. We designed the Archer VariantPlex NGS assays to detect single nucleotide variants (SNVs), insertions and deletions (indels) and copy number variants (CNVs) with a customizable set of panel targets. **Results:** We assess the performance of the VariantPlex Solid Tumor Focus panel using reference materials titrated from 50ng to 1ng total input. We show that this panel is able to detect CNVs, as well as SNVs and indels with allele frequencies down to 5%. Furthermore, we compare per variant sensitivity and CNV sensitivity across input amounts for this panel alone and in the context of additional content that substantially increases the total targeted genomic sequence. Our data show that these assays maintain similar abilities to detect variants in small and large panels. **Conclusions:** Our results demonstrate that AMP-based NGS assays are sensitive while maintaining flexibility in panel design.

### ST134. Targeted Next-generation Sequencing of Solid Tumors versus Comprehensive Genomic Profiling Using Large Gene Panels: A Comparative Study

J. Routh, R.R. Gullapalli, M. Vasef  
University of New Mexico Health Sciences, Albuquerque NM.

**Introduction:** As the cost of genomic sequencing continues to fall, the larger gene panel (LGP) testing or comprehensive genomic profiling of solid tumors continues to grow. However, the clinical utility of LGPs compared to customized and focused targeted sequencing strategies remains unclear. Studies comparing the outcome between the 2 testing strategies are sparse. We sought to determine the potential clinical value of performing LGP compared to a smaller but more focused in-house targeted next generation sequencing (NGS) algorithm. **Methods:** Our archived database was searched for the solid tumor cases that were sent to Foundation One for LGP testing between January 2016 and May 2018. For each case, the results were reviewed and annotated for the following factors: availability of FDA approved therapy for the specific tumor type,

availability of FDA approved therapy for another tumor type, and general clinical trial availability. These results were then compared to results of our internal NGS 50 gene panel (Ion AmpliSeq Cancer Hotspot Panel v.2, Thermo Fisher). In cases where internal testing had not been performed, we assessed the likelihood of detection of the given mutations based on our in-house NGS test assay coverage. In addition to a 50 gene panel, our internal testing algorithm includes MSI testing; fluorescence *in situ* hybridization (FISH) for *ALK*, *ROS1* and *RET* rearrangements and *MET* amplification; and *HER2* by IHC or FISH as indicated. Finally a chart review was performed for all cases where actionable variants were identified by LGP but not by our 50 gene panel. **Results:** A total of 54 cases were reviewed. 12 of 12 cases with FDA approved therapies identified by LGP would have been identified by the internal testing algorithm. 21 of 34 cases with FDA approved therapy for a different tumor type and 32 of 46 cases with available clinical trials would have been identified by the internal testing algorithm. However a chart review revealed that neither treatment nor clinical trial enrolment directed by LGP were pursued in any of the 12 cases. **Conclusion:** Among 54 cases with available LGP data, 100% concordance was present between the 2 strategies in detecting the FDA approved therapies. For cases where actionable variants were identified by LGP only and clinical information was available, none of the patients were enrolled in the suggested trials or started on the recommended targeted therapies at our cancer center. These results call into question a significant add on value of performing LGP compared to a more cost-effective focused targeted NGS panel testing. Due consideration must be provided to the menu of clinical trials available in making the determination of the utility of the use of large gene panels.

### ST135. T-cell Receptor Beta Immune Repertoire Sequencing in Several FFPE Tissue Types: Interrogation of the Tumor Microenvironment in Archived Tissue Samples

G. Lowman<sup>1</sup>, T. Looney<sup>2</sup>, A. Zheng<sup>2</sup>, L. Miller<sup>1</sup>, E. Linch<sup>1</sup>, D. Topacio-Hall<sup>1</sup>, F. Hyland<sup>2</sup>, M. Andersen<sup>1</sup>

<sup>1</sup>ThermoFisher Scientific, Carlsbad, CA; <sup>2</sup>ThermoFisher Scientific, South San Francisco, CA.

**Introduction:** Immune repertoire sequencing is a valuable tool for studies of the tumor microenvironment and potential immune responses to cancer immunotherapy. Here we describe a T-cell receptor beta (TCR $\beta$ ) sequencing assay that leverages the low sample input requirements of AmpliSeq library preparation technology to extend the capability of targeted immune repertoire sequencing to include formalin-fixed, paraffin-embedded (FFPE) samples which can often be degraded and in short supply. **Methods:** Evaluation of the highly diverse CDR3 region of TCR $\beta$  allows for T-cell clone identification and frequency measurement. We demonstrate assay functionality with input of RNA or DNA samples, as well as flexibility in sequencing throughput and sample multiplexing capability. T-cell repertoires were evaluated from as low as 10ng to 1ug of input material of varying repertoire diversity, such as sorted T-cells, peripheral blood leukocytes, fresh-frozen tissue, and FFPE tissue from a variety of normal and cancerous tissues such as lung, colon, brain, spleen, lymph node, and thymus. **Results:** Accuracy is demonstrated through the evaluation of samples comprised of known numbers of sorted T-cells or spike-in experiments using 30 well-studied lymphoma rearrangements. In order to test functionality of the assay with a range of degraded input material, RNA was controllably degraded with heat treatment at 90-95°C. In these systematically degraded samples we observe a strong correlation ( $r = 0.97$ ) between the percentage of RNA molecules over 200bp in length and the amount of productive repertoire reads that the assay produces, while maintaining performance levels with samples with RIN values of below two. T-cell richness and diversity in repertoires measured from FFPE tissue samples vary, as expected, depending on sample quality, disease state, and tissue of origin. In parallel, we present a complimentary qPCR assay, specific for T-cell markers, which allows for sample T-cell quantification and acts to guide optimal sample input ranges for library construction. Finally, we present this CDR3 TCR $\beta$  sequencing assay including a dual barcoding approach to extend sensitivity for detection of rare clones in deep sequencing experiments. **Conclusions:** These data introduce a T-cell immune repertoire sequencing solution for applications in a wide range of sample



types including challenging FFPE preserved tissues. This assay is capable of profiling repertoire metrics from samples over a large range of input amounts from several tissue types. In addition, we demonstrate use of a qPCR assay for quantification of sample T-cell content to guide sample input for TCR $\beta$  immune repertoire sequencing with samples with highly variable T-cell content.

### ST136. Next Generation Sequencing for Quantitative Measurement of Human Epidermal Growth Factor Receptor-2 (HER2) Amplification: A Comparative Study

C. Reyes Barron<sup>1</sup>, B. Buscaglia<sup>2</sup>, A. Campbell<sup>2</sup>, D.G. Hicks<sup>2</sup>, Y. Ding<sup>2</sup>

<sup>1</sup>University of Rochester Medical Center, Victor, NY; <sup>2</sup>University of Rochester Medical Center, Rochester, NY.

**Introduction:** The over-expression of Human Epidermal growth factor Receptor-2 (HER2)/Erb-B2 receptor tyrosine kinase 2 (ERBB2) in breast cancer is associated with an aggressive clinical course and benefit from HER2 targeted therapy. Assessing HER2 status in breast cancer is imperative for treatment planning and patient care. Current clinical assays include immunohistochemistry (IHC) to detect protein over-expression and fluorescence *in situ* hybridization (FISH) to detect gene amplification. The HER2 protein may also be quantitatively measured using streptavidin-coated Phosphor Integrated Dot fluorescent nanoparticles (PID) with immunofluorescence measured by computer assisted image analysis. IHC, FISH, and PID have limitations and may have discordant results that are difficult to interpret. Our aim was to compare next generation sequencing (NGS) with these methods in the determination of HER2 amplification/over-expression. **Methods:** Seventeen well characterized cases of breast invasive ductal carcinoma, which had previously undergone IHC, FISH, and PID testing, were selected. NGS study was performed using the OncoPrint Comprehensive Assay on an Ion Torrent S5 XL sequencer. HER2 amplification/over-expression was considered positive by IHC with a value of 3+, by PID with a value  $\geq 30$ /cell and by NGS with a copy number  $\geq 5$ . HER2 copy number results by NGS were compared with IHC, FISH (ratio and HER2 copy number) and quantitative PID results. **Results:** Three cases with HER2 amplification by NGS were identified (copy number range 11.26 to 16.92). All three were designated classical amplified by FISH (ratio  $> 2$ , HER2  $> 6$ ), had IHC scores of 3+ and were positive by PID (range 155.5 to 343.5). One case was classical amplified by FISH, had IHC score of 3+, was positive by PID (148.1) and had a copy number of 2.94 by NGS. Of the remaining 13 cases with FISH results including low level amplified (3 cases; ratio  $> 2$  HER2  $> 4$   $< 6$ ), polysomy (4 cases; ratio  $< 2$  HER2  $> 6$ ), equivocal (3 cases; ratio  $< 2$  HER2  $> 4$   $< 6$ ) and non-amplified (3 cases); all had IHC values 0 to 2+, PID score range 3.7 to 21.4, and copy number range 1.12 to 4.18 by NGS. There was no correlation between copy number determined by NGS and PID score. **Conclusions:** NGS provided highly concordant results (75%) with established methods of HER2 amplification for breast cancers found to be classic amplified by FISH. Non-classic FISH results including low-level amplified, polysomy, equivocal as well as negative cases, had negative classification by NGS, IHC and PID. The potential for NGS utility in further classifying the nature of the HER2 genetic alterations remains to be explored and may help to further elucidate cases with non-classical FISH results or amplification by FISH with incongruous protein results by PID.

### ST137. Clonal Outgrowth and Microsatellite Shift in Paired Endometrial Intraepithelial Neoplasia and Endometrial Carcinoma

S.A. Patil, A. Plagov, D.B. Chapel, R.R. Lastra, S. Kadri, J.P. Segal, L. Ritterhouse

University of Chicago Medicine, Chicago, IL.

**Introduction:** Endometrial intraepithelial neoplasia (EIN) is a histopathologically distinct, precursor lesion to endometrial endometrioid carcinoma (EC). Microsatellite instability (MSI), wherein the number of repeats of microsatellites is altered via impaired mismatch repair (MMR) gene function, is seen in a subset of EC. Even though the progression of EIN to EC is a well-established phenomenon, studies examining the molecular and clonal evolution for cases with MSI are limited. **Methods:** Seven paired EIN and EC samples with known mismatch repair deficiency were used for this study. Next generation sequencing (NGS) based, UCM-OncoPlus assay (1,213 genes) was performed to detect mutations, copy number variations, MSI and tumor mutational burden. A UCM in-house

developed MSI module assesses stability of a microsatellite locus by comparing the homopolymer length distribution to a baseline distribution, across a set of selected 336 loci. MSI was also independently confirmed by immunohistochemistry (IHC). Clonal evolution of molecular alterations and microsatellite unstable loci was evaluated. **Results:** The mutational spectra of both EIN and EC in the present study were similar to those previously reported in the literature. EINs harbored on average approximately one-third the number of mutations than the paired ECs, across a subset of recurrently mutated genes in EC (PTEN, PIK3CA, ARID1A, CTNNB1, KRAS, PIK3R1). In all 7 paired cases, the EC samples were found to harbor a higher proportion of microsatellite unstable loci (mean=38%) compared to the EIN samples (mean=16%). While all 7 EC cases were determined to have MSI by NGS, only 3 EIN samples met the threshold for calling MSI by NGS. To elucidate the mechanism of microsatellite shifts during neoplasm evolution, individual microsatellite loci were analyzed and a progression of instability was identified in the majority of loci. **Conclusion:** This study demonstrates the utility of NGS-based MSI analysis in offering quantitative results as well as the ability to probe molecular events at individual loci. This limited study provides evidence for increasing levels of MSI from precursor lesions to established endometrial carcinomas and sheds light on the clonal evolution of these tumors.

### ST138. "PleSSision": A Pathologist Edited Multigene Genomic Test Promotes Cancer Precision Medicine in Japan

E. Aimonio<sup>1</sup>, E. Yanagita<sup>1</sup>, R. Matsuoka<sup>2</sup>, Y. Kato<sup>1</sup>, H. Hayashi<sup>1</sup>, M. Imai<sup>1</sup>, S. Tanishima<sup>3</sup>, H. Nishihara<sup>1</sup>

<sup>1</sup>Keio University, Tokyo, Japan; <sup>2</sup>International University of Health and Welfare, Narita, Japan; <sup>3</sup>Mitsubishi Space Software, Amagasaki, Japan.

**Introduction:** Development of genomic medicine enables us to perform contemporary clinical sequencing, while the acquisition of high quality biospecimens and the appropriate handling of these materials are indispensable. In Japan, several types of multigene genomic testing for cancer have been ratched as a clinical examination since around 2016. We herein developed a novel cancer clinical sequencing system by amplicon exome sequence targeting 160 cancer genes; PleSSision (Pathologists edited, Mitsubishi Space Software supervised clinical sequence system for personalized medicine) collaborating with Mitsubishi Space Software Co. Ltd (MSS). **Methods:** In our system, genomic DNA was extracted from both tumor FFPE (formalin-fixed paraffin embedded) tissue and peripheral blood mononuclear cells as a normal control. The quality of extracted DNA was evaluated by TapeStation 2000 (Agilent) and the library made with GeneRead Comprehensive Cancer Panel (QIAGEN) was deeply sequenced using MiSeq. FastQ files were analyzed by MSS using original bioinformatics pipeline within 3 business days, and we identified cancer-specific somatic gene alteration such as single-nucleotide variant (SNV), Ins/Del and CNV. Finally, cancer board conference consisting of medical oncologists, pathologists, clinical laboratory technologists, bioinformaticians and clinical geneticists proposes the personalized treatment strategies based on the gene profiles. **Results:** For 24 months, we examined approximately 300 solid cancer patients and performed targeted exome sequence. As a result, the success rate of sequence was 98%, and detection rate of actionable gene mutation was over 90%. In addition, 20 out of 159 (12 %) patients underwent "Genotype-matched treatment" based on our sequence report, and response rate (CR + PR) and disease control rate (CR + PR + SD) are 44% and 67%, respectively. **Conclusion:** Here we revealed the clinical significance of our "PleSSision" system to identify actionable gene alteration. Moreover, utility of genotype matched treatment was also confirmed. We believe that our novel clinical sequencing system will vigorously promote cancer individualized medicine throughout Japan.

### ST139. Next Generation Sequencing (NGS) Testing with the OncoKids Panel Identifies Clinically Significant Findings in the Majority of Pediatric Sarcomas/Soft Tissue Tumors

M.C. Hiemenz, J. Buckley, G. Raca, R.J. Schmidt, F. Navid, R. Shah, S.M. Thomas, N. Shillingford, S. Zhou, L.J. Helman, L. Mascarenhas, M. Warren, D.M. Parham, J.A. Biegel, T.T. Triche  
Children's Hospital Los Angeles, Los Angeles, CA.

**Introduction:** The OncoKids panel is an amplification-based next-generation sequencing assay designed to detect diagnostic, prognostic, and therapeutic markers across the full spectrum of pediatric malignancies. This assay uses low input amounts of DNA and RNA and is compatible with formalin-fixed, paraffin-embedded (FFPE). This study sought to examine the clinical utility of OncoKids testing of pediatric solid tumors including sarcomas and soft tissue tumors. **Methods:** Sequencing was performed using the Ion S5 platform. This study was a retrospective analysis of non-CNS solid tumors (including sarcomas and soft tissue tumors) assayed with OncoKids between June 20, 2017 and May 20, 2018. **Results:** Of over 300 pediatric malignancies assayed to date there were 110 non-CNS solid tumors including 15 neuroblastomas, 14 osteosarcomas, 13 rhabdomyosarcomas, nine sarcoma NOS specimens, six Ewing sarcomas, six sex cord stromal tumors, and a variety of less common tumors. A total of 59 samples (54%) demonstrated at least one variant of strong clinical significance while nine samples had at least one variant of potential clinical significance (8%). Gene fusions of strong clinical significance were observed in 24 samples (22%). Nine of 24 fusion-positive samples demonstrated a fusion that would be undetectable with standard fluorescence *in situ* hybridization (FISH) testing at our institution. This group included therapeutically actionable fusions with targeted therapies such as larotrectinib (*TPM3-NTRK1* and *LMNA1-NTRK1*) and crizotinib (*TFG-ROS1*) as well as diagnostic fusions for a variety of tumor types (e.g., *CIC-DUX4* in Ewing-like sarcoma, *MYB-NFIB* in adenoid cystic carcinoma, and *CCDC6-RET* in papillary thyroid carcinoma). Notably, three of 10 fusion-positive samples by OncoKids NGS testing were negative with the corresponding FISH probe; this may be due to sample quality or other limiting factors. Testing of tumor tissue revealed potential underlying germline mutations in cancer predisposition genes in 27 of 110 patients (25%). A recommendation for targeted germline testing resulted in screening 16 patients. Six of the 16 patients had a germline pathogenic or likely pathogenic variant, including three children with *DICER1* mutations, and one patient each with mutations in *TP53*, *CBL*, and *WT1*. **Conclusions:** Routine molecular profiling of pediatric malignancies in the front-line setting by OncoKids provided a significant improvement in the diagnosis and the opportunity for targeted therapy in children with sarcomas and other solid tumors and prompted screening and counseling in patients and families with cancer predisposition.

### ST140. EWSR1-ATF1 Fusion in Solid Tumors: Next-generation Sequencing Analysis by MSK-IMPACT

G. Zhu, R. Benayed, M. Ladanyi, N. Katabi, M. Hameed  
Memorial Sloan Kettering Cancer Center, New York City, NY.

**Introduction:** Precise diagnosis of translocation-driven solid tumors is augmented by identification of the underlying gene fusions. Next-Generation Sequencing technology has greatly improved and accelerated the identification and discovery of recurrent and novel gene fusions. *EWSR1-ATF1* has been found to be the oncogenic driver in a variety of solid tumors. Here we summarize the clinicopathological and molecular findings of the solid tumors with *EWSR1-ATF1* fusion identified at our institution during 2014-2018. **Methods:** MSK-IMPACT is an FDA approved customized targeted Next-generation Sequencing assay offered at our institution. In addition to 468 genes, the introns of multiple genes including *EWSR1* are tiled by targeted probes in order to identify recurrent fusions. **Results:** A database search of more than 20000 solid tumors analyzed by MSK-IMPACT found 15 cases that harbor *EWSR1-ATF1* fusion. The tumor types include one case of angiomatoid fibrous histiocytoma (AFH), one case of clear cell odontogenic carcinoma, 5 cases of hyalinizing clear cell carcinoma of salivary gland (HCCC), 2 cases of clear cell sarcoma-like tumor of the gastrointestinal tract (CCSLGT) and 6 cases of clear cell sarcoma of soft tissue (CCS). The patients, ranging from 13 to 68 year old in age, were 5 males and 10

females. Eight patients developed local or distant metastases which include one case of AFH, two cases of HCCC, one case of CCSLGT and four cases of CCS. The metastatic sites include lymph node, lung, bone and soft tissue. Nine cases had an alternative molecular assay, fluorescence *in situ* hybridization (FISH) or targeted RNA sequencing, which confirmed the fusion. The genomic breakpoints of *EWSR1* fall within g.29683656-29694628 on chromosome 22, which correspond to intron 7 to intron 13 (NM\_005243). All the breakpoints are located within the introns except one case of CCS where the breakpoint is located in the middle portion of exon 9. The genomic breakpoints of *ATF1* fall within g.51160825-51207953 on chromosome 12, which correspond to intron 2 to exon 5 (NM\_005171). All the breakpoints are located within the introns. Genomic alterations in *CDKN2A*, *TERT* or *TP53* are identified in 7/8 metastatic cases, but not in cases without metastasis. **Conclusion:** Next-generation Sequencing is a robust molecular assay for detection of *EWSR1-ATF1* fusion. This promiscuous fusion can be seen in many different tumor types whose biological behavior is driven by cell type where fusion occurs. Genomic alterations in *CDKN2A*, *TERT* or *TP53* might be involved in the progression of disease driven by *EWSR1-ATF1*.

### ST141. Identification of COL1A1-USP6 and ANGPTL2-USP6 Gene Fusions in Myositis Ossificans-like Aneurysmal Bone Cyst (MO-like ABC)

G. Zhu<sup>1</sup>, R. Benayed<sup>1</sup>, S. Hwang<sup>1</sup>, N. Agaram<sup>1</sup>, J. Bridge<sup>2</sup>, M. Hameed<sup>1</sup>  
<sup>1</sup>Memorial Sloan Kettering Cancer Center, New York City, NY; <sup>2</sup>University of Nebraska Medical Center, Omaha, NE.

**Introduction:** MO-like ABC is a rare soft tissue lesion which can pose a diagnostic challenge due to overlapping morphological and imaging features with myositis ossificans. Approximately 70% of primary ABC of bone exhibit rearrangement of the *USP6* gene. This study is undertaken to analyze clinical, imaging, pathological and molecular characteristics of a series of MO-like ABC cases. **Methods:** Nine cases to include 5 resections and 4 biopsies of MO-like ABC diagnosed between 2009 and 2018 were retrieved. H&E slides for archival formalin-fixed paraffin-embedded sections were reviewed and radio-pathological correlation was performed. Specimens that were not subjected to acid decalcification were submitted for fluorescence *in situ* hybridization (FISH) for *USP6* gene rearrangement. Archer RNA Fusion Plex targeted RNA sequencing technology was used to identify fusion partners after FISH confirmation. **Results:** Patients included 6 females and 3 males with a median age of 29 years. The tumor sites were thigh, buttock, axilla, hand and clavicular soft tissue. Tumors ranged in size from 2.1 to 5.0 cm in greatest dimension. Radiological imaging showed that all the tumors were located in the soft tissue including two cases that were juxtacortical. Radiological differential diagnoses included myositis ossificans, solid ABC, secondary ABC, nodular fasciitis, juxtacortical osteoblastoma, juxtacortical chondroma, Langerhans cell histiocytosis and osteosarcoma. Histologically, the predominant pattern was a cellular spindle cell lesion with admixed osteoclast-type giant cells in association with abundant new bone formation. FISH showed *USP6* gene rearrangement in 8 out of 9 cases (one case had insufficient cells for evaluation). Targeted RNA sequencing performed on 7 of the *USP6* rearranged cases revealed *COL1A1-USP6* fusions in 6 cases and a novel *ANGPTL2-USP6* in one. All the breakpoints in *USP6* were located in the 5' UTR. **Conclusions:** 1) Our study confirms the neoplastic nature of MO-like ABC. 2) *USP6* gene rearrangement analysis is a useful test to identify this lesion in biopsy material. 3) We identified a novel fusion *ANGPTL2-USP6* in addition to the recurrent *COL1A1-USP6* fusion in MO-like ABC.

### ST142. Differential Detection of BRAF V600E and V600K Using a Simple and Sensitive Droplet Digital PCR Assay

L. Landry<sup>1,4</sup>, L. Bellizzi<sup>2</sup>, J. Bruce<sup>1</sup>, V. Rojas-Rudilla<sup>1</sup>, S. Diegue<sup>1</sup>, A. Duchesne<sup>1</sup>, Y. Kuang<sup>3</sup>, C.P. Paweletz<sup>3</sup>, N. Lindeman<sup>1</sup>, L. Sholl<sup>1</sup>

<sup>1</sup>Brigham and Women's Hospital, Boston, MA; <sup>2</sup>University of Connecticut, Storrs, CT; <sup>3</sup>Dana Farber Institute, Boston, MA; <sup>4</sup>Harvard Medical School, Boston, MA.

**Introduction:** The *BRAF* V600 codon is a well-characterized oncogenic hotspot and certain mutations at this position predict therapeutic response to RAF and MEK inhibitors in a variety of tumor types. *BRAF* V600E is the most common somatic variant in *BRAF*, accounting for > 90% of

variations at this codon and has been shown to have diagnostic, prognostic, and therapeutic relevance in glioma, melanoma, hematologic malignancies, lung cancer, and colon cancer, among others. *BRAF* V600K is less common and represents ~ 10% of codon 600 activating mutations in melanoma and is therapeutically relevant in that context. *BRAF*-mutated patients, particularly melanoma, can have rapidly progressive disease that can respond rapidly to treatment. As such, rapid standalone *BRAF* testing can be critical to support urgent clinical treatment decisions. We developed and validated a droplet digital PCR (ddPCR) assay for simultaneous, differential detection of V600E/K mutations from tumor tissue specimens. **Methods:** ddPCR primers and probe (Applied Biosystems) were designed against nucleotide sequences encoding for *BRAF* V600 wild type and V600E. The V600E probe binds both the E and K substitution sequences but generates distinct post-PCR mutant signal amplitude. The validation study included 55 tumor samples with known V600E (n=21), V600K (n=11), other exon 15 non-V600E/K variants (n=9) and wild-type (n=14) based on pyrosequencing or next generation sequencing. Specimen types included formalin-fixed paraffin embedded tissue, aspirate smears, ThinPrep, and fresh or frozen tissue. The tumor types included glioma, melanoma, histiocytic, lymphoid and myeloid neoplasms, and carcinomas of the colon, lung, thyroid, and miscellaneous sites. Purchased engineered heterozygous cell lines were used as controls (Horizon Discovery). ddPCR results were compared to previous known results. **Results:** Average signal amplitude for V600E was 9300 [range 7500-11000] vs. V600K 3300 [2500-4000] fluorescence units. Samples generated between 500-4000 total amplified events per replicate. The ddPCR results were 100% concordant with prior results. The assay achieved perfect inter-run and inter-operator agreement and was linear from 50% - 1% mutant allele fraction. Limit of detection was empirically determined to be >1 mutant event, however a conservative cutoff of >3 mutant events was established for reporting.

**Conclusion:** This novel assay design permits highly accurate, sensitive and simultaneous, differential detection of two clinically important *BRAF* variants with a single primer/probe design. This assay may complement other more time consuming and expensive molecular methods such as NGS, particularly for critically ill patients.

#### ST143. Development of a Targeted NGS Panel for Solid Tumor Actionable Gene Targets using Multiplex PCR-based Enrichment in an Integrated Fluidic Circuit

P. Chen<sup>1</sup>, X. Wang<sup>1</sup>, H. Gong<sup>1</sup>, A. Lespagnol<sup>2</sup>, S. Meyers<sup>1</sup>, D. Do<sup>1</sup>, M. Gonzalez<sup>1</sup>, L. McLean<sup>1</sup>, T. Goralski<sup>1</sup>, B. Hunt<sup>1</sup>

<sup>1</sup>Fluidigm, South San Francisco, CA; <sup>2</sup>Centre Hospitalier Universitaire de Rennes, France.

**Introduction:** Next-generation sequencing (NGS) has been being rapidly adopted in clinical research to align actionable variants in tumors to targeted therapies or clinical trials. Comprehensive NGS-based tumor profiling assays are an efficient and effective method to characterize a variety of clinically relevant somatic mutations. We have developed a targeted NGS cancer gene panel that focuses on actionable gene mutations and employs a NGS library preparation method based on multiplex PCR enrichment in an integrated fluidic circuit (IFC) and the automated Juno System. **Methods:** Fifty-three actionable cancer genes were selected based on both clinical and research knowledge that covers 15 solid tumor types. Assays for the selected regions of the 53 genes were designed by an internal assay design pipeline. Multiplex PCR using target-specific primers is conducted in an LP—48.48 IFC on Juno (Fluidigm), where up to 48 DNA samples can be processed simultaneously with unique barcodes for individual samples. To evaluate the assay performance, three sets of cell line gDNA samples were identified from commercial sources: Set 1: 22 reference samples; Set 2: 12 samples for single nucleotide variants (SNVs) and insertion/deletions (indels), and Set 3: 12 samples for copy number variations (CNVs). Each sample was tested on the IFC in 4 replicates. PCR products harvested from the IFC were pooled and purified. A second PCR step using a universal primer pair was performed to add sequencing adapters. After a second purification, the DNA library was sequenced on a NextSeq 500 system. The data analysis was performed by a service provider partner (GenomOncology). **Results:** A total of 1,508 primer pairs with an average insert size of 155 bp were selected to cover SNVs, indels, and CNVs. The

assays were separated into 44 pools to minimize the interaction between assay primers and improve performance. The percentage of reads mapped to the genome was 98.9%, and the percentage of reads that mapped to amplicons was 96.8%. Mutation detection sensitivity was 4% variant allele frequency. In 47 selected samples, the total SNVs, CNVs and indels represented are 182, 154 and 28, respectively. For SNVs, the positive percent agreement (PPA) is 1.0 and positive predictive value (PPV) is 0.974. For CNVs, the PPA is 1.0 and PPV is 0.969 and for indels, the PPA and PPV are 1.0 and 0.966, respectively. Overall concordance is 0.99. **Conclusions:** A targeted NGS cancer panel employing PCR-based enrichment on an IFC has been developed and yielded high quality of libraries for detecting SNVs, indels and CNV in solid tumor samples. This panel covers actionable targets in 53 cancer genes and utilizes a microfluidic device to provide a simple streamlined workflow for library preparation of up to 48 DNA samples per IFC.

#### ST144. Prognosis Determined by Tumor Mutational Burden (TMB) Using Whole Exome Sequencing (WES)

E. Fernandez<sup>1</sup>, K. Eng<sup>1</sup>, A. Sboner<sup>1</sup>, O. Elemento<sup>1</sup>, W. Song<sup>1,2</sup>

<sup>1</sup>Weill Cornell Medicine, New York, NY; <sup>2</sup>New York-Presbyterian, New York, NY.

**Introduction:** Tumor mutational burden (TMB) has been explored as a potential biomarker for immunotherapy for several types of cancer. TMB is commonly defined as the total number of somatic mutations in cancer genome, and it can also be represented as mutation density - dividing the total TMB by DNA coding region. It is believed that those mutations will lead to translation of novel peptide epitopes, or neoantigens, which further elicit T cell repertoires that recognize these antigens as "foreign" and infiltrate the tumor microenvironment (TME). However, no consensus currently has been established to define high vs. low TMB. To continue moving forward with our understanding of TMB in a clinical setting, a more contextualized and dynamic view of the TMB landscape must be investigated, especially through more comprehensive sequencing approach, such as whole exome sequencing (WES). **Methods:** Mutation Annotation Format (MAF) files were used for calculating TMB. Expected germline mutations from the ExAC database were filtered out, as well as variants with VAF < 0.1. TMB was calculated for our own WES (EXACT-1) cohort, as well as for TCGA samples, including bladder, prostate, endometrial, breast, stomach, and colorectal cancers. **Results:** First, the distribution of TMB in EXACT-1 samples (n = 979) and TCGA (n = 6591) were similar, with both showing a long tailed distribution, with similar mean and standard deviation. Second, we examined the distribution of TMB in different cancer types and explored potential different clinical outcomes between high and low TMB values. To define a cutoff value discriminating high vs. low TMB, we have used four different mechanisms: a) static cutoff of 20 mutations/Mb, b) median (TMB) + 2\*IQR(TMB), c) the 75<sup>th</sup> percentile, and d) mean(TMB) + 2\*IQR(TMB). It appeared that only d), e.g. mean(TMB) + 2\*IQR(TMB); resulted in significant improved progression-free survival and overall survival separation between high and low TMB groups in some cancer types: bladder cancer (p-value = 0.018; log-rank test) and endometrial cancers (p-value = 0.048; log-rank test). However, such separation was not observed in other types of cancers, including kidney, prostate, breast, colorectal. By adopting this approach to define TMB high, we found that most cancers had a frequency of TMB high samples of 3%-10% in different cancer types. **Conclusions:** By using our classification threshold for TMB high of mean(TMB) + 2\*IQR(TMB), bladder and endometrial cancer patients with high TMB show significantly better prognosis compared to patients with low TMB. However, this result is not seen in other types of cancer. We hope this result will help to evaluate and identify patient groups with better response to immunotherapy.



#### ST145. Streamlined Next-generation Sequencing Assay Development Using a Highly Multiplexed FFPE Quality Control Technology Based on the Genome in a Bottle

L. Liu, A. Lau, K.L. Norman

Thermo Fisher Scientific, Fremont, CA.

**Introduction:** Next generation sequencing (NGS) assay development for solid tumor sequencing requires characterization of variant calling directly from formalin-fixed paraffin embedded (FFPE) tissue samples. However, cell line based FFPE and human FFPE samples only contain 2 to 20 variants, which require laboratories to invest significant resources in sample sourcing and preparation when developing assays to detect 100+ variants. This challenge is further compounded by the inherent heterogeneity of human FFPE samples. In this study, we demonstrate the development of a standardized full process (controlling from extraction to result) FastFFPE quality control material that contains more than 500 variants commonly found in lung, breast, colon, and other solid tumors. Notably, all 500+ variants are contained within a single FFPE section. To ensure predictable performance and enable accuracy applications, all variants were Sanger sequenced and cells from the Genome in a Bottle (GIAB) Consortium were used as a background genome. The performance of the FastFFPE samples was evaluated for variant allelic frequency (AF) using next-generation sequencing (NGS) and droplet digital PCR platforms. The extraction yield was also determined.

**Methods:** FastFFPE quality control material was generated to contain 500+ cancer hotspot variants (including SNP, MNP, and insertion/deletions or indels) at various AF in the background of the GIAB GM24385 cells. FastFFPE blocks were sectioned into 10 micron scrolls and stored at 2-8°C. The DNA was extracted in triplicate from each block using the QIAamp DNA FFPE tissue kit, and concentration was determined using the Qubit 3.0 dsDNA HS assay kit. The Ion AmpliSeq Cancer Hotspot Panel v2 was used for library preparation and tested on Ion Torrent Personal Genome Machine (PGM). AF results determined using the PGM instrument were compared to the AF by Bio-Rad droplet digital PCR (ddPCR) platform. **Results:** The FastFFPE blocks showed an average AF of  $62 \pm 2\%$ ,  $12.5 \pm 1.3\%$ ,  $6.0 \pm 0.7\%$  and  $1.1 \pm 0.3\%$  by ddPCR. The AF obtained using NGS was similar to ddPCR results, which suggests that the performance of FastFFPE is platform agnostic. Each section at the respective AF (except 1%) showed a greater than 70% hotspot mutation detection rate by NGS, indicating that each scroll has consistent performance. DNA extraction yields were all >100 ng/section, indicating that each section, indicating that each section provides sufficient material for multiple replicate testing.

**Conclusions:** A highly multiplexed FastFFPE sample has been generated and tested on both NGS and ddPCR platforms. This external quality control material could provide reliable and consistent testing results for NGS solid tumor assay development, from DNA extraction through to variant calling.

#### ST146. Evaluation of ctDNA Extraction Methods and Amplifiable Copy Number Yield Using Standardized Human Plasma-based ctDNA Control Materials

L. Liu, R. Hu, K.L. Norman

Thermo Fisher Scientific, Fremont, CA.

**Introduction:** The use of cell-free circulating tumor DNA (ctDNA) for non-invasive cancer testing has the potential to revolutionize the field. However, emergence of an increasing number of extraction methods and detection assays is rendering laboratory workflow development much more complex and cumbersome. The use of standardized, well characterized ctDNA control materials in human plasma could facilitate the evaluation of extraction efficiency and assay performance across platforms. In this study, we use a full process ctDNA quality control material in true human plasma to demonstrate the variability of extraction yield between different ctDNA extraction kits. We also examine the correlation between the amplifiable copy number and DNA concentration post-extraction. **Methods:** DNA materials that carry cancer hotspot mutations were spiked into the NIST Genome in a Bottle (GM24385) reference gDNA as background DNA at various allelic frequencies. The DNA was then fragmented and spiked into AcroMetrix Normal Human Plasma matrix to mimic human samples, enabling full process evaluation from extraction to data analysis. Nucleic acids were extracted using 3

different commercially available kits to evaluate extraction efficiency. DNA concentration was determined with Qubit 3.0 using dsDNA HS assay kit. The absolute copy number of ctDNA was evaluated using Bio-Rad droplet digital PCR system. **Results:** The bead-based MagMAX nucleic acid isolation kit resulted in up to 97% recovery efficiency while the column-based QIAamp circulating Nucleic Acid kit resulted in >100% recovery rate. However, the amplifiable copy number correlated well with the amount of MagMAX extracted DNA input, whereas less correlation was shown with the Qiagen column-based extracted DNA. This suggests that this column based extraction method may capture some DNA fragments that are not amplified by the same assay. The combination of bead- and column-based QIAamp MinElute ccfDNA mini kit showed similar recovery rate and amplifiable copy number to the MagMAX extraction kit, suggesting that the bead-based methods may have similar DNA size selection functions. No extracted samples showed changes in the allelic frequency, which indicates that the extraction step does not preferentially select the wild-type or mutant DNA. **Conclusions:** This study demonstrated that the commercially available ctDNA extraction kits have various extraction efficiencies, and yield different correlations between DNA input and amplifiable copy number. The ctDNA quality control material in normal human plasma with known DNA input serves as a useful tool for evaluation of different extraction kits and assay performance.

#### ST147. Gene Expression Analysis for Predicting the Response of Anti-PD-1 Therapy in NSCLC Patients from Longitudinal Data

Y. Cheung<sup>1</sup>, J. Wu<sup>1</sup>, W. Huang<sup>2</sup>, C. Yin<sup>2</sup>, M. Fanucchi<sup>2</sup>, J.T. Fallon<sup>2</sup>, N. Dimitrova<sup>3</sup>

<sup>1</sup>Philips Research North America, Cambridge, MA; <sup>2</sup>New York Medical College, Valhalla, NY; <sup>3</sup>Philips Healthcare, Valhalla, NY.

**Introduction:** Programmed death 1 (PD-1) is a key immune checkpoint receptor expressed by activated T-cells. It mediates immunosuppression when T-cells encounter the PD-1 ligands PD-L1 and PD-L2 expressed by cancer, stromal or other immune cells. While measures such as PD-L1 expression and neoantigen burden in tumor tissue may help predict the response of anti-PD-1 immunotherapy, the biopsy procedure is not without risk. The goal of this study is to evaluate the potential use of gene expression profile of peripheral blood mononuclear cells (PBMCs) as a non-invasive biomarker for predicting the response of anti-PD-1 therapy in non-small cell lung cancer (NSCLC) patients. **Methods:** Peripheral blood of four NSCLC patients treated with an anti-PD-1 monoclonal antibody was collected prior to treatment (week 0) and subsequent infusions (weeks 4, 8, 12 and 24). All patients had a partial response to the therapy according to standard RECIST 1.1 criteria. Total RNA was extracted, and libraries were prepared using TruSeq Stranded mRNA LT Sample Prep Kit and sequenced by Illumina NextSeq. The paired-end reads were mapped to human reference hg38 using STAR and gene expressions were calculated using RSEM. Preliminary analysis of differential gene expression (DE) was performed to identify transcripts that were significantly up-/down-regulated respectively at 4 (4 samples) and 8 weeks (3 samples) after treatment compared with the baseline (week 0). Genes with Bonferroni-adjusted p values less than 0.05 were selected for pathway enrichment analysis with DAVID. **Results:** For samples collected in week 4, the DE signal was relatively weak, with log2 fold change (LFC) of gene expression ranging from -1.87 to 1.64, and seven significant DE genes with adjusted p < 0.05 out of 24,150 genes. Whereas for samples from week 8, LFC ranged from -4.39 to 3.30, and 1,588 significant DE genes were identified, of which the Pearson correlation of LFC with samples from week 4 is high at 0.94. The top five KEGG pathways associated with these 1,588 DE genes were *Ribosome* (73/136 genes, p =  $7.3 \times 10^{-39}$ ), *Toxoplasmosis* (36/118,  $3.5 \times 10^{-10}$ ), *Leishmaniasis* (27/72,  $4.3 \times 10^{-10}$ ), *T cell Receptor Signaling* (33/103,  $5.5 \times 10^{-10}$ ) and *Chemokine Signaling* (44/186,  $1.8 \times 10^{-8}$ ). **Conclusions:** Blood samples collected in week 8 has much stronger DE with respect to the baseline compared with samples collected in week 4, suggesting they are likely to be more effective for immunotherapy response prediction. The results of these analyses show promise of developing a predictive signature for the response of anti-PD-1 immunotherapy in NSCLC patients based on the gene expression profile of PBMCs. While these are preliminary results, we

continue to recruit patients, and will present the up-to-date results at the conference.

**ST148. Performance Evaluation of Methylation-Specific Real-time PCR (MSP) Assay for the Detection of O (6)-methylguanine-DNA Methyltransferase (MGMT) Promoter Methylation in Glioblastoma Multiforme**

J. Barry, S. Talwalkar  
CPA Lab, Louisville, KY.

**Introduction:** Methylation of MGMT promoter is associated with increased response to alkylating agents in patients with glioblastoma multiforme (GBM). Objective of this study was to test performance characteristics of the EntroGen MGMT assay in a mid-sized reference laboratory. **Methods:** Genomic DNA extracted from macrodissected formalin-fixed, paraffin-embedded (FFPE) slides from patient samples (N=31), control materials (N=6), and the 2017 CAP GLI-B survey (N=3) were tested. All patient samples were diluted to 200-500ng and processed with the EZ DNA Methylation-Lightning Kit according to manufacturer's protocol with the modification of eluting into a 30uL volume. Control material with known methylation was tested to establish analytical measurement range (AMR). Assay includes 7 MGMT and ACTIN-B standards that cover the AMR as well as a no template control each tested in triplicate. Samples are also tested in triplicate with ACTIN-B (amplification control) and the average of three wells was used for result calculation. Samples used for validation were evaluated between performing technologists, shipments, multiple days, and correlation with a reference laboratory. **Results:** AMR for methylated MGMT was determined to be between 10-9,335 copies per 4uL (2.5-2,333.75 copies/uL). Due to the broad analytical range, a greater variability of methylation was observed at higher levels. Three CAP survey samples (negative, low, and high positive) were tested for AMR verification. These demonstrated 100% correlation with CAP's intended responses for these specimens. Some discrepancies were observed between the reference lab methodology (pyrosequencing) and the methylation-specific PCR (MSP), especially for samples which showed low level of methylation very near the lower limit of quantification. Using the resulting guideline helped eliminate this inconsistency for the MSP assay. MSP assay had 100% specificity as tested using 4 DNA samples extracted for other molecular assays (non-small cell lung carcinoma and melanoma) found to be negative for MGMT promoter methylation. **Conclusion:** The EntroGen MSP assay can be reliably used for evaluation of MGMT promoter methylation on DNA from FFPE tissues. Assay is robust and has capability to provide fast turn-around time. MSP assay also provides quantitative assessment of methylation which can be used for assessment of treatment responsiveness and correlation with clinical outcome. The resulting guideline can help in interpretation especially for samples with low level methylation.

**ST149. T-cell Receptor Repertoire Profiling in High Grade Serous Ovarian Cancer Using Next-generation Sequencing**

B. Lo<sup>1</sup>, S. Smiley<sup>2</sup>, N. Wright<sup>2</sup>, H. Sekhon<sup>1</sup>, J. Weberpals<sup>1</sup>

<sup>1</sup>The Ottawa Hospital, Ottawa, Canada; <sup>2</sup>Ottawa Hospital Research Institute, Ottawa, Canada.

**Introduction:** In this study, we explored whether T-cell receptor (TCR) repertoire profiling can predict extreme responses to carboplatin/taxol chemotherapy in high grade serous ovarian cancer (SOC) patients. **Methods:** Suboptimally debulked SOC patients with advanced disease (stage III-IV) treated at The Ottawa Hospital were stratified by response to first-line carboplatin and taxol chemotherapy. There were 20 extreme platinum-sensitive (PS) responders (>12 mo progression-free interval (PFI)) and 19 platinum-resistant (PR) responders (<6mo PFI) whose formalin fixed paraffin embedded tissue specimens were available for analysis. For each of these 39 cases, using RNA extracted from macro-dissected tumor sections, TCR repertoire profiles were generated using the ArcherDx Immunoverse assay. **Results:** We performed clonotype quantification and diversity analysis using the ArcherDx bioinformatics pipeline and present comparisons of this data to other representations of the immune response derived from transcriptomics and Nanostring experiments. **Conclusions:** Through this study, we aim to further our understanding of the heterogeneity of immune responses in high grade

serous ovarian cancer as well as provide performance characteristics for the ArcherDx Immunoverse assay on a large set of formalin fixed archival specimens.

**ST150. Evaluation of the AMP/ASCO/CAP Classification Guidelines for Reporting Sequence Variants in Cancer**

B.A. Parikh, L. Love-Gregory, E.J. Duncavage, J.W. Heusel  
Washington University School of Medicine, St. Louis, MO.

**Introduction:** The utility of next generation sequencing (NGS) data in precision medicine relies, in part, on the consistency and accuracy of variant interpretation. Guidelines governing the assessment of germline variants in constitutional diseases have been instituted and undergone revisions; standardization of somatic variants is less well established. In 2017, the AMP/ASCO/CAP published a 4-tiered classification system for the interpretation and reporting of sequence variants in cancer. In this algorithm, somatic variants with clinical significance are further subdivided based on supporting evidence, resulting in sub-categories for both Tiers I and II. To implement these recommendations, our laboratory initiated a mapping of these guidelines to our current and broadly applied 5-level classification for somatic variants. Described here are two approaches to evaluate the correlation between these reporting guidelines.

**Methods:** We used two methods to assess the implementation of these classification systems. (1) 150 variants assigned to our top 3 levels identified in 40 consecutive solid tumor and hematologic cancer cases were selected for retrospective assessment by a single qualified reviewer. Based on prior interpretive information and an evaluation of the clinical case, these variants were scored according to the AMP/ASCO/CAP tiers I-IV. (2) A variant classification survey tool was developed to evaluate concordance within the group of sign-out faculty. Five variants were chosen to represent a continuum of expected difficulty. **Results:** The results of the retrospective reanalysis of 150 variants demonstrated that correlation between these reporting guidelines was consistent for >75% of clinically relevant variants. However, reassignment of certain variants, particularly TP53, was an area of notable discordance. The faculty survey results showed minimal variability in benign classification levels between the two guidelines. Conversely, reassignment of pathogenic cancer variants resulted in an unexpected variability when correlated with the top 2 tiers in AMP/ASCO/CAP guidelines, even when the evidence categories were combined. **Conclusions:** Our preliminary analysis demonstrated that a transition to the AMP/ASCO/CAP guidelines is likely to correlate well with current classification systems. Although similar sources of evidence were used to guide clinical interpretations, the strength of the evidence affected the AMP/ASCO/CAP variant assignment to a greater degree. A standardized approach to evaluating the literature with discretely defined literature sources could reduce the observed variability. As such, we anticipate adoption of the AMP/ASCO/CAP guidelines in parallel with an ongoing somatic variant assessment quality assurance program.

**Technical Topics**

**TT001. Clinical Validation of a Comprehensive DNA/RNA Panel (170 Genes) for Single Nucleotide Variants (SNVs), Small Insertions/Deletions (Indels), Copy Number Variations (CNVs) Splice Variations (SVs), and Gene Fusions (GF) on an NGS Platform in a CLIA Setting.**

R. Kolhe<sup>1</sup>, A.K. Mondal<sup>1</sup>, C. Pundkar<sup>1</sup>, P.K. Ahluwalia<sup>1</sup>, C. Bloomer<sup>2</sup>, A. Chaubey<sup>1</sup>

<sup>1</sup>Medical College of Georgia, Augusta, GA; <sup>2</sup>Augusta University, Augusta, GA.

**Introduction:** Currently next generation sequencing (NGS) techniques are being widely used as a tool in routine oncology workflows. Majority of labs use either DNA based small hotspots panels or two separate DNA and RNA panel. Unfortunately, this methodology can lead to an incomplete mutation profile because it lacks comprehensive screening of all known hotspots and tumor-suppressor genes. Many heterogeneous tumors carry multiple mutations and gene function can be altered by several types of variations including SNVs, insertion/deletions (indels),

SVs and GFs. The assay is a targeted next-generation sequencing (NGS) assay designed to detect genetic alterations in 170 genes, including 148 genes for substitutions (single nucleotide and multinucleotide variants) and indel detection, 55 genes for fusion and splice variant detection, and 59 genes for CNV detection. Assessment of fusions, splice variants, indels and SNVs, and CNVs in one assay using DNA and RNA creates efficiencies in sample usage, time, and cost. **Methods:** The validation was guided by the joint consensus recommendation for validation of NGS assays by the AMP and CAP. The validation included evaluations of precision, analytic sensitivity, analytic specificity, accuracy, reportable range, and reference range. RNA and DNA from 188 samples [mixture of known patient specimens, known CAP proficiency specimens as well as known synthetic reference standards (Acrometrix hotspot panel and Seracare fusion V2 panel)] was used. These clinical samples were earlier tested on gold standard orthogonal methods like PCR, fluorescence *in situ* hybridization (FISH), CDx NGS, and karyotype (n=181 clinical and reference materials formalin-fixed, paraffin-embedded (FFPE) tumor samples from lung, GI, skin, CNS, breast, kidney, uterus, ovarian, brain tumors, head and neck. Dental cancer, salivary gland tumor, thyroid gland tumor, lung cancer, GI and colon cancer. GB cancer, soft tissue cancer, bone cancer, endometrial and other uterine cancers, ovarian cancers, melanoma etc). Libraries for were prepared using the Illumina TruSight Tumor 170 (TST170) kit and sequenced on NextSeq 550. Sequencing analysis and variant calling were performed using the BaseSpace TST170 App. **Results:** We were able to validate manufacturers analytical claims for all the variants groups in our validation, e.g. for small variants, ≥98% sensitivity and 100% specificity at 5% allele frequency at positions with coverage ≥250x was obtained. **Conclusions:** DNA and RNA libraries are prepared, sequenced, and analyzed simultaneously for efficient assessment of numerous types of somatic variants. We anticipate that this approach of obtaining high-resolution data from an FFPE samples at reduced sequencing cost will facilitate testing in different malignancies which was not previously possible.

#### TT002. A Process for New Clinical Laboratory Test Implementation

J. Brock, W. Nedlik, D.H. Farkas  
Cleveland Clinic, Cleveland, OH.

**Introduction:** A plan that included the addition of six highly complex laboratory developed tests (LDT) was implemented to reinvigorate a molecular pathology service. New tests were 1) next generation sequencing (NGS)-based molecular oncology panels for sarcomas, hematologic neoplasms, and solid tumors; 2) expanded carrier screening by multiple technology platforms; 3) pharmacogenomics panel; and 4) non-invasive prenatal testing (NIPT). Test development occurred concurrently with implementation of a new laboratory information management system (LIMS). **Methods:** Tests under consideration were chosen based on a combination of clinicians' expressed need, financial issues, feasibility, and competitiveness. To optimize planning and stakeholder engagement, a phase-based test development process was established using a cross-functional matrix mapping deliverables associated with feasibility, optimization, verification, and validation (FOVV). Core teams were formed for each initiative that included medical sponsorship and work-stream leads for project management, assay development, clinical operations, and bioinformatics. Based on test requirements, extended core team members were appointed, e.g., genetic counselors, medical technologists, clinical stakeholders, and support area representatives from reference laboratory, marketing, administration, and finance. Core team meetings provided a forum for members to report on key deliverables, and align on pre-analytical, analytical, and post-analytical processes. Project management software was employed to assign task ownership, track progress, monitor timelines, and identify potential delays for core team reaction. **Results:** All test (and LIMS) implementations benefitted from the process to facilitate bringing these online by elevating the culture of a production laboratory that had developed inefficient processes for new test development. One test has launched, four more are nearly ready to launch, and all will be incorporated into production within the new LIMS. One test (NIPT) underwent significant delay but the core team meeting minute notes and project management software retain the "institutional memory" required to re-ignite the process quickly should implementation hurdles be overcome.

**Conclusion:** A new test implementation process added efficiency, rigor, and stakeholder input to a process that had previously lacked those characteristics and enabled implementation of four highly complex tests and a new LIMS in a relatively short amount of time with minimal disruption to a functioning, production-level molecular pathology laboratory within a busy Pathology and Laboratory Medicine service at a major academic medical center.

#### TT003. Validation and Development of a Data Analysis Pipeline for a Customized Hematological Neoplasm Next-generation Sequencing Panel

Z.J. Tu, M.A. Jakubowski, J.E. Brock, D.H. Farkas, J. Cook, Y. Cheng  
Cleveland Clinic, Cleveland, OH.

**Introduction:** Disease associated DNA sequence changes have been reported in hematological disorders, including myeloproliferative neoplasms, myelodysplastic syndromes, chronic myelomonocytic leukemia, and acute myeloid leukemia. Identification of these variants in patient specimens provides information important to diagnosis and prognosis, and that may guide appropriate therapeutic choices and strategies. We describe a customized next generation sequencing (NGS) panel that targets 62 genes involved in hematologic malignancies.

**Methods:** The panel is intended to identify single nucleotide variants (SNVs) and insertion/deletions (indels) in genomic DNA isolated from peripheral blood and bone marrow specimens. Targeted regions are enriched by nested PCR. The validation cohort included 32 specimens containing large indels (>10 bp), 48 specimens containing SNVs and small indels (≤10 bp), and 4 wild type specimens. Unique molecular barcodes were used in NGS library construction to mitigate duplicate reads. A bioinformatics approach was developed to determine the efficiency of the data analysis pipeline in identifying large indels. **Results:** During validation, the panel and data analysis workflow delivered an average of 500X coverage per specimen and >98% of targeted regions with over 100X read-depth. Results of the validation exercise demonstrated 95.2% sensitivity and 99.9% specificity in identifying SNVs and small indels of >5% variant allele fraction (VAF). For the JAK2 V617F variant, the limit of detection was 1%. For the identification of large indels >5% VAF, we demonstrated 87.5% sensitivity and 99.9% specificity. In the bioinformatic simulation study, we have found that the data analysis pipeline can detect insertions up to 48 bases at 98.6% efficiency and deletions up to 52 bases at 96.4% efficiency. **Conclusions:** The nested PCR and unique molecular barcode approach provides a robust method to enrich gene regions of interest. We showed adequate (100X) coverage in >98% of the targeted genes. During validation, we detected the 52 bp CALR exon 9 deletion and demonstrated full coverage of the GC-rich CEBPA gene.

#### TT004. Comparing a Template-independent Next-generation Sequencing Assay with Standard Genomic Assays for HCV Subtyping and Sequencing

B. Wei<sup>1</sup>, M. Kibukawa<sup>1</sup>, L. Chen<sup>1</sup>, J. Kang<sup>2</sup>, D. Levitan<sup>1</sup>, E. Asante-Appiah<sup>1</sup>, H. Huang<sup>1</sup>, H.L. Platt<sup>3</sup>

<sup>1</sup>Merck & Co., Inc., Kenilworth, NJ; <sup>2</sup>Merck & Co., Inc., Rahway, NJ; <sup>3</sup>Merck & Co., Inc., North Wales, PA.

**Introduction:** To overcome challenges posed by high mutability and sequence diversity of HCV genomes, we developed a template-independent (TI)- next generation sequencing (NGS) assay that utilized RNA-seq of random primed cDNA to perform HCV whole genome sequencing. We demonstrated with 5 commercial (GT1a/1b/2b/4a/6a) and 17 clinical (GT1b/6a/6e/6q/6t) samples that the assay could simultaneously perform HCV subtyping, detect subtype-level mixed infection, and identify resistance-associated variants. To further evaluate assay's accuracy, we compared subtyping and sequencing results to the ones obtained with standard HCV genomic assays using samples from the MK-5172-062 study, a Phase 3 clinical trial in treatment naïve subjects on opiate substitution therapy, a population with a potential higher prevalence of mixed infections of different HCV lineages due to likely transmission etiology resulting from injection risk behaviors. **Methods:** A total of 322 samples, including all 297 baseline samples and 25 follow-up samples from treatment failure subjects with viral load ≥10,000 IU/mL, were analyzed by TI-NGS. The results were compared to standard HCV



genomic assays including VERSANT HCV Genotype 2.0 LiPA and genotype or subtype-specific PCR based NGS assays for NS3/4A and NS5A, 2 viral targets of the study. A NS5B small amplicon sequencing based subtyping assay was used for follow-up samples or for samples with ambiguous LiPA results. **Results:** Only one baseline sample had discrepant subtyping calls between LiPA and TI-NGS, with the NS5B subtyping assay confirming TI-NGS. One sample was called a GT1a/1b mix by LiPA, but GT1a only by TI-NGS due to the presence of GT1b at 2%, below the 5% minor subtype cutoff. Twenty-four of the 25 follow-up samples were subtyped by the NS5B assay and all matched TI-NGS. The study's subtypes included 1a/1b/3a/4a/4d/6a/6p. Except for 1 GT1a sample, full-length sequences were obtained for all samples for both targets by TI-NGS. For 20 samples (4 1a/1 1b/1 4a/12 6a/2 6p), the GT or ST-specific PCR based NGS assays failed to amplify 1 target or to obtain the last 1,177 to 1,444 base sequences at the 3' end of NS3/4A. These assays also failed to sequence stretches of bases in NS5A for 7 of 9 GT4d samples. Nucleotide sequence comparison was performed for 69 samples, including the ones that tend to be more discrepant between TI-NGS and GT/ST-based NGS, such as lower viral loads (<100,000 IU/mL) or GT3/4/6, and all samples from treatment failure subjects. The concordance rates range from 94.3% to 100.0%, with an average of 99.3%. **Conclusions:** A TI-NGS assay is able to perform multiple standard HCV genomic tests with comparable results. High accuracy in HCV subtyping and variant detection was observed with this assay for samples with viral load  $\geq 10,000$  IU/mL, regardless of genotype.

#### TT005. NGS Library Quantification Using the Quantabio Q and PerfeCTa NGS Quantification Kit

S.J. Deharvengt, G.J. Tsongalis

Dartmouth Medical Center, Lebanon, NH and Geisel School of Medicine at Dartmouth, Hanover, NH.

**Introduction:** Library DNA quantification is an important and necessary step for most Next-Generation Sequencing (NGS) assays. Different modes of library quantification are available, including qPCR-based methods. Quantabio developed a new qPCR instrument, the Quantabio Q, that uses a patented magnetic induction technology to rapidly heat samples and fan forced air to cool in order to acquire data more rapidly. Available in two or four channel models, the optical system reads all channels simultaneously and allows for fast, multiplexed assays. Q's size and lightweight design make it a portable and versatile qPCR cyclers that does not need to be calibrated. Q can process up to 48 samples per run and up to 10 Q's can be connected to a single computer wirelessly via Bluetooth enabling up to 480 samples to be processed simultaneously. A key difference is that Q incorporates a unique spinning aluminum rotor providing superior temperature uniformity of  $\pm 0.05^\circ\text{C}$  versus traditional block-based real time cyclers, which rely on multiple Peltier heating blocks that can create edge effects resulting in sample variation. The aim of this study was to assess the Q instrument for library quantification.

**Methods:** NGS Libraries were generated using Illumina's TruSight Myeloid Sequencing Panel. Each library was quantified using a validated, established qPCR method in our lab - the KAPA Library Quantification Kit (which contains the SYBR FAST qPCR MasterMix) (Roche) on the 7500 fast (ThermoFisher) - and the Q instrument using the PerfeCTa NGS Quantification Kit - Illumina (Quantabio). **Results:** We quantified 8 library preps with the KAPA Library Quantification kit and the PerfeCTa NGS Quantification kit. The concentration of undiluted library using the KAPA kit ranged from 1.92 nM to 2.50 nM and 1.74 nM to 2.48 nM with the PerfeCTa kit. Variability between samples with the two methods was less than 0.35 nM. In addition, we evaluated a standard and "fast" protocol on the Q instrument and each gave very similar and reproducible results with respect to nM concentrations with  $R^2 = 0.9994$  and  $0.9998$ , respectively. **Conclusion:** The Quantabio Q in conjunction with the PerfeCTa NGS Quantification Kit gave comparable and reproducible library quantification results when compared to the KAPA Library Quantification kit. The Q results obtained were reproducible and achieved in 50 minutes when using the fast cycling protocol vs 1 hr and 25 minutes with the standard cycling protocol without any loss in performance or assay sensitivity.

#### TT006. Whole Exome Sequencing of Matched Tumor Normal Cancer Specimens can Reliably Determine Microsatellite Instability

I. Baek<sup>1</sup>, E. Fernandez<sup>1</sup>, J.S. Martinez<sup>2</sup>, K.W. Eng<sup>1</sup>, H. Park<sup>1</sup>, Q. Pan<sup>1</sup>, A. Sboner<sup>1</sup>, O. Elemento<sup>1</sup>, W. Song<sup>1</sup>

<sup>1</sup>Weill Cornell Medicine/New York-Presbyterian, New York, NY; <sup>2</sup>Universidad de los Andes, Bogotá, Columbia.

**Introduction:** Microsatellite instability (MSI)/mismatch repair (MMR) status is increasingly important in the management of patients with cancer to predict response to immune checkpoint inhibitors. Traditionally, MSI testing has been done via PCR focusing on five predefined microsatellites (mono- or dinucleotide microsatellites). With the advent of next generation sequencing (NGS) with targeted gene sequencing or whole exome/genome sequencing, numerous microsatellites can now be investigated simultaneously with computational tools. **Methods:** At the Engländer Institute for Precision Medicine/Department of Pathology and Laboratory Medicine (Weill Cornell Medicine), we analyzed 1,062 Whole Exome Sequencing (WES) matched normal-tumor pairs across 13 tumor types at more than 5,000 microsatellite loci for each case by using MSIsensor, a program for automatically analyzing somatic microsatellite changes. This program analyzes the aligned DNA sequences reads in matched tumor and normal BAM files and calculates a score consisting of the percentage of simple-repeat sites that show evidence of MSI. For validation, MSI-PCR was performed by using the Promega kit.

**Results:** MSI high (MSI-H) was defined by a score of 3.5. In total, 6 of 58 (10.3%) in colorectal cancers (CRCs) and 4 of 116 (3.5%) in prostate adenocarcinoma (PRAD) were found to be MSI-H. Three more MSI-H cases were identified in stomach, thymic, and endometrial cancers. Thirteen of MSI-H cases and 21 of MSI stable (MSS) cases predicted by MSIsensor were also analyzed by MSI-PCR. Results from both methods showed a concordance of 98.4%. Only one case showed discrepancy which was MSI-H by MSIsensor and MSS by MSI-PCR. In addition, out of 885 cases classified as MSS by MSIsensor in non-CRC/PRAD tumors, 18 (2.5%) had elevated scores ( $\geq 1$  and  $< 3.5$ ) and were confirmed to be MSS by MSI-PCR. **Conclusions:** These results indicate that MSIsensor is an accurate tool to evaluate MSI status of tumor-normal paired exome sequencing data. MSI status assessment through Whole Exome Sequencing data by using MSIsensor yield about 99% concordance comparing to MSI-PCR. Therefore, comprehensive sequencing of patient's cancer specimens not only can provide potential markers for targeted therapy, but also provides reliable information on MSI status, a key feature for selection of treatments based on checkpoint inhibitors.

#### TT007. Influence of Centrifugation Conditions for Plasma Processing on ccfDNA Yield

D. Groelz<sup>1</sup>, T. Krenz<sup>1</sup>, E. Provencher<sup>1</sup>, T. Voss<sup>2</sup>

<sup>1</sup>PreAnalytiX GmbH, Hombrechtikon, Switzerland; <sup>2</sup>PreAnalytiX GmbH, Hilden, Germany.

**Introduction:** Circulating cell-free DNA (ccfDNA) in blood has become widely accepted for biomarker analysis in academic and clinical research and beyond. Considering the usual low sample input and required high sensitivity for target detection, optimal outcomes require validation and control of the entire workflow. The PAXgene Blood ccfDNA System (RUO) integrates preanalytical steps from sample collection to ccfDNA extraction for subsequent analysis by quantitative PCR (qPCR), digital PCR (ddPCR) and next-generation sequencing (NGS). Here we present the influence of centrifugation conditions for plasma processing on ccfDNA yield and compatibility with primary tube handling on the QIAAsymphony SP instrument using the QIAAsymphony PAXgene Blood ccfDNA Kit and protocol. **Methods:** Blood from healthy consented donors was processed into plasma directly after phlebotomy or stored for 7 days at room temperature (15–25°C) to simulate a typical processing delay in a routine setting. Different plasma processing protocols were tested including double versus single centrifugation, centrifugation for 15 minutes at 1,600  $\times g$ , 1,900  $\times g$  and 3,000  $\times g$ , and with full, medium and no braking. Yield and stability of ccfDNA in PAXgene Blood ccfDNA Tubes were measured by qPCR and ddPCR with assays for 18s rDNA and DYS14 Y-chromosomal fragments in plasma. Compatibility with NGS was evaluated by sequencing on the QIAGEN GeneReader instrument using the GeneRead QIAact Actionable Insights Tumor (AIT) Panel and data management with the QIAGEN Clinical Insight (QCI) Analyze tool.

**Results:** The PAXgene Blood ccfDNA System (RUO) is compatible with the GeneReader NGS workflow. Sixty out of 60 samples passed acceptance criteria for target enrichment, library preparation and sequencing, including coverage of bases >500× (99.8%) and coverage of bases >200× (99.99%). Analyses by qPCR and ddPCR showed no significant differences in the stability of ccfDNA level between double and single centrifugation protocols with full, medium and no braking. However, full braking led to a sloped buffy coat, which may impact retrieval of clean, uncontaminated plasma. In addition, no significant differences were found for primary tube handling when plasma was generated by a single centrifugation step for 15 minutes at 1,600 versus 3,000 ×g.

**Conclusions:** The PAXgene Blood ccfDNA System workflow for ccfDNA stabilization in blood and extraction from plasma is robust with regard to centrifugation conditions for plasma separation and suitable for sensitive ccfDNA applications such as qPCR, ddPCR and NGS.

#### TT008. Evaluation of a Novel Formalin/Methanol-free FNA Fixative for Study of Lung Cancer

T. Krenz<sup>1</sup>, D. Groelz<sup>1</sup>, T. Voss<sup>1</sup>, E. Provencher<sup>1</sup>, M. Southwood<sup>2</sup>, D. Rass<sup>2</sup>, E. Shaw<sup>3</sup>, M. Ashton-Key<sup>3</sup>

<sup>1</sup>PreAnalytiX GmbH, Hombrechtikon, Switzerland; <sup>2</sup>Royal Papworth Hospital NHS Foundation Trust, Cambridge, United Kingdom; <sup>3</sup>University Hospital Southampton NHS Foundation Trust, Southampton, United Kingdom.

**Introduction:** Lung cancer remains a leading cause of cancer mortality worldwide. Identification of actionable mutations is a key strategy for guiding personalized care and highly dependent on nucleic acid quality. Currently, formalin and methanol-based fixation methods for pathology samples are a rate-limiting step for tumor molecular analysis. STRATFix is an Innovate UK-funded collaborative project between QIAGEN and NHS pathology teams across the UK. A major aim was to compare PAXgene (a novel formalin/methanol-free fixative) to traditional fixation approaches and evaluate performance in preserving cellular morphology and DNA/RNA, which are now crucial for the molecular study of lung cancer.

**Methods:** Paired PAXgene and CytoLyt fixed fine needle aspirates (FNAs) of lung tumor resections were collected from 8 subjects undergoing surgery for non-small cell lung carcinoma. At a second site, an additional 20 paired PAXgene and CytoRich Red fixed lung tumor FNAs were collected. All samples were centrifuged, encapsulated in a cell clot and processed into a paraffin block. Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) was performed and sections were blind-scored by pathologists. DNA and RNA were extracted using QIAGEN kits and assessed for quantity with Qubit Fluorometer and for fragmentation with Agilent TapeStation and Bioanalyzer. RT-PCR performance was tested using a β-Actin assay and the QIAseq DNA QuantIMIZE Kit. DNA was further tested for suitability with molecular methods using the *therascreen* EGFR RGQ PCR Kit. **Results:** Despite some eosinophilia of PAXgene fixed samples, adequate and comparable H&E staining was observed. IHC was successful and comparable to CytoLyt/CytoRich Red in all samples scored. PAXgene samples showed less fragmentation and higher average yield of DNA compared to alternatives using Qubit. PAXgene fixation also had lower C<sub>T</sub> values resulting in better QuantIMIZE PCR scores and significantly higher amplifiable DNA concentration. *EGFR* mutations were detected in 2 of 8 subjects, in both PAXgene and CytoLyt-fixed matched samples. For all PCR-based methods, significantly less DNA input was required for PAXgene fixed samples. **Conclusions:** The majority of lung cancer patients present with advanced stage and inoperable disease resulting in limited availability of tissue for subsequent molecular analyses. PAXgene fixed samples show comparable morphology to formalin/methanol fixation, are adequate for histopathology and IHC and are more suitable for molecular studies, which is important for low yield samples. We conclude that PAXgene fixation is beneficial for the molecular research of lung cancer samples to advance precision medicine.

#### TT009. Validation of a Targeted Variant Genotyping Assay for Personalized Oncology Therapy

W.M. Stansberry, M. Swart, E.B. Medeiros, T.C. Skaar, V.M. Pratt  
Indiana University School of Medicine, Indianapolis, IN.

**Introduction:** Genetic variants exist in genes coding for enzymes that are targets of oncology medications or responsible for their metabolism and transport. Interpretation of pharmacogenomic test results for patients with cancer is particularly complex because both germline and somatic DNA alterations could inform therapy: somatic mutations can be used to select a targeted therapeutic agent while germline DNA changes can highlight possible risk of toxicity or inefficacy. A generalized approach to drug selection and dosage has not proven effective in reducing the incidence of these conditions. **Methods:** We selected 17 genes with corresponding gene-drug based recommendations and used Coriell DNAs (Camden, NJ) from the pharmacogenetics reference material projects (Pratt *et al.*, 2010 and 2016) for the analytical validity studies. DNA was amplified by real time PCR on the ThermoFisher QuantStudio 12K Flex (software v1.2.2; Waltham, MA) and subjected to Taqman allele discrimination using ThermoFisher (Waltham, MA) reagents and software (Genotyper software, v1.3) in a custom designed open array. **Results:** The analytical sensitivity was 100% for the detection of variant alleles, with no reported false negative results. The analytical specificity was 100% for detection of non-variant alleles, with no false positive results. DNA samples were also run for intra- and inter-assay variation. In all, 14 samples were included in intra-assay validation and 18 samples were included in the inter-assay validation. The intra- (within) assay variation studies showed that all three replicates of the 14 samples ran on the same plate, were concordant with expected results. The inter- (between) assay variation studies showed that the 18 samples consistently yielded the same result across three separate runs. Since there were no known reference materials for most variants, selected DNA samples were Sanger sequenced with custom designed primers (Integrated DNA Technologies, Coralville, IA) for 100% accuracy. **Conclusions:** Genetic testing may serve as an important tool for clinicians who embrace precision medicine. Increasing scientific evidence has supported the utilization of genomic information to select efficacious, antitoxic therapy for oncology patients. Given the wide array of treatments available and diversity of genetic predictors, a panel-based approach to genotyping may be an efficient means of establishing an individualized toxicity response profile for patients with various cancers.

#### TT010. Employment of Digital Droplet PCR as a Confirmatory Testing of Next-generation Sequencing for Tumor Profiling

Y. Li, I. Baek, H. Park, H.V. Tran, E.C. Merzier, D. Pancirer, G. Cheang, G. Raja, J. Catalano, Q. Pan, W. Song  
Weill Cornell Medicine, New York, NY.

**Introduction:** Next generation sequencing (NGS) is bringing precision medicine into the clinical realm. The NGS-based testing is becoming of unprecedented prevalence in many clinical molecular diagnosis laboratories. Therefore, it is important to validate a highly sensitive, reliable and robust analytical method suitable for confirming some uncertain data of NGS due to the low coverage or different quality issues in the NGS process. Sanger sequencing (10% to 20% of sensitivity) and pyrosequencing (1% to 5% of sensitivity) are the acceptable confirmatory assays. However, they are not ideal methods for confirmation of all variants. The challenge we faced, was the relatively high input of samples, low sensitivity and not easy to use. Here, we validated the droplet digital PCR (ddPCR) in order to mature them into reliable analytical method suitable for confirmation of NGS results. The validation data are presented. **Methods:** We selected clinically relevant mutations: EGFR L858R, T790M, exon19 deletion as well as BRAF V600E as detection targets. For every mutation, HDx reference standard genomic DNA was used to measure the sensitivity and the triple test was applied to every dilution of mutation to measure the repeatability of precision. We tested 60 negative patient samples with 10 positive patient samples (approximately 70% formalin-fixed, paraffin-embedded (FFPE) and 30% frozen samples) to estimate the false positive ratio and analyze the accuracy in every assay. The reference DNAs, three positive patient and negative patient samples were repeated three times to detect the reproducibility of precision. **Results:** Our data showed that limit of detection (LOD) can reach 0.1% with 10 ng input, 0.3% with 4 ng input,

1% with 2 ng input as well as 2% with 1 ng input (95% confidence interval). The false positive ratio was 0% with 1-4ng input and approximately 0.03% with 10ng input. In intra-run, Coefficient of variation (CV) was within 4%-20% of means; in inter-run, CV was within 2-14% of means. Moreover, pretreatment of FFPE samples with uracil DNA glycosylase prevented false-positive signal of EGFR T790M.

**Conclusions:** ddPCR proves to be an ultra-sensitive, reliable method for accurately and precisely detecting the specific mutations in different genes in various types of samples. We provide a realistic view of the factors affecting the measurement results for routine use of the method, as well as covering the concentration ranges and sample types within the scope of the method.

#### TT011. Dual Extraction of DNA and Total Nucleic Acid (TNA) from Single Specimens Enables Evidence-based Therapeutic Strategy for Minute Samples

R.T. Sussman, A. Oran, C. Paolillo, D. Lieberman, J. Rosenbaum  
University of Pennsylvania, Philadelphia, PA.

**Introduction:** As the target list for genomic testing in oncology expands, the pressure to interrogate limited specimens also increases. In our laboratory, we recently developed and implemented a clinical RNA-based assay to detect fusion transcripts and oncogenic isoforms. This assay is often performed in parallel with DNA-based sequencing, requiring the validation of a protocol capable of extracting both DNA and Total Nucleic Acid (TNA) from the same (often limited) specimen. **Methods:** Twelve formalin-fixed paraffin embedded (FFPE) samples were obtained in triplicate and extracted using the existing protocol, a modified protocol, or using Beckman's FormaPure DNA with a split protocol (new protocol). The yield and DNA degradation (measured as % of gDNA <1,000bp long) were compared between protocols. Eight cytology specimens were split and DNA was extracted using either the existing protocol or the new protocol, and yield was compared between the two groups. Six additional cytology specimens were split and TNA was extracted using either the existing protocol or the new protocol. Five intraoperative gliomas in PreservCyt were tested using the new protocol. **Results:** For FFPE samples, an ANOVA analysis revealed that one group had significantly higher yield than the others ( $p=0.038$ ) and a Bonferroni test established that the new protocol resulted in a significantly higher yield than the current protocol ( $p=0.046$ ) without significant difference in DNA degradation between the protocols ( $p=0.57$ ). A student's t-test indicated no significant difference in TNA yield ( $p=0.229$ ) between the current protocol and the new protocol. Libraries made from DNA extracted using the new protocol produced identical results. For cytology samples, a student's t-test suggested no significant difference in DNA yield between the new and current extraction protocols ( $p=0.77$ ). Of the 6 cytology specimens used to compare TNA extraction methods, 3 did not have yield from either protocol, 2 had comparable yields from both protocols, and one yielded TNA only from the new protocol. **Conclusions:** We extracted DNA and TNA from FFPE, cytology, and tissue in PreservCyt using the new FormaPure DNA with a split protocol. This protocol results in a higher DNA yield for FFPE specimens and comparable TNA yield to our existing extraction protocols. Experiments with cytology and tissue (gliomas) suggest a single extraction workflow for three specimen types received by the laboratory will not impair existing sequencing protocols, and will permit additional assays to be performed as necessary.

#### TT012. Synthetic Serial Dilution Samples Derived from Normal Plasma as a Specimen for Analytical Validation of a Commercial ctDNA Kit

R. Ringle, S. Verma, A. Ghosal, P. Cotter, M. Moore, S. Gunn  
ResearchDx/PacificDx, Irvine, CA.

**Introduction:** Analytical validation of a commercial ctDNA kit requires adequate samples to demonstrate limit of detection, sensitivity, and reproducibility of variants at varying allelic frequencies. However, for many clinical laboratories real patient material is unavailable or uncharacterized, and the cost of relying only on purchased reference standards is prohibitive. In the current study, we performed analytical validation of a commercial ctDNA kit using a combination of purchased reference standards and internally synthesized samples from purchased human plasma. **Methods:** Eight normal human plasma samples were purchased

from Biological Specialty Corporation followed by extraction of multiple cfDNA replicates. DNA libraries were created from each donor and sequenced using the Avenio ctDNA Expanded Kit (Roche, Pleasanton CA). The samples were from normal healthy donors, thus somatic mutations were not detected using the DefaultExpandedPanelWorkflow, DefaultFilter setting (variant-report-DefaultFilterSet) of the kit's bioinformatics pipeline (version 1.0.0). In order to identify variants that could be tracked in a dilution series, we instead relied on variants reported using DefaultExpandedPanelWorkflow, Unfiltered Filter Set (variant-report-Unfiltered). Variants reported in the unfiltered data set were used to identify 22 "mutations" present in one of the normal plasma samples at ~50% allele frequency, designated as the "mutant" sample. Selected mutations were identified in the following genes: *NTRK1*, *FGFR2*, *BRCA1*, *BRCA2*, *ALK*, *FGFR3*, *PDGFRA*, *CSF1R*, *PMS2*, *PTCH1*. Three normal plasma samples lacking these 20 mutations were designated as the normal wild type (WT) and mixed to create a background normal sample. Serial dilutions were prepared by spiking the mutant into the WT with resultant expected allele frequencies of: 5.0%, 1.0%, 0.5%, and 0.1%. Dilutions were made such that the total amount of cfDNA for each reaction was a total of 12 ng. Libraries were constructed and sequenced using multiple replicates of the resultant cfDNA dilutions. **Results:** The observed variant-report-Unfiltered reports revealed expected results with >95% sensitivity and specificity at allele frequencies 0.5% and above.

**Conclusions:** Serial dilutions of synthetic mutations derived from normal plasma provide a robust, economical alternative sample for development and validation of ctDNA commercial kits. These internally next generation sequencing (NGS)-characterized samples can be mixed at known allele frequencies to evaluate end-to-end ctDNA kit workflow and performance from library preparation through bioinformatics analysis.

#### TT013. the Clinical Significance of 391 Fusion Genes Identified in 911 Pediatric Cancers Using Custom-designed RNA-Seq

F. Lin, L.F. Surrey, M. Luo, G.B. Wertheim, F. Chang, K. Cao, R. Aplenc, R. Bagatell, T.R. Bhatti, S.P. MacFarland, J.M. Maris, Y.P. Mosse, M. Santi, P.B. Storm, S.K. Tasian, A.J. Waanders, M.M. Li  
Children's Hospital of Philadelphia, Philadelphia, PA.

**Introduction:** Chromosomal rearrangements resulting in *de novo* fusion genes play important roles in tumorigenesis. Pediatric cancers harbor a lower mutation rate compared to adult cancers, and fusion genes likely serve as an alternative oncogenic mechanism. Many fusions are tumor-specific and can be used as biomarkers for cancer diagnosis, prognosis and treatment. The emergence of next generation sequencing (NGS) technology and modern computational tools allows sequencing numerous genes in parallel and facilitates the identification of known and novel fusions. **Methods:** The CHOP Fusion Panel utilizes anchored multiplex PCR technology combined with high-throughput RNA-Seq. The panel interrogates 110 consensus fusion partners and covers more than 600 recurrent fusion transcripts. The technology also enables the detection of novel fusions. Since April 2016, 911 consecutive tumor samples were analyzed. Identified fusions were categorized and reported according to the AMP/ASCO/CAP guidelines for somatic variant interpretation and reporting. **Results:** A total of 349 tumors (38.3%) were positive for 391 fusions, including 166 of 333 leukemia/lymphomas (49.8%), 103 of 306 non-CNS solid tumors (33.6%), and 80 of 272 CNS tumors (29.4%). The most common fusions were *ETV6-RUNX1* and *KMT2A*-associated fusions in leukemia, *EWSR1-FLI1* in non-CNS solid tumors, and *KIAA1549-BRAF* in CNS tumors. A total of 140 unique fusions were detected, of which 70 (50%) were previously unrecognized. 83.9% (328/391) of the fusions were deemed clinically significant with a direct impact on patient care. In 304 tumors, the identified fusions were diagnostic for specific type of cancers. In two patients, novel fusion partners of *EWSR1* were identified and completely altered the original diagnosis. Many fusions were potential therapeutic targets including 58 *BRAF*-associated fusions predicted to activate the MAPK pathway; 25 *CRLF2*-, *JAK2*-, or *EPOR*-associated fusions predicted to activate the JAK-STAT pathway, and 19 *NTRK*-associated fusions and 16 *RET*-associated fusions predicted to activate Ras/MAPK and PI3K/AKT pathways. Other targetable fusions included 11 *ABL*-associated fusions and 7 *FGFR*-associate fusions. Clinical trials using FDA approved or investigative therapies targeting these pathways have shown clinical benefit in certain pediatric cancers.



Identification of actionable fusion genes are crucial for pediatric clinical trials. The panel also provides sequence information of the fusions, enabling personalized testing for monitoring treatment response to targeted therapies. **Conclusion:** We have identified a large cohort of known and novel fusion genes in pediatric cancers using a custom-designed RNA-Seq panel. These findings have shown significant impact on clinical management of cancer patients.

#### TT014. Assessment of the Performance of a Hybridization-based NGS Enrichment Panel with as Little as 10ng of Severely Formalin-compromised DNA

J. Chan<sup>1</sup>, J. Forster<sup>1</sup>, A. Hecke<sup>2</sup>, V. Pullabhatla<sup>1</sup>, C. Proud<sup>1</sup>, D. Cook<sup>1</sup>, G. Speight<sup>1</sup>

<sup>1</sup>Oxford Gene Technology, Begbroke, Oxfordshire, United Kingdom; <sup>2</sup>Oxford Gene Technology, Tarrytown NY.

**Introduction:** Formalin-fixed, paraffin-embedded (FFPE) storage is a standard method for archiving samples from solid tumors and these can be analyzed by next generation sequencing (NGS), for example to study cancer development and progression. However, the amount of FFPE material is often limited and the quality of DNA adversely effected through exposure to formalin, resulting in degradation and damage. Consequently, it may be difficult to distinguish between true low frequency mutations and damage-induced false positives. In this study reference standards with different levels of formalin-induced damage were hybridized and sequenced with a SureSeq custom NGS panel in conjunction with the SureSeq FFPE DNA Repair Mix. **Methods:** We utilised the SureSeq FFPE DNA Repair Mix, Library Preparation Kit and a SureSeq myPanel Custom Cancer Panel design to assess the impact of the repair mix on three levels of formalin compromised DNA (fcDNA) - mild, moderate and severe, at 4 different DNA input amounts down to 10 ng. We then compared the uniformity and coverage of the enriched targets. We also assessed the concordance to the allele frequencies of the variants in the reference standards. **Results:** Using the custom panel with 16 samples per MiSeq lane, we achieved (post repair) a mean target coverage (MTC) of over 1000x from all types of fcDNA at 100 and 200 ng input and over 500x at 50 ng input. Improvements in MTC in treated samples varied from 1.2x in mildly compromised samples to 1.5x in severely affected samples, showing the positive effect of the repair mix. All 100% of known variants were detected in samples with >500x coverage, including three *EGFR* variants at allele frequencies of 1, 2, and 3%. Overall 99.6% of the expected variants were detected, and 91.3% of the reported frequencies were within 5 percentage points of their expected values.

**Conclusions:** Enrichment enables the parallel sequencing of several cancer-related genes. We have shown a superior depth of coverage generated from FFPE repair mix treated samples which enables the successful detection of variants in formalin compromised reference samples. The utility of this hybridization-based enrichment approach, even at 10 ng sample inputs, permits analysis of somatic variation in genes associated with solid tumors from difficult to use samples.

#### TT015. Evaluation and Troubleshooting of the Chemagic 360 Instrument Used for the Extraction of High Quality and High Yield DNA Utilized for Molecular Clinical Testing

H.L. Sellers<sup>1</sup>, R. Veith<sup>1</sup>, U.K. Jaentges<sup>2</sup>, T. Weissbach<sup>2</sup>, M. Honickel<sup>2</sup>, J. Bedbur<sup>2</sup>, A. Lopez<sup>3</sup>, K. Rumilla<sup>1</sup>, W.E. Highsmith<sup>1</sup>

<sup>1</sup>Mayo Clinic, Rochester, MN; <sup>2</sup>PerkinElmer Chemagen Technologie GmbH, Baesweiler, Germany; <sup>3</sup>PerkinElmer, Waltham, MA.

**Introduction:** We sought to evaluate whole blood extractions of DNA on the chemagic 360 (PerkinElmer, magnetic bead-based method) to verify the extraction platform as a viable replacement for high yield and quality extractions currently performed on the Autopure LS (Qiagen, salt precipitation method). We worked with the manufacturer to troubleshoot and customize the extraction protocol. Adaptation of the extraction protocol for the chemagic 360 (CM360) to an Autopure-optimized analysis platform was successful and led to maximized performance on multiple analysis platforms. **Methods:** We performed initial extractions on the CM360 instrument per manufacturer's protocol using 2 mL WB collected in EDTA/ACD anti-coagulants. DNA quantification was performed on the Trinean. We tested 196 samples on multiple platforms including: Multiplex ligation-dependent probe amplification, Fragment Analysis, Matrix

Assisted Laser Desorption/Ionization Time of Flight, Sanger sequencing, Next Generation Sequencing and long-range PCR. Sample performance was evaluated on pass/fail basis and underperformance of some assays spurred comprehensive troubleshooting. We performed experiments in collaboration with PerkinElmer which included spiking DNA with various concentrations (to replicate downstream issues) of cerium, iron, and bead supernatant as well as customization of existing instrument protocols.

**Results:** Initial evaluation of the CM360 DNA showed good yields of high quality DNA. Post-optimization DNA quality values increased from initial results: A260/A280: 1.87 to 1.88, A260/A230: 2.34 to 2.46; and yields increased from 60-70 to 80-90 µg. Initial clinical assay performance was acceptable for most platforms; however, several assays failed testing. Initial testing of Sanger assays (n=79) showed an increased failure rate for 50% of the assays. After protocol customization, a particular troublesome Sanger assay (CASR gene), had a decreased failure rate from 53% (n=9) to 3.3% (n=6). Likewise, initial results of the AMPLiEx assay (Asuragen) showed reduced amplification of the repeat prime peaks, failing testing. Extensive troubleshooting revealed high residual levels of iron present in the DNA causing interference and low/no amplification for PCR in some assays, and was resolved using customized protocols involving additional bead reagent preparations. **Conclusions:** Initial failure of the validation of a new extraction instrument and method in our laboratory led to extensive troubleshooting and optimization resulting in DNA elutions that are more compatible with the many existing protocols covering several assay platforms in our laboratory. The chemagic 360 instrument utilizing the customized protocol is now a viable option as an extraction platform for our laboratory.

#### TT016. Comparison of Manual High Pure PCR Template Preparation Kit Versus the Automated Magna Pure 24 and 96 Systems for DNA Extraction with the Cobas Factor II And Factor V Test

J.W. Longshore<sup>1</sup>, P.M. Das<sup>2</sup>, K. Yu<sup>2</sup>, S. Chaudhuri<sup>2</sup>, S. Chugh<sup>2</sup>, S. McCune<sup>3</sup>, J. Engstrom-Melnyk<sup>3</sup>, S. Karwowska<sup>2</sup>

<sup>1</sup>Carolinas Pathology, Charlotte, NC; <sup>2</sup>Roche, Pleasanton, CA; <sup>3</sup>Roche, Indianapolis, IN.

**Introduction:** In today's clinical laboratories, workflow is continually increasing in complexity to meet the demands of expanding testing menus and often, increased testing volumes. Although both manual and automated nucleic acid (NA) extractions are used extensively within clinical workflows, automated systems have advantage to mitigate burdens associated with manual processes and assay throughput needs, yet these extraction methods may vary in the efficiency, quality, and the amount of NA yield. Thus, the purpose of this study was to compare the performance of the manual High Pure PCR Template Preparation Kit (HP) and the automated MagNA Pure 24 (MP24) and MagNA Pure 96 (MP96) Systems when applied for extraction of NA to be used with the recently IVD cleared cobas Factor II and Factor V Test (cobas Factor II/V Test) that offers the user choice of NA extraction methodology. **Methods:** DNA from 200 K2EDTA whole blood clinical samples (previously frozen and banked) with known Factor II and Factor V Leiden genotype status, as determined by Sanger sequencing, was obtained using the manual HP and automated MP24 and MP96 methods, within two different and independently operated laboratories. Extracted NA was tested for the presence of mutations using the cobas Factor II Factor V Test.

**Results:** Based on Sanger sequencing, the samples were classified as: wild type (WT), heterozygous A or G (HET) and homozygous (MUT). Extracted DNA obtained from either the manual or automated extraction processes yielded correct results for 103 WT, 94 HET and 3 MUT Factor II genotypes, as well as for the 87 WT, 96 HET and 17 MUT Factor V Leiden genotypes, demonstrating 100% concordance between the three extraction methods and Sanger sequencing reference method. The CT values obtained using the DNA from either of the two automated extraction methods (MP24 and MP96) were within tighter ranges across the clinical specimens than what was observed with the manual method; the difference in CT between the manual and automated methods for individual samples were not significant. The Standard Deviation (SD) in the CT values noted in Factor II genotypes were highest for HP (1.04-1.21) while for automated MP 24 and MP 96 methods SD ranged from 0.48-0.54 and 0.36-0.53, respectively. **Conclusion:** There was a minimal difference between manual versus automated extractions when Ct values

were compared. The Roche manual HP or automated MP24 and MP96 nucleic acid extraction methods can be reliably used for cobas Factor II Factor V Test, which provides flexibility of user choice for nucleic acid extraction methodology, to generate accurate genotype results.

#### TT017. Allele-Specific Real Time PCR Versus Peptide Nucleic Acid Clamping for Low Copy Epidermal Growth Factor Receptor (EGFR) Mutation Detection in Liquid Biopsy

W. Yang, H. Tran, J. Tsai, P. Das, C. Litterst

Roche, Pleasanton CA.

**Introduction:** Clinical data indicate that advanced non-small cell lung cancer (NSCLC) patients harboring *EGFR* activating mutations exhibit good response and prolonged progression-free survival when treated with anti-*EGFR* Tyrosine Kinase Inhibitors. A surrogate tissue, such as plasma which contains circulating cell-free tumor DNA, can be used to reliably detect *EGFR* mutations. Current guidelines recommend *EGFR* mutation testing of NSCLC patient plasma, making it critical that the assay accurately detects low copy number mutations. There are several available *EGFR* test kits. This study compares the performance of Roche's IVD cobas *EGFR* Mutation Test v2 and PANAGene's RUO PANAMutyper R *EGFR* Kit. The PANAGene test consists of six 20 µL reaction mixes, only one with IC included, to which 5 µL of sample is added; the cobas test consists of three 25 µL reaction mixes, all containing an IC, to which 25 µL of sample is added. Both kits cover the same groups of *EGFR* mutations and claim similar analytical sensitivity: ≤ 0.1% mutant allele frequency. **Methods:** Contrived cell line DNA spiked with plasmids containing eight different *EGFR* mutations at mutation frequencies as low as 0.1%, as well as healthy donor plasma samples spiked with mutant plasmid DNA, were tested. DNA was extracted using cobas cfDNA Sample Preparation Kit and assays performed in duplicate according to manufacturer's instructions. **Results:** Cell line DNA samples: The PANAGene kit missed multiple mutations at or below 1%. The cobas test detected all mutations down to 0.1%. Plasma samples: The PANAGene kit missed multiple mutations, e.g. G719A at all copy levels tested and T790M at 50 copies/mL plasma. The cobas kit detected all mutant copies except one replicate of G719A at 250 copies/mL and one replicate of L858R at 50 copies/mL, demonstrating 0.25% analytical sensitivity for 17 of 18 mutations tested. The PANAGene IC, present in only one of the six tubes, failed for 3 of 43 samples tested. The cobas IC, incorporated into each of the 3 reaction mixes, did not fail for any samples. In general, the PANAGene workflow involves more handling steps and lower throughput, with analysis requiring skilled user evaluation. The cobas *EGFR* Mutation assay has fewer steps and higher throughput, with fully automated results analysis; it also provides a Semi-Quantitative Index (SQI) which reflects the amount of mutant DNA in the blood. **Conclusions:** Performance of the PANAGene kit was less robust relative to cobas *EGFR* Mutation Test v2, one potential reason being the lower sample input volume. Sensitivity for PANAGene was not as high as product claims with contrived samples.

#### TT018. An Automated Solution for DNA Extraction and Bisulfite Conversion of High Volume Liquid Biopsy Specimens – Application to Colorectal Cancer Detection Using Methylated *SEPT9*

S. Rausch, O. Hasinger, T. König, A. Schlegel, G. Weiss  
Epigenomics AG, Berlin, Germany.

**Introduction:** Aberrant DNA methylation detected in liquid biopsies is a clinically useful biomarker for the colorectal cancer screening (*SEPT9*) or as an aid in diagnosis of lung cancer (*SHOX2*, *PTGER4*). Key technology elements are: efficient DNA extraction from high volume plasma samples; complete bisulfite conversion; extensive DNA purification; and a highly sensitive real-time PCR assay. A detailed description of a manual workflow to derive bisulfite-converted DNA of sufficient quality and quantity from 3.5 mL plasma for downstream PCR applications is provided with the Epi BiSKit. Here, we describe automation of this workflow on a liquid handling platform. **Methods:** The Tecan Freedom EVO 200 platform was customized to enable magnetic bead-based DNA extraction, bisulfite conversion at high temperature, and efficient DNA washing. For the high volume portion of the workflow, a phased protocol for 2x 24 sample DNA extraction was implemented. Thereafter, the 48 samples were assembled on a 96-well plate and further processed for an output of purified bisulfite-

converted DNA in a 96-well storage plate, ready for PCR set-up. The Epi proColon Sensitive PCR kit was used to compare DNA yield and sensitive detection of methylated *SEPT9* DNA for the automated and manual workflows. **Results:** The automated workflow processes 48 samples within 6 hours and 30min. Comparison of analytical sensitivity and specificity, repeatability, precision, and concordance of test results was performed by repeatedly processing biological specimens of various DNA concentrations in a total of 6 automated batch runs (6 x 48 = 288 samples) and 8 batches run manually (8 x 16 = 144 samples). Finally, for validation of the automated workflow, a method comparison study was conducted using 92 CRC plasma pools. Equivalence of analytical sensitivity was demonstrated by overlapping 95% confidence intervals for the limit of detection, and analytical specificity by 0% positivity of blank samples. A modest improvement in precision was noted for the automated version (p-value > 0.2). Variance component analysis of precision data revealed a slight shift towards within-run variation. Percent agreement of test results was 97% (CI95%: 91 - 99%) validating the equivalence of the two workflow versions. **Conclusions:** The automated workflow demonstrated equivalent performance in the analytical parameters investigated. The comparison study results met a standard criterion for migration studies as outlined in the respective FDA guidance document. The results of this project provide a safe and effective solution for the preparation of bisulfite-converted DNA from high volume liquid biopsy samples via an automated high throughput protocol.

#### TT019. Evaluation of Low Coverage Regions of an Exome Dataset Generated By Hybridization Capture-based Sequencing Approach

N.H. Muto<sup>1</sup>, M.C. Cervato<sup>1</sup>, P.M. Oliveira<sup>1</sup>, M.S. Basqueira<sup>1</sup>, R.

Sitnik<sup>1</sup>, R.A. Santana<sup>1</sup>, R.M. Minillo<sup>1</sup>, J.B. Oliveira Filho<sup>2</sup>, J.R. Pinho<sup>1</sup>, C.L. Mangueira<sup>1</sup>

<sup>1</sup>Hospital Israelita Albert Einstein, São Paulo, Brazil; <sup>2</sup>Genomika, São Paulo, Brazil.

**Introduction:** Whole exome sequencing (WES) is a powerful technique with potential to identify genetic variants relevant for disease diagnosis and therapy. In spite of extensive use of next-generation sequencing in diagnostic context, one of the main challenges is the achievement of accurate coverage of all target regions, once low coverage regions potentially results in missed variant calls, even in samples with high average read depth. By sequencing samples prepared with a widely used hybridization capture-based library preparation kit, we obtained a dataset of 450 samples. In order to evaluate low coverage regions in our exome dataset, we performed a comprehensive search for regions with less than 10x read coverage in at least 50% of analyzed samples. **Methods:** To perform coverage analysis, all regions reported in the exome kit manufacturer's BED file were evaluated for all 450 samples using the same pipeline. Coverage was computed using bedtools software with the option to report coverage depth at each position according to BED file, representing 60,456,963 positions. All positions with less than 10x coverage were selected as low coverage positions. All adjacent low coverage positions were grouped into a single region. We next developed an algorithm able to identify low coverage regions in at least 50% of samples. The systematic failure regions were then annotated with the respective gene, transcript and exon, using bedmap tool. Finally, to evaluate the clinical relevance of low coverage regions, we searched for known variants inside those regions using ClinVar and dbSNP databases. **Results:** We found an average of 21,318.4 low coverage regions per sample, after consolidating bedtools position output into single regions. The search for failure regions in relation to the minimum 10x coverage in at least 50% of samples resulted in 1,017 low covered exons of 243,190 total exons contained in the kit (0.4%). Those exons represent 357 genes unable to cover the entire exonic region. Two interesting findings were found: 1) 251 of 357 genes have phenotype described in OMIM (70%); 2) 110 of 357 low coverage genes (30%) presented failure to cover the first exon. We searched for variants in ClinVar and dbSNP databases, and didn't find any known variants in low coverage regions. **Conclusions:** In summary, we found systematic low covered regions corresponding to 0.4% of all exons in our dataset. We are not able to identify variants catalogued by ClinVar and dbSNP in those regions, suggesting that no known relevant clinical data is missed, although 110 genes have loss of function as disease mechanism. However, according to genotype-

phenotype correlation, the missed region(s) of the gene(s) can be covered using Sanger sequencing or custom next generation sequencing (NGS) protocol.

#### TT020. Immune Repertoire Sequencing Enables Clonality Determination and Minimal Residual Disease Assessment

C. Song, P. Liu, A. Barry, B.W. Langhorst, F.J. Stewart, S. Russello, T.B. Davis, E.T. Dimalanta  
New England Biolabs, Ipswich, MA.

**Introduction:** The study of complex immunological diseases and tumor microenvironment has progressed through recent developments that enable the sequencing of the immune repertoire. Using this approach, the interrogation of disease progression is facilitated through analysis of millions of V(D)J combinations from both B cell antibodies (IG) and T cell Receptors (TCR). One major challenge of immune repertoire sequencing is to capture the structural and sequence complexities of antibody and TCR genes. **Methods:** We have developed and optimized a method for accurate sequencing of full-length immune gene repertoires of B-cells and T-cells. RNA extracted from tissue and PBMCs were used to generate immune sequencing libraries. Using a unique molecule index (UMI) to discretely barcode each mRNA molecule, PCR copies of each mRNA fragment can be collapsed into a single consensus sequence. The B cell genes were enriched during library preparation by isotype-specific primers (IgM, IgD, IgG, IgA and IgE). Full-length heavy chain antibody sequences were aligned to germline genes from reference databases. TCR libraries were generated from RNA from tumor/normal tissues and matched PBMC samples, and analyzed to determine patient/tumor specific clones and clonal expansion. Jurkat RNA was obtained from a homogenous population of Leukemic Jurkat T cells and spiked into a PBMC RNA sample at varying proportions of Jurkat RNA (10%, 1%, 0.1%, 0.01% and 0.001%). The RNA mixtures were made into TCR libraries and analyzed for TCR repertoire. **Results:** Utilization of the UMIs enabled absolute quantification of input RNA molecules and accurate ranking of the B cell antibody/TCR clone abundance. For B cell repertoire sequencing, the use of isotype-specific primers enabled measurement of the heavy chain isotype proportions within the samples. And full-length heavy chain antibody analysis enabled quantitation of the mutation level of each antibody sequence, providing information on the overall maturity and mutational profile of the sample repertoire. For TCR repertoire sequencing, distinct and shared clonal sequences were quantitatively detected in tissue and blood samples, which enabled identification of tumor specific clones to evaluate immunotherapy effect. Our method also accurately and sensitively detected the spiked Jurkat TCR clone down to 0.01%, enabling minimal residual disease assessment. **Conclusions:** Our immune repertoire sequencing approach allows accurate clonal determination for both IG and TCR. The information obtained enables the possibility for *in vitro* synthesis and expression of complete antibody chains, as well as investigation of T cell infiltration in tumor microenvironment and as a method to track of minimal residual disease.

#### TT021. PCR in Less than Six Minutes for Accurate Genotyping of Pathogenic Mutations

L. Xu, W. Hooker, K.R. Dawkins, L.A. Mull, A. Capobianchi, M. Cai, D.B. Sullivan, J.J. Huuskonen  
Canon US Life Sciences, Inc., Rockville, MD.

**Introduction:** Nucleic acid amplification using polymerase chain reaction (PCR) is one of the most sensitive and powerful tools for clinical laboratories, precision medicine, forensic science, basic research laboratories, and other fields. Ultrafast PCR represents a key development in such fields. Conventional thermocyclers raise and lower the temperature in a single thermal zone, requiring ramp times to achieve the necessary temperature for each step of the PCR. The ramp times are based on the rate of temperature change. Using conventional PCR instruments, we developed the Novallele genotyping assays that use high-resolution melting (HRM) analysis to detect relevant human genomic variations in nuclear and mitochondrial genomes. In this study, we evaluated the same assays using an innovative, end-point PCR thermocycler called NEXTGENPCR, manufactured by Molecular Biology Systems, B.V. (MBS). NEXTGENPCR rapidly heats and cools samples by using three separate, distinct thermal zones and moving the sample plates

between the temperature zones. The 35-cycle PCR for the Novallele genotyping assays was completed in 70% to 80% less time compared to conventional PCR instruments. Subsequent HRM analysis on commercially available instruments showed accurate genotyping results from the ultrafast PCR reactions. **Methods:** Targeting mutations in the *NDUFV3*, *L2HGDH*, *ITPA*, *IFNL3*, and *IKBKAP* genes, 9 cell-line genomic DNA samples as well as 10 synthetic double-stranded DNA constructs specifically designed as assay controls were amplified using NEXTGENPCR and the Novallele genotyping assays. Amplicons, which ranged in size from 42 to 50 bps, were transferred from the 96-well, ultrathin microplates to an HRM-compatible microplate for detection on a commercially available HRM-enabled thermocycler. HRM analysis was performed using a melt rate of 0.3°C per second. Genotypes were determined using the Novallele HRM Analyzer software for melt curve analysis. Genotyping accuracy was determined by comparing the results to synthetic DNA constructs and data generated from the same assays and samples on other commercially available thermocyclers. **Results:** Using NEXTGENPCR, the PCR cycling time was reduced from the typical 20 to 40 minutes to 5 to 5.5 minutes, a 70% to 80% reduction as compared to conventional thermocyclers. All assays showed 100% genotyping accuracy during HRM analysis after cycling on NEXTGENPCR. **Conclusion:** We demonstrated that the Novallele genotyping assays provide accurate genotyping results when amplification is performed with NEXTGENPCR, an ultrafast PCR in less than 6 minutes, without modifying the reaction chemistry.

#### TT022. Nanopore Sequencing for Clinical Fusion Oncogene Detection

W. Jeck, J. Lee, H. Robinson, L. Le, J. Iafrate, V. Nardi  
Massachusetts General Hospital, Boston, MA.

**Introduction:** Fusion oncogene detection is a cornerstone of diagnosis in some neoplastic entities. Fusion detection often requires focused search fluorescence *in situ* hybridization (FISH), live tissue (karyotype analysis), or extended turn-around time (deep sequencing). Nanopore-based deep sequencing has opened the possibility of rapid detection of a broad set of fusion genes from frozen or fixed tissues with rapid turn-around time. Previously, we reported successful and rapid identification of fusions in a cell line specimen using nanopore sequencing. We sought to perform an initial validation of nanopore fusion detection in clinical specimens in a manner that permitted serial and parallel multiplexing. **Methods:** We identified 16 specimens that previously underwent sequencing with our clinical assay and sequenced on an Illumina NextSeq device. Additional PCR amplification was performed on the existing libraries to add a nanopore barcode sequence, and specimens were sequenced with 4-fold multiplexing on an Oxford Nanopore MinION sequencer for up to 12 hours. Fusion calling was performed by an investigator blinded to all specimen information. Following this initial validation, additional assay validation was performed on unblinded samples. Additional pilot library preparation methods were also tested to further reduce total assay time. **Results:** Of 16 true fusion events analyzed, 12 were successfully detected by nanopore sequencing in the context of four-fold parallel specimen multiplexing. The assay was sensitive to fusions in specimens with as low as 40% tumor cellularity, as assessed histologically, with additional limiting dilution tests planned to further define lower limits of detection. One reference lab specimen with a very low level *PML-RARA* fusion was called negative. However additional testing with clinical *PML-RARA* positive cases demonstrated positive identification of this event. Testing of alternative library preparation techniques showed substantial decrease in library preparation time, but with decreased sequencing depth of the targeted fusion for some methods. **Conclusions:** Our results suggest that nanopore sequencing for fusion detection is a promising approach with beneficial features for laboratories with low volume or for samples requiring rapid turn-around time, with <12 hours of sequencing for 4-fold multiplexed specimens. With further optimization of library preparation technique, the assay could feasibly have a 24 hour total turn-around time, from specimen acquisition to reporting.



### TT023. Laser Capture Microscopy for Microsatellite Instability Testing

L. Dubeau, J. Pettersson, L. Du, T.I. Long, M. Campan, P.M. Ward  
University of Southern California, Los Angeles, CA.

**Introduction:** Tissues submitted for microsatellite instability testing may contain only small proportions of either cancer or normal cells, complicating accurate data interpretation and leading to test cancellations. We tested the validity of laser capture microdissection for microsatellite instability testing of such cases. **Methods:** Formalin-fixed paraffin embedded sections from ten colorectal cancers mounted on PEN membrane slides (ThermoFisher) and previously tested for microsatellite instability by macro-dissection (6 microsatellite stable, 2 with low instability and 2 with high instability) were subjected to laser capture microdissection using an Arcturus XLT instrument. Dissections of cancer and normal cells (100 to several thousand cells per dissection) were performed on unstained sections to avoid interference with PCR due to presence of chemical stains. Micro- and macro-dissected samples were amplified by PCR using primers for 5 mononucleotide repeats and 2 pentanucleotide repeats (Promega MSI kit v1.2) and analyzed on a 3500 genetic analyzer. Microdissections and downstream analyses were repeated 6 more times on a case with high instability to assess inter-run variability. The dissection was repeated using a stained section of one microsatellite stable case to evaluate the impact of staining artifacts. With both approaches, an effort was made to maximize the purity of each cell population. **Results:** Robust biomarker stutter profiles were generated for all cases with both approaches. The predominant peak sizes and stutter profiles generated by micro- versus macro-dissection matched exactly in 5/6 microsatellite stable cases, 1/2 cases with low instability, and 2/2 cases with high instability. Discrepancy in one microsatellite stable case was due to absence of a 170bp Penta C allele in the tumor sample in a case harboring 170bp and 180bp alleles in the normal cells, most likely reflecting loss of heterozygosity not appreciated in the macrodissected sample due to normal cell contamination. Discrepancy in one case with low instability was due to a one-base pair shift in the NR-21 biomarker profile of the macro-dissected tumor sample. Absence of this shift in the microdissected sample changed the diagnosis to microsatellite stable. Stutter patterns and predominant peaks were replicated exactly in all repeats of the case used to evaluate inter-run variability. Performance of microdissection on H&E stained sections led to electrophoretic artifacts. **Conclusion:** LCM is a valid approach to capture low abundant cancer and normal cells for microsatellite instability testing.

### TT024. Comparison of Roche 6800 System, Roche AmpliPrep/cobas TaqMan, and Hologic Tigris: Quantifying Efficiencies in a Growing Laboratory

K. Curless, A. McClain, V. Winn, R. Relich  
Indiana University Health, Indianapolis IN.

**Introduction:** As part of a growing health care system, the Indiana University Health Molecular Pathology Laboratory recognized a need to increase automation and efficiency in order to keep up with growing demand. The Roche cobas 6800 system was implemented for HCV, HIV, HBV and CMV testing, replacing the previous AmpliPrep/cobas TaqMan (CAPTAQ), with the goal to improve workflow by increasing automation and testing efficiency. The laboratory is also investigating if a switch from the Hologic Tigris to the cobas system will have similar outcomes for *Chlamydia* and *Gonorrhea* testing. This study attempts to quantify the efficiencies gained by moving testing to the cobas 6800 system. **Methods:** Metrics including number of tests and controls run, number of lot to lot conversions, overall turnaround time, and hands on time were compared between the Roche cobas 6800 system and the Roche AmpliPrep/cobas TaqMan and (HIV, HCV, HBV, CMV) and the Hologic Tigris (*Chlamydia*, *Gonorrhea*). **Results:** During the observation period, Indiana University Health saw an overall increase in virology testing volume of 12.9%. Despite this increase in volume, Indiana University Health was able to run 23.3% fewer controls with the cobas 6800 as well as 30% fewer lot to lot conversions. Run time and hands on time were also decreased by varying margins depending on test and initial instrumentation. **Conclusions:** Overall, Roche 6800 system demonstrated an increase in efficiency for all metrics analyzed in this study. The switch to this system proved to be advantageous for Indiana University Health

during its growth for the HIV, HCV, HBV, and CMV assays and this study has shown a similar result can be expected for the impending implementation of this instrument for *Chlamydia* and *Gonorrhea* testing in the future.

### TT025. Automated Primer Design for Single-tube Multiplex PCR for Tiled Amplicon Resequencing – TP53 Assay Design and Characterization as a Pilot Study

M. Zillmann, Y. Ke, N. Lodato, M. Prasol, Z. Wang  
Pillar Biosciences, Natick, MA.

**Introduction:** Target enrichment by PCR is widely used to prepare libraries for next generation sequencing (NGS), especially for formalin-fixed, paraffin-embedded (FFPE) specimens, where DNA amount may be low and quality compromised. Amplicon tiling (overlap) is often needed to cover continuous sequences of Regions of Interest (ROI). Pillar's SLIMamp technology is unique in enabling amplification in a single reaction with amplicon tiling. In this work, we use a customized primer design tool (ampPD) for rapid primer design to the TP53 oncogene and evaluate the performance of the resulting assay. **Methods:** The 11 coding exons from all TP53 transcripts were targeted for amplification. In the ampPD design tool, nominal amplicon length, amplicon overlap, and primer parameters (%GC, T<sub>m</sub>) are user specified. In the target characterization step, regions to be excluded as primer sites due to high polymorphism or repetitive regions were identified. In the candidate primer generation step, all primers with properties within design constraints were created forming a large number of combinatorial possibilities for each ROI. In the primer selection and pooling stage, all primer-primer interactions were considered, and an interaction minimized pool with full coverage of the ROI regions was selected. SLIMamp tags were added to the primers in this pool. The performance of the TP53 assay design was evaluated by synthesizing the output primers, mixing at equal concentration, and preparation of libraries from DNAs extracted from 10 clinical FFPE samples obtained from Dana Farber Cancer Institute. For each sample, library preparations using 10 ng, 5 ng and 1 ng of input DNA were performed. Resulting libraries were normalized, pooled and sequenced on an Illumina MiSeq. **Results:** Automated TP53 primer design generated an initial pool of 627 compatible primers that was reduced to an optimized pool of 19 amplicons varying between 138 and 180 bp and 16 overlapping amplicons. The total design took roughly 10 seconds to complete. For the 10 FFPE samples at 5 and 10 ng of input, variant detection was highly reproducible and 100% concordant with known outcome. At 1 ng DNA input all positive variants were detected but with increased variant noises at allele frequencies of 1-3%. The median on-target rate was 86% for 10 ng and 5 ng samples and 31% for the 1 ng samples. The assay displayed high coverage uniformity with 100% of targeted bases covered above 0.2x mean coverage for all samples. **Conclusion:** We have demonstrated a rapid and robust primer design pipeline using the TP53 oncogene as a model system. The resulting primers were shown to provide uniform coverage across TP53 and sensitive variant detection at FFPE-derived DNA inputs greater than 1 ng. Future work will focus on extending the design pipeline to larger and more complex panels.

### TT026. Sample Identification and Tracking Using a Nebnext Direct Target Enrichment SNP Panel

C. Hendrickson<sup>1</sup>, A. Emerman<sup>2</sup>, K. Patel<sup>1</sup>, S. Bowman<sup>1</sup>, S. Adams<sup>1</sup>, J. Dunn<sup>1</sup>, B. Desmond<sup>1</sup>, A. Barry<sup>3</sup>, E. Mauceli<sup>1</sup>  
<sup>1</sup>Directed Genomics, LLC, Ipswich, MA; <sup>2</sup>Directed Genomics, Essex, MA; <sup>3</sup>New England Biolabs, Ipswich, MA.

**Introduction:** Next-generation sequencing is a powerful and widely used tool for genetic screening and clinical diagnostics. As the throughput of sample processing and data analysis increases, the risk that samples may become switched or contaminated with each other becomes a concern, as such instances could lead to false results reported to a patient. Thus, samples should be robustly tracked throughout each stage of processing. A reliable method to track patient samples is to monitor single nucleotide polymorphisms (SNPs) that have a high discriminatory power among individuals; thus, the tracking method is embedded in the sequencing data itself. Work from others has identified 24 SNPs with a strong discriminatory power across diverse populations using whole exome sequencing data. Here we developed and validated an NEBNext Direct

target-enrichment bait set to those 24 SNPs to be used as a stand-alone or spike-in panel for sample identification. We demonstrate that the panel is an effective and robust tool for the confirmation of sample identity and the validation of sample integrity. **Methods:** Baits were designed to the 24 sample identification SNPs and bait performance was tested against a blend of 24 HapMap DNA samples from Coriell Institute using NEBNext Direct technology. A 12-base unique molecular identifier was used to remove PCR duplicates from the sequencing reads. The concentrations of overperforming and underperforming baits were adjusted to produce uniform sequencing read coverage of the SNPs. The final panel was used as either a stand-alone panel or spiked into a larger, cancer-focused panel to confirm the identity of 16 HapMap samples across a range of populations. Additionally, HapMap samples were mixed to simulate low levels of cross-contamination to test detection of contamination by the sample identification panel. **Results:** The final sample identification panel resulted in coverage of the 24 SNPs within a normalized range of 0.4 to 1.7. Greater than 95% of the sequencing reads mapped to the targeted SNPs. Sufficient coverage was achieved to confirm the identity of 16 HapMap samples when using the sample identification panel either as a stand-alone or spike-in panel. Additionally, the panel was able to detect down to 1% cross-contamination of samples. **Conclusion:** Our 24 SNP sample identification panel for NEBNext Direct technology provides a simple and robust tool to cross-validate the source of a DNA sample for sequencing. This panel can be used on its own as a secondary screen or can be spiked into another target-enrichment panel to be processed and sequenced together. Interrogation of these markers with target-enrichment panels aids in the correct assignment of a patient to a sample and reduces the risk of reporting false results.

**TT027. Analytical Performance of TruSight Oncology 500 on Small Nucleotide Variations and Gene Amplifications Using DNA from Formalin-fixed, Paraffin-embedded (FFPE) Solid Tumor Samples**

*D.M. Chou, T. Jiang, A. Purdy, J.L. Silhavy, B. Crain, T. Du, Y. Fu, Y. Guo, R. Haigis, S. Johansen, J. Ju, S. Kaplan, S. Katz, P. Le, D. Lee, L. Liu, J.S. LoCoco*  
*illumina, San Diego, CA.*

**Introduction:** Innovative applications of next generation sequencing (NGS) for genomic characterization of tumors have emerged as a powerful and disruptive force in oncology. The advent and proliferation of targeted therapies require interrogation of an ever-increasing number of actionable biomarkers that, combined with immunotherapy biomarkers such as tumor mutation burden (TMB) and microsatellite instability (MSI), are critical for patient stratification and therapy selection. Despite the strengths of next generation sequencing (NGS) technology in this diagnostic landscape, the high analytical sensitivity and specificity requirements for both driver and passenger mutations across broad regions of tumor DNA remain a formidable challenge. Here, we describe TruSight Oncology 500 (TSO500) from Illumina, an NGS-based solution designed to meet this challenge of biomarker identification in formalin-fixed, paraffin-embedded (FFPE) samples. **Methods:** TSO500 incorporates unique molecular identifiers for error suppression and a panel targeting 523 cancer-relevant genes. After sequencing on the Illumina NextSeq 550 instrument, the software detects small nucleotide variants, gene amplifications, and determines TMB and MSI status. Moreover, systematic errors and FFPE artifacts are removed to enable highly accurate calls of both cancer and passenger mutations. Together, these advances that maximize small variant calling sensitivity with extremely high analytical specificity across a large panel size form the basis from which an accurate tumor mutation burden measurement could be made. **Results:** Testing of a panel of samples consisting of cell line DNA, FFPE cell line DNA, and formalin compromised DNA with variants near our target limit of detection showed that TSO500 consistently detected key pathogenic small variants at allele frequencies  $\leq 5\%$ . Confirmation with FFPE samples derived from cancer patients were performed by mixing FFPE samples to dilute variants to near 5% allele frequency. The data showed that the assay correlated highly with droplet digital PCR (ddPCR) results and accurately detected small variants at allele frequencies as low as 3%. Testing of reference samples demonstrated robust detection of gene amplifications down to a 2.2-fold increase. Testing of normal FFPE samples showed that TSO500 can

achieve a false positive rate that is 100-fold lower than the typical rate from standard NGS bioinformatics pipelines. **Conclusions:** Together, our results demonstrate 95% analytical sensitivity and 99.995% specificity for variants down to 5% allele frequency coupled with robust gene amplification detection. These TSO500 capabilities derived from key innovations in assay design and bioinformatics are critical to the detection of actionable cancer biomarkers and form the foundation from which robust TMB and MSI outputs can be derived and leveraged.

**TT028. Optimization of a Custom Pharmacogenomic Panel**

*D. Thach, A. Penta, Q. Phan, G. Jo, N. Plaussiou, P. Kothiyal, X. Liu, R. Iyer*  
*Inova, Fairfax, VA.*

**Introduction:** Our current pharmacogenomics panel interrogates 40 genes. To interrogate more genes, a larger pharmacogenomics panel was designed by adding more loci to a total of 272 SNPs mapping to 63 genes. Herein, we optimized this larger panel for deployment as a clinical diagnostic assay. **Methods:** The multiplex PCR was designed in Ampliseq Designer. Libraries were generated via Ampliseq technology and sequenced on the Ion Torrent platform. Bioinformatics analyses were performed in Torrent Suite and open source tools. **Results:** The initial run of the new panel with 64 DNA samples showed low on-target coverage of <17% compared to the ~80% in the original panel. Analysis of unaligned reads revealed off-targets products, suggesting non-specific multiplex PCR. Analysis of the primer sequences suggested 2 primer pairs with some homology to human repeat sequences. Removal of these 2 primer pairs from the pool increased on-target coverage to >73%. Analysis of sensitivity of genotype calls across the 64 samples revealed No Call percentages ranging from 1% to 100%, with 150 of hotspots showing >5% No Calls. Increasing the total reads from 3 million to 14 million decreased the number of hotspots showing >5% No Call to 10. Bioinformatics quality control analysis of the hotspots BED file revealed 3 SNP RS identifiers have been removed from dbSNP. **Conclusions:** These results demonstrate that expanding established panels is not trivial, and the importance of optimizing both the wet-lab and bioinformatics steps. Increasing sensitivity by increasing total reads also increased the cost of the assay. These factors contribute to decisions whether to proceed with validation and clinical deployment.

**TT029. Validation of Reporting Metrics for ASXL1 C.1934dupG Variant in Hematologic Malignancies Based on the Illumina TruSight Myeloid Sequencing Platform**

*L. Ramkissoon, A. Arreola, N. Strande, C. Edgerly, M. Li, J. Booker, N. Montgomery, K. Weck*  
*University of North Carolina Medical Center, Chapel Hill, NC.*

**Introduction:** Somatic mutation in the additional sex combs-like 1 (ASXL1) chromatin remodeling gene has been implicated in several hematologic malignancies including acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) and is associated with an inferior overall survival. The most commonly detected ASXL1 mutation is the c.1934dupG (p.Gly646Trpfs\*12) variant located in a homopolymer region that results in a frameshift and premature protein truncation. However, this variant has been controversial due to frequent sequencing artifacts, likely due to slippage of the DNA polymerase, resulting in false duplication or deletion variant calls. Reflecting these challenges, ASXL1 c.1934dupG has been reported as both a pathogenic variant as well as a population variant (2.58% frequency in the overall population). During initial validation of the Illumina TruSight Myeloid (TSM) next generation sequencing panel, ASXL1 c.1934dupG was seen as a recurring artifact at <10% variant allele fraction (VAF) in multiple samples including normal controls. Given the significant prognostic implications for this variant in myeloid malignancies, we sought to determine metrics for reporting ASXL1 c.1934dupG as pathogenic using the Illumina TSM panel. **Methods:** Retrospective analysis was performed on data generated from clinical samples submitted at our institution for TSM sequencing between July-December, 2017. The VAF for both ASXL1 c.1934dupG and c.1934delG were recorded, as well as VAFs of other clinically relevant variants detected in the samples. Samples with ASXL1 VAF greater than 10% were confirmed by Sanger sequencing. Normal controls (n=20) were also subjected to Sanger

sequencing. **Results:** Our cohort included 26 AML, 43 MDS, and 31 myeloproliferative neoplasms (MPN). The average VAFs for the ASXL1 c.1934dupG were 6.4%, 6.8%, and 4.8% for the AML, MDS and MPN samples, respectively, while the average VAFs for ASXL1 c.1934delG were 4.4%, 4.4% and 4.5%. Of the 100 samples analyzed, six had evidence of ASXL1 c.1934dupG at a VAF greater than 10% including four MDS and two AML specimens, with VAFs ranging from 10.5% to 33.1%. ASXL1 c.1934dupG was confirmed by Sanger sequencing in all samples analyzed with VAF >10%, demonstrating ~10% sensitivity for Sanger analysis. Two samples with ASXL1 c.1934dupG VAFs at 5.5% and 6.5% and 20 normal control samples demonstrated wildtype sequence by Sanger sequencing. **Conclusions:** Our experience with the TSM platform resulted in a bimodal distribution of VAFs for ASXL1 c.1934dupG. We determined that VAF <10% should be considered a sequencing artifact and greater than 20% reported as a pathogenic variant for hematologic malignancies. For samples with a 10-20% VAF, our policy is to confirm by an orthogonal method.

#### TT030. The Use of Engineered DNA as Standards in Next-generation Sequencing Assays

P. Mann<sup>1</sup>, S. McNulty<sup>1</sup>, E. Duncavage<sup>1</sup>, C. Huang<sup>2</sup>, B. Anekella<sup>2</sup>, R. Garlick<sup>3</sup>, J. Pfeifer<sup>1</sup>

<sup>1</sup>Washington University School of Medicine, St. Louis, MO; <sup>2</sup>SeraCare Life Sciences, Inc., Gaithersburg, MD; <sup>3</sup>SeraCare Life Sciences, Inc., Milford, MA.

**Introduction:** Next generation sequencing (NGS) approaches for DNA analysis can detect all four major classes of sequence variants. External standardized samples of known sequence and variant allele frequency (VAF) are necessary for NGS assay validation, proficiency testing (PT), and comparison of results between laboratories. Traditional standardized samples (e.g., cell lines or tumor samples) have limited utility because they are not available for the full range of sequence variants, combinations, and VAFs. Engineered DNA segments have been proposed as standardized samples, but their utility in clinical NGS assays is uncertain; this study aimed to explore their performance for variant detection. **Methods:** Constructs harboring human gene inserts ~600 bp long with variants centered in the insert were cloned in a pUC vector. Linearized plasmids bearing 40 variants (single-nucleotide variants (SNVs), insertion/deletions (indels), and structural variants) were added to genomic DNA from the GM24385 cells to achieve 10% target VAF. The same linearized plasmids were electroporated into the GM24385 cell line and used to produce formalin-fixed paraffin-embedded (FFPE) cell pellets with 10% target VAF. DNA extracted from the FFPE scrolls by different methods was sequenced by a hybrid capture-based comprehensive cancer NGS assay that is in routine clinical use, and the VAFs determined by digital PCR were compared with the VAF determined by NGS.

**Results:** Using DNA mixed at 10% VAF (allele-specific digital PCR range 8.5% to 10.7%) all variants were detected by NGS with an average VAF of 8.3%. Larger indels (e.g., EGFR p.E746\_A750delELREA, NPM1 p.W288fs\*12) showed greater variance from expected VAF (3.0% and 3.9% VAF, respectively). Using DNA extracted from FFPE, all variants were also detected by NGS. However, the difference in measured versus expected VAF was dependent on extraction method. Extraction optimized for short DNA fragments (Qiagen QIAamp DNA FFPE Tissue Kit) produced measured VAFs within -0.46% to 11% of the expected VAF, whereas extraction optimized for mid-length fragments (Qiagen QIAamp DNAeasy) produced measured VAFs within 14% to 34% of the expected VAF. The differences in VAF did not correlate with variant type.

**Conclusions:** Engineered DNA segments have potential for use as standardized samples in NGS assays. However, when short engineered DNA fragments are used, the DNA extraction method must be optimized to achieve concordance between measured and expected VAF, indicating that engineered fragments will need to be optimized for use with existing clinical workflows. The variation between measured and expected VAFs when plasmid DNA was directly mixed with cell line DNA indicates that other issues, potentially related to the hybridization and/or bioinformatic methods, are also involved.

#### TT031. Beyond FISH, SNVs and Indels: Improved Resolution of Translocation Detection using Next-generation Sequencing (NGS)

L. Georgieva<sup>1</sup>, E. Uddin<sup>1</sup>, A. Heckel<sup>2</sup>, J. Reid<sup>1</sup>, J. Holdstock<sup>1</sup>, D. Cook<sup>1</sup>, G. Speight<sup>1</sup>

<sup>1</sup>Oxford Gene Technology, Begbroke, Oxfordshire, United Kingdom; <sup>2</sup>Oxford Gene Technology, Tarrytown, N.Y

**Introduction:** Large DNA rearrangements are known to be associated with the initiation and progression of multiple haematologic malignancies. While karyotype, fluorescence *in situ* hybridization (FISH), RT-PCR and microarrays are routine research techniques for variant discovery, they all have limitations, and with improvements in panel design, DNA- and RNA-sequencing using next generation sequencing (NGS)-based methods are rapidly becoming established as the method of choice. NGS panels facilitate the simultaneous discovery of novel alterations alongside known mutations and structural alterations in genomic research. The rapid growth in the development of this mutation detection capability has moved beyond single-nucleotide variants (SNVs) and insertion/deletions (indels) to now include translocations. Chronic myeloid leukaemia (CML) is a myeloproliferative neoplasm resulting from a t(9;22)(q34;q11) translocation. It is characterized by the presence of the BCR-ABL1 fusion gene encoding the BCR-ABL oncoprotein resulting in a deregulated tyrosine kinase activity. In this study, we tested the capability of a SureSeq myPanel NGS Custom Cancer Panel to detect known t(9;22)(q34;q11) translocations. **Methods:** We utilized a SureSeq myPanel NGS Custom panel and associated library preparation kit to determine whether this approach can be used for detection of the t(9;22)(q34;q11) translocation. We used a hybridization-based enrichment approach for library preparation and analyzed well characterized DNA samples (cell lines and research samples) with breakpoints across multiple sites in the major breakpoint area of BCR (ex13-ex15). To mimic the BCR-ABL translocation with different frequencies we created a serial dilution using cell line K562 (homozygous for BCR-ABL translocation). The resulting libraries were sequenced using a 2x150 bp read length protocol on an Illumina MiSeq. Resulting data was analyzed using OGT's proprietary translocation detection software. **Results:** We have achieved high depth (>1000x) and uniformity of coverage across the targeted regions which enabled successful detection of all translocations events. Furthermore, the confident detection of a BCR-ABL fusion event was reported at frequencies as low as 5%. NGS data from research samples revealed the presence of complex re-arrangements within the breakpoint region which would have been difficult to detect with FISH. **Conclusion:** We have demonstrated the capability of a SureSeq myPanel NGS Custom panel to detect complex rearrangements such as translocations. Our approach allows for the simultaneous evaluation of numerous translocations by NGS using a single panel as a simpler and more convenient alternative to the analysis of multiple single-gene or FISH assays on oncology specimens.

#### TT032. Comparison of cfDNA Reference Material Prepared Using Enzymatic Fragmentation or Sonication for the Validation of Liquid Biopsy Assays

H. Child, A. Mele, K. Wilczynska, J. Wickenden  
Horizon, Cambridge, United Kingdom.

**Introduction:** The growing field of liquid biopsies has huge potential to transform the clinical oncology space; allowing more genetic tests to be carried out on smaller amounts of tumor DNA without the need for invasive deep-tissue surgical biopsies. The genotype of multiple actionable loci can now be determined from as little as 10ng DNA extracted from a routine patient blood sample, helping to direct therapeutic decision making and improve overall clinical outcome faster for the patient. However, in order to realize the full potential of this emerging technology, sequencing labs need to ensure accuracy by validating a range of challenging new techniques. This includes the ability to extract cfDNA from blood samples, sequence it at new levels of sensitivity (down to 0.1% limit of detection) and establish effective bioinformatics pipelines. Reference materials that closely mimic real cfDNA samples are critical to support this effort. This study investigates the use of sonicated or enzymatically sheared cell-line derived DNA as alternative methods to create the most commutable cfDNA reference material for the validation of liquid biopsy assays. **Methods:** DNA was extracted from well-



characterized cancer cell lines and fragmented to 160bp using either sonication or enzymatic shearing, as assessed by TapeStation. In addition, a size selection step was included for investigation into the ability to purify the fragment peak to a size distribution profile most similar to real cfDNA samples. Test samples were validated for the presence of 8 onco-relevant mutations by droplet digital PCR (ddPCR), allowing for accurate variant allele frequency to be determined. The sample set was tested by next generation sequencing (NGS) on Thermo breast cfDNA assay, and Illumina TST-15 assay, following the manufacturer's instructions. **Results:** TapeStation analysis confirmed that both sonication and enzymatic shearing produced cfDNA with an average fragment size of 160-170bp. A size selection step proved useful to concentrate the amount of DNA within the desired fragment size range. Variant detection by ddPCR confirmed the presence of 8 mutations across 4 genes (*EGFR*, *KRAS*, *NRAS* and *PIK3CA*) at either 0.1% or 5% variant allele frequency. Analysis by NGS confirmed that all variants were detectable at the correct allele frequency as expected. **Conclusion:** Results demonstrate that both sonication and enzymatic shearing can be used to create cfDNA reference material with an average fragment size of 160-170bp to closely mimic cfDNA from real patient samples. This study has allowed a new manufacturing method to be investigated; utilizing enzymatic shearing and a size selection step in order to further improve the commutability of high quality cell-line derived cfDNA reference material for the validation of liquid biopsy assays.

#### TT033. Highly Stable and Commutable NIPS Reference Materials for Validation, Proficiency Testing and Quality Control

F.L. Tomson, Y. Konigshofe, C. Huang, R. Santhanam, R. Garlick, B. Anekella

SeraCare, Gaithersburg, MD.

**Introduction:** Plasma-based DNA Next Generation Sequencing (NGS) diagnostics for non-invasive prenatal screening (NIPS) has exploded in popularity in recent years. However, sufficient volumes of patient-derived reference materials for assay validations, proficiency testing and daily quality checks remain difficult if not impossible to obtain. We developed a technology and formulation that stabilized biomimetic NIPS reference materials derived from fragmented genomic DNA (gDNA). Stabilization is essential because fragmented DNA degrades in blood or blood-like matrices. Several different formulations for NIPS aneuploidy controls (Trisomy 13, 18 and/or 21) were tested for stability by NGS at two different temperatures: 42°C for up to 9 months and 4°C for up to 36 months. **Methods:** DNA formulations for NIPS were prepared by mixing normal female gDNA with aneuploid or euploid male gDNA. The mixture was verified by dPCR for copy number and then sheared using Covaris ultrasonic shearing before incorporation into a stabilizing biomimetic. The lipophilic encapsulated reference materials were then blended into a synthetic plasma. Samples were stored at 4°C for up to 36 months or at 42°C for up to 9 months. Over the time course, samples were tested by quantitative NIPS using a Verinata-derived NGS assay. **Results:** Real-time stability for a Trisomy 21 aneuploidy reference material, a multi-analyte aneuploidy reference material (including Trisomy 13, 18 and 21) and a euploid reference material (an aneuploidy negative control) were called correctly by NGS over thirty-six months. The multi-analyte reference material had NCVs (Normalized Chromosome Values) within 3% of the average for NCV 13, 1% of the average for NCV 18, and 9% of the average for NCV 21. Stressed stability studies were conducted comparing encapsulated DNA and free DNA in modified MatriBase at 4°C and 42°C. The encapsulated DNA samples, using Trisomy 13 reference materials, were called as Trisomy 13 samples by NGS and were within 13% of the average NCV 13 value after 275 days at both temperatures. In contrast, free DNA samples failed library preparation at 47 days at both temperatures. **Conclusions:** We have developed a commutable technology to stabilize small DNA fragments spiked into a synthetic plasma to generate NIPS reference materials that can be used for validations, proficiency testing and as daily run controls. These serve as true process controls and can be inserted into existing workflows similar to patient plasma samples. Both real-time and stressed stability data show that the protection conferred by the encapsulation creates a stable, refrigerated reference material for up to 3 years. Stable reference materials will enable improved quality monitoring of NIPS NGS diagnostic assays.

#### TT034. New Technology to Generate Commutable and Comprehensive Circulating Tumor DNA (ctDNA) Reference Materials for Next-generation Sequencing (NGS)

D. Ruminski Lowe, Y. Konigshofer, F.L. Tomson, C. Huang, R. Garlick, B. Anekella

SeraCare, Gaithersburg, MD.

**Introduction:** The need for commutable and comprehensive ctDNA reference materials is evident from the increasing number of liquid biopsy companion diagnostics and comprehensive panels on the market. Assay development, validation, verification, and QC testing benefit from consistent ctDNA reference materials. Commutable materials formulated with single variants are required for companion diagnostics while comprehensive panels must be validated with complex, multianalyte reference materials containing mutations, translocations, and copy number variation (CNV). We have developed custom VariantPlex library and new manufacturing technology making highly flexible materials for any ctDNA profiling workflow possible. **Methods:** Single EGFR variants or a complex mixture of 25 synthetic variants were mixed at defined allelic frequencies (AF) from 0.1 to 5% into gDNA from a reference cell line (GM24385). Digital PCR (dPCR) was used to verify allele frequencies and gene copy number. The DNA mixtures were sheared following an optimized protocol and new processing methods were used to improve behavior of the DNA during library preparation by repairing damage that inhibits ligation of adapters, a critical step in many next generation sequencing (NGS) assays. Then automated size-selection and novel purification steps were used to obtain ctDNA that is ideal for NGS library preparation. The size distribution was confirmed by Agilent Bioanalyzer analysis and then the manufactured ctDNA was encapsulated and diluted in artificial plasma to provide stability and enhance commutability. Extraction of ctDNA from the encapsulated plasma-like matrix was carried out using the QIAamp Circulating Nucleic Acid kit, and variant AF and CNVs were verified by digital PCR (Bio-Rad QX200) and by NGS (ArcherDx Reveal ctDNA 28/Illumina MiSeq Reagent Kit v2).

**Results:** Greater than 85% of the ctDNA fragments were between 100 and 225 bp, exhibiting a profile more like native ctDNA than sheared gDNA. The average AF of each variant measured by NGS was within 24% of the AF measured by dPCR for all mixes targeted above 1%; more variability was seen when approaching the lower limit of detection. *ERBB2* CNVs were detected by NGS within 17% of those detected by dPCR in all mixes, while *MET* CNVs were more variable at up to 39%. The new approach improved library yield and the number of unique molecules compared to unprocessed sheared gDNA.

**Conclusions:** Novel ctDNA reference materials were designed with an emphasis placed on size distribution and variant complexity. Both single variant-containing materials and complex multianalyte controls are demonstrably more commutable than materials composed only of sonicated gDNA and perform consistently across several platforms, highlighting the flexibility and value of this new technology.

#### TT035. Nucleic Acid Quantification and Smear Analysis for Fully Automated Next Generation Sequencing (NGS) Workflows

C.N. Paxton<sup>1</sup>, D. Rhodenizer<sup>2</sup>, J. Raney<sup>2</sup>, R. Bastien<sup>2</sup>, D.W. Close<sup>2</sup>, H. Best<sup>3</sup>

<sup>1</sup>ARUP Laboratories, Bountiful, UT; <sup>2</sup>ARUP Laboratories, Salt Lake City, UT; <sup>3</sup>University of Utah, Salt Lake City, UT.

**Introduction:** Next generation sequencing is increasingly being used in high volume clinical laboratories, which due to the complexity of library preparation and targeted enrichment would benefit tremendously by adopting fully automated workflows. Automation enables increased throughput and sample prep consistency while reducing technical error. Identifying fully automated quantitation and smear analysis instrumentation for continuous sample processing has been a challenge. The Agilent TapeStation run process is automated, but requires technical intervention for sample setup. Concentrations calculated on the TapeStation are based on the peak area of an upper marker, which is problematic if there is sample bleed into the upper marker. Accurate solution based quantification can be performed on the Qubit (Invitrogen), which is not amenable to integration into an automated process. Coastal Genomics recently released the LightBench, which integrates well as a

component of a fully automated solution for both direct quantitation and smear analysis. This study compared the performance of the LightBench to the TapeStation and Qubit—our current methodologies. **Methods:** Libraries were constructed from either whole blood or formalin-fixed, paraffin-embedded (FFPE) samples using the KAPA HyperPrep kit and capture based enrichment. Libraries were quantified and analyzed using the LightBench, Qubit and the TapeStation. Smear analysis covered 100-1,000 bp regions and we compared fragment size and concentration across the entire cohort, and as individual cohorts of each sample type. **Results:** There was no significant difference between concentrations measured on the LightBench and the TapeStation either as a whole group or by individual sample type. We did observe a significant difference in fragment size between the two instruments, with the LightBench yielding slightly shorter fragment sizes as compared to the TapeStation. Individual sample type cohorts showed similar results. However, there was no significant difference in subsequent molar concentrations calculated using the shorter fragment sizes. There was no significant difference in total DNA quantification when comparing the Qubit to the LightBench. **Conclusions:** LightBench, Qubit and TapeStation concentration results are comparable. Fragment sizes, determined to calculate molar concentrations, were shorter as measured by the LightBench, but molar concentrations were not significantly impacted. Throughput is similar on both the LightBench and TapeStation, with capability to analyze a full 96-well plate in a single run. The time required per run is sample-dependent on the TapeStation (~1 min/sample), but fixed on the LightBench (~30 min/run). The LightBench is walk away automation friendly allowing for continuous sample processing.

#### TT036. Tandem Single Nucleotide Polymorphism (SNP) Analysis Using Next-generation Sequencing (NGS) for Sample Contamination Detection and Chimerism Assessment

K. Halverson, S. Dasari, M. Mai, R. He, D. Viswanatha  
Mayo Clinic, Rochester, MN.

**Introduction:** Large volume next generation sequencing (NGS) clinical labs seek to optimize efficiency and lower cost by maximizing batch sample throughput using molecular indexes to identify specific patient samples at analysis. However, indexing of samples for hybrid capture-based libraries occurs at later steps in the chemistry protocol. Sample mixes (contamination) occurring early in the process would therefore be undetected. SNP fingerprinting is a method for post-sequencing evaluation of sample contamination, but is limited by the requirement of large numbers of informative SNPs. Microhaplotypes (MH) are stable sets of highly informative SNPs occurring over small regions of DNA (<300 bps) distributed throughout the human genome (Kidd K, *et al.*, 2014, 25038325). We evaluated MH in the context of sample mixes, including in the setting of post-stem cell transplant (SCT) engraftment (chimerism). **Methods:** Blood samples from healthy donors, samples tested for chimerism, a NGS sample with known sample contamination, a known SCT relapse sample and cell line DNA samples (NA12878, NA19240; Coriell) were evaluated using a 42 gene myeloid-targeting NGS panel (NGSHM). Sheared adapter-ligated DNA was used for targeted hybrid capture library preparation (Agilent SureSelect, Santa Clara, CA) and additional coverage for 31 MH regions was included in the bait design. NGS was performed on Illumina instruments (Illumina, San Diego, CA) and sequence data processed using a custom bioinformatics analysis pipeline (Mayo NGS Workbench). Microhap analysis was performed as a separate step for this study. **Results:** Addition of capture probes for the 31 MH regions did not affect the analytical features of the NGS assay. MH regions were adequately covered with >250x depth. DNA from healthy donors and cell lines demonstrated close to expected germline SNP variant allele fractions (>0.4 to <0.6). Some deviations were present in a small subset of loci, but these appeared random and more apparent in the cell line DNA. The chimerism samples, SCT relapse specimen and the sample with known contamination were all identified as abnormal by consistently skewed SNP VAF distributions at >2 MH loci. Quantitative assessment of MH-determined contamination or chimerism percentage closely aligned with the results of previous short tandem repeat (STR) PCR analysis. **Conclusions:** Inclusion of MH loci in the standard NGS panel build (either capture or PCR based) can help to identify potential sample mixes in high volume NGS applications and does not require the

addition of exogenous “tracking” reagents. MH analysis is also potentially useful in the setting of post-SCT engraftment monitoring, especially if combined with minimal residual disease evaluation using NGS methods.

#### TT037. Spike-in NGS Controls for Copy Number Assessment and Improved LOD and VAF Confidence

F.B. deAbreu<sup>1</sup>, S.J. Deharvengt<sup>1</sup>, B. Austermiller<sup>2</sup>, T. Morrison<sup>2</sup>, G.J. Tsongalis<sup>1</sup>, J.A. Lefferts<sup>1</sup>

<sup>1</sup>Dartmouth-Hitchcock Medical Center, Lebanon, NH; <sup>2</sup>AccuGenomics, Inc., Wilmington, NC.

**Introduction:** Spike-in controls are a crucial component of many real-time PCR assays and rule out PCR inhibition. Similarly, next-generation sequencing (NGS) spike-in controls could help monitor the NGS process in individual samples. AccuGenomics is developing Standardized Nucleic Acid Quantification (SNAQ) spike-in controls for molecular testing that are designed to co-vary with the measured target and have the potential to improve the traditional performance limits by providing direct sensitivity and specificity control for every target in every sample. For targeted NGS, the controls mimic the NGS library sequencing errors and yield variation of the sample and allow for LOD and VAF confidence for every base in every sample. In this proof-of-principle study, we examine the application of SNAQ-SEQ in the estimation of CNV and LOD for actionable mutations in tumor formalin-fixed, paraffin-embedded (FFPE) samples. **Methods:** The spike-in control mixture is synthetic DNA that can be distinguished from genomic DNA (gDNA) by unique dinucleotide alterations every 50bp. The controls are spiked into the gDNA being sequenced at a 1:1 molar ratio and the library preparation and DNA sequencing proceed as normal. In this study, sequencing was performed using the AmpliSeq Cancer Hotspot Panel (CHP) v2 (ThermoFisher). The control and genomic sequences are separated at the FASTQ file stage using a Perl script that identifies the dinucleotide sites. For copy number analysis the sequence counts of gDNA are normalized to the counts of the control at individual loci. To estimate limits of detection, the false positive rate of actionable variants in the control was measured. The confidence of VAF was estimated based on the previously published formula of Blomquist *et al.* (2015). Two normal tissue samples were sequenced in duplicate along with three tumor samples with known single-nucleotide variants (SNVs) and CNV findings. **Results:** CNV findings using the SNAQ-SEQ controls with the tumor sample correlate well with known CNVs, specifically EGFR amplifications of different levels in two different tumor samples. False-positive SNV rates were low in the control and gDNA sequences but slightly higher in the gDNA, presumably due to formalin fixation. The false positive rate was significantly higher in both the control and gDNA sequence for one NRAS variant, suggesting an isolated sequencing error. Confidence interval estimates for these VAFs ranged from 2.5- to 18-fold. **Conclusion:** These initial data suggest that SNAQ-SEQ controls are a useful spike-in control for NGS that not only help to more accurately define the accuracy of individual samples but can also improve the ability to identify CNVs.

#### TT038. Anchored Multiplex PCR Sequencing with *de novo* Contig Assembly Successfully Identifies BCOR Internal Tandem Duplication in Pediatric Tumors

A. Church, S. Duraisamy, J. Putra, T. Restrepo, S. Cano, J. Plunkitt, R.S. Pinches, V.A. Paulson, A. Al-Ibraheemi, M.H. Harris  
Boston Children's Hospital, Boston, MA.

**Introduction:** Clear cell sarcoma of kidney (CCSK) and the related primitive myxoid mesenchymal tumor of infancy (PMMTI) are rare tumors affecting young children. The histology is varied and these rare diagnoses can be challenging. Internal tandem duplications in BCOR (BCOR ITD) have been reported in a majority of cases of CCSK and PMMTI; a minority of CCSK cases have YWHAE fusions. Other pediatric tumors in the differential diagnosis also have characteristic rearrangements, including FUS, EWSR1, PLAG1, HMGA2, NTRK3, TFE3 and TFEB. The identification of these characteristic alterations is important in making an accurate diagnosis. **Methods:** All available archived cases of CCSK diagnosed over the preceding 17 years as well as 3 prospectively diagnosed cases of CCSK (1) and PMMTI (2) were collected, for a total of 9 CCSK and 2 PMMTI cases. Total nucleic acid (DNA and RNA) was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue. Each case underwent anchored multiplex PCR library preparation with a custom

designed panel of 64 genes, including *YWHA*E, *FUS*, *EWSR1*, *PLAG1*, *HMGA2*, *NTRK3*, *TFE3*, and *TFEB*; BCOR was targeted for both fusions and ITDs. Eight cases were also analyzed by standard PCR. In order to evaluate components of the informatics pipeline critical to the detection of ITDs, parallel analyses were conducted with and without *de novo* contig assembly. **Results:** All 11 cases were successfully sequenced, with 9 of 9 (100%) CCSK cases and 2 of 2 (100%) PMMTI cases demonstrating BCOR internal tandem duplication sequences in exon 15, ranging from 85-128bp in length. Analysis performed without the *de novo* contig assembly component of the pipeline, however, failed to accurately detect the ITD, with the duplicated sequence aligning to the reference sequence with mismatches at the ends of reads. All 11 cases were negative for other fusions including *YWHA*E, *TFE3*, *TFEB* and *NTRK3*. Eight CCSK cases had sufficient material for parallel analysis by PCR and the ITD was confirmed in 8 of 8 (100%) cases. **Conclusions:** Multiplex targeted RNA sequencing from FFPE tissue is successful at identifying BCOR internal tandem duplications in CCSK and PMMTI. The prevalence of BCOR ITDs and the size of the duplications are consistent with previously published reports. The *de novo* contig assembly is a critical component of the analysis pipeline. This analysis represents the first published description of the use of this technique for the identification of BCOR ITDs. Furthermore, the BCOR analysis is multiplexed with several other gene fusions that are diagnostically relevant for pediatric tumors. A robust multiplexed sequencing assay with multiple targets for pediatric tumors that works well with FFPE tissue is a valuable diagnostic tool.

#### TT039. Improvement of RNA Extraction Yield from Formalin-fixed Paraffin-embedded Tissue Specimens by RNAstorm

X. Qiu<sup>1</sup>, H. Tang<sup>1</sup>, E. Kool<sup>2</sup>, M. Chen<sup>1</sup>

<sup>1</sup>UT Southwestern Medical Center, Dallas, TX; <sup>2</sup>Stanford University, Stanford, CA.

**Introduction:** Formalin-fixed paraffin-embedded (FFPE) tissue specimens are important materials for performing retrospective studies on patients with complete pathology and clinical records. Comprehensive gene expression profiling analysis by microarray or next-generation sequencing ((RNA-Seq) of FFPE tissue samples is indispensable for cancer research and provides the opportunity to evaluate links between molecular and clinical information. However, RNA in FFPE specimens is extensively cross-linked and fragmented. Extraction of RNA from FFPE samples is technical challenging, which often results in low yield and poor quality. In the current study, we aim to optimize the process of RNA isolation from FFPE tissue samples by comparing multiple kits. **Methods:** Total RNAs were isolated from sections of different tumor (lymphoma, metastatic renal cell carcinoma and colon cancers) and control tissues (tonsils and placenta). The quantity and quality of isolated RNA were evaluated. Real-time RT-PCR to analyze the genes related to immune-oncology including PD-1/PDL-1 pathways were performed to compare the integrity of isolated RNA. **Results:** Total RNAs were isolated by multiple commercial RNA purification kits, including RNeasy FFPE kit (Qiagen) and RNAstorm kit (Celldata). Both kits performed well and resulted in the isolation of high quality RNA. The yield of RNAs isolated by RNAstorm kit was higher than these by RNeasy kit, as measured by OD reading on a NanoDrop (18.55±2.176 ng/slide vs 15.46±2.887 ng/slide, n=6). No significant differences were seen in A260/A280 or A260/A230, indicating the purities were equivalent quality. The expression levels of two genes, PD-L1 and beta-actin, were analyzed by real-time RT-PCR. Significant improvements in RNA yield and quality (as measured by amount of RNA) are seen using a prototype of the RNAstorm kit on FFPE samples from various tissues. **Conclusions:** RNA isolated from FFPE tissue samples using the RNAstorm kit showed a higher real-time RT-PCR sensitivity than RNA isolated using two other commercially available kits. There is no significant difference between the kits in term of RNA quality or downstream analysis by real-time RT-PCR. However, RNA extracted from RNAstorm kit results in higher yield, which may be better while handling limited samples. Our pilot study provide evidence for further high-throughput transcriptional analysis by optimizing FFPE tissue sample processing from RNA isolation to comprehensive transcriptome next generation sequencing (NGS) analysis.

#### TT040. Assessing the Performance of Automated Library Preparation in a Pan-solid Tumor NGS Workflow

J.E. Brock, L. Yin, R. Johnson, D.H. Farkas  
Cleveland Clinic, Cleveland, OH.

**Introduction:** Library preparation steps in next generation sequencing (NGS) workflows can be complex and lead to inefficiencies in the lab. Automation may simplify lab operations, minimize operator fatigue, and improve turnaround time without sacrificing data quality. We describe performance criteria used to compare automated versus manual library prep in a pan-solid tumor NGS workflow that will be applied in routine molecular pathology testing. **Methods:** DNA and RNA from multiple formalin-fixed, paraffin-embedded (FFPE) tissue specimens and reference materials were prepared for sequencing by both automated (Biomek NXP liquid handler) and manual processing in this next generation sequencing (NGS) workflow. The two methods' performances were compared using these data quality metrics: Quantity of the final libraries by Qubit, pass filter rate (%PF), median insert size, % region of interest (ROI) bases ≥100x for small nucleotide variants (SNVs), coverage MAD and median of normalized raw read counts for copy number variants (CNVs), and average median CV value of ROI coverage >1000x for fusions and splice variants. Data were statistically analyzed by the 2 tailed paired t test (p) and correlation coefficient (r). A no template control (NTC) was processed in the automated library preparation workflow and sequenced to assess cross-contamination. **Results:** Data from DNA and RNA libraries prepared in both automated and manual workflows were compared. The average quantities of the final libraries were 23.3 ng/μL and 23.9 ng/μL, respectively, with p=0.68 and r=0.92. The average %PFs for DNA libraries were 9.6% (automated) and 8.9% (manual), and RNA libraries were 1.9% and 2.0%, respectively, with p=0.46 and r=0.97. Median insert sizes ranged from 83-139 bp and 84-131 bp, respectively, with p=0.23 and r=0.97. All expected variants were detected by both methods at similar allele frequencies. For SNVs, the %ROI bases ≥100x was 99.9% for both methods, with p=0.88 and r=-0.1. For CNVs, the coverage MAD was 0.1 (r=1), and the median of the normalized raw read counts was close to 21 for both methods, with p=0.32 and r=0.96. For fusions and splice variants, the average median CV values of ROI coverage >1000x were 0.51 and 0.59 in the automated and manual methods, respectively, with p=0.03 and r=0.92. The concentration of the NTC library was <0.5 ng/μL, suggesting no cross-contamination. **Conclusions:** In studying automated versus manual library preparation, data quality for both methods was equivalent, leading to adoption of the automated method and resultant increased operational efficiency for a pan-solid tumor NGS laboratory developed test.

#### TT041. Incidental "Metagenomic" Findings Using a Next-generation Sequencing (NGS) Panel for Hematologic Malignancies

K. Bessonon, M. Hubbard, L. Frederick, D. Devine, R. He, D. Viswanatha  
Mayo Clinic, Rochester, MN.

**Introduction:** Metagenomic profiling by next generation sequencing (NGS) methods enables deep molecular profiling of bacterial or viral organisms in environmental samples. Similarly, DNA of microbial origin can also be detected in human tissue samples. During routine application of a custom NGS panel for myeloid neoplasms, we identified rare patient samples harboring varying quantities of bacterial genomic DNA. Although incidental, identification of microbial species in human blood or bone marrow samples has both technical and possible medical implications. We present a small number of cases that were identified and characterized in our laboratory practice. **Methods:** Blood or bone marrow samples were evaluated using a 35 gene myeloid-NGS panel (NGSHM). A minimum of 50 ng of sheared adapter-ligated DNA was used for targeted hybrid capture library preparation (Agilent SureSelectXT, Santa Clara, CA). NGS was performed on an Illumina platform (Illumina, San Diego, CA) and sequence data processed using a custom bioinformatics analysis pipeline (Mayo NGS Workbench). Clipped reads identified in this process are reviewed in Alamut Visual (Rouen, France) for insertions/deletions with an approximate detection sensitivity of 1%. Clipped reads not matching the GRCh37 human reference sequence were analyzed separately using NCBI Nucleotide BLAST to identify the origins of the non-reference DNA. **Results:** Fastq files processed and mapped to the human genome reference revealed a lower than expected percent match identity ranging



from 75% to 99% in rare patient samples. Unmapped reads were mainly identified in "extended" .bam files because the analysis pipeline filtered the reads out within the standard .bam files, unless the clipped reads were present at a high percentage. Only certain genes from the targeted panel were found to be affected by suboptimal mapping, including *CEBPA*, *RUNX1*, *TERT*, *TET2*, and *WT1*. The atypical clipped reads in several cases from one institution were found to be from the Gram negative species *Stenotrophomonas maltophilia*. Two additional samples containing *Bosea vaviloviae* and *Agrobacterium tumefaciens* were also identified from different facilities. **Conclusions:** In our experience, a small percentage of total analyzed cases (<1%) can have detectable microbial contamination in a high volume NGS setting. Sources include sample contamination at time of collection, sample transfer or aliquoting, or possibly evidence of true infectious disease in a patient. These sequences are identified by lower than expected mapping identity to human reference and can be resolved precisely. The significance of these findings is uncertain; unusual or rare pathogenic species may be difficult to distinguish from commensal or non-pathogenic contaminants.

#### TT042. Fast and Accurate *SMN1* and *SMN2* Copy Number Determination Using High-resolution Melting Analysis

L. Jiang, Z. Kabir, Y. Machida, S. Paquerault, J. Huuskonen  
Canon U.S. Life Sciences, Rockville, MD.

**Introduction:** Spinal muscular atrophy (SMA) is a neuromuscular disorder leading to loss of motor neurons and progressive muscle wasting. SMA is largely caused by deletion of the *SMN1* gene. SMA severity can be tempered by low-level survival motor neuron (SMN) protein expression from the 99% identical, alternatively spliced *SMN2* gene. With recent treatment advancements, fast and accurate SMA screening is imperative. Our research demonstrates that the Novalelle copy number assays for both the *SMN1* and *SMN2* genes using high-resolution melting (HRM) analysis are accurate and simple. **Methods:** Genomic DNA was extracted from 9 blood samples using 4 commercially available kits. The *SMN1* and *SMN2* copy numbers of these samples were independently assessed using multiplex ligation-dependent probe amplification (MLPA), digital PCR, and the Novalelle copy number assays. The samples, along with cell-line controls, were tested with the Novalelle copy number assays on 5 HRM-enabled, commercially available thermocyclers. The reaction chemistry, DNA amount, and PCR and melt protocols were uniform for all the thermocyclers. Additionally, another 34 cell-line samples were tested using the *SMN1* Copy Number Assay, and representative samples from each copy number group were confirmed by digital PCR. **Results:** For *SMN1*, 7 of the 9 samples were reported as having two *SMN1* copies by both MLPA and the Novalelle *SMN1* Copy Number Assay. For the two remaining samples, MLPA called one sample as two copies and the other as no call because the sample fell between two and three copies; repeated experiments (n=16) with the Novalelle *SMN1* Copy Number Assay reported three copies. Digital PCR resolved the discordance by confirming both samples as having three copies. For *SMN2*, MLPA and the Novalelle *SMN2* Copy Number Assay determined concordant copy numbers for all 9 samples – zero (n=1), one (n=6), or two (n=2) copies. The Novalelle copy number assays had 100% agreement for all 5 thermocyclers and all 4 DNA extraction kits. Four thermocyclers provided results within one hour. Additionally, the 34 cell-line samples were identified as two copies (n=27) or three copies (n=7) using the Novalelle *SMN1* Copy Number Assay, and digital PCR confirmed representative samples from each copy number group. **Conclusions:** For this study, the Novalelle copy number assays had greater accuracy than the MLPA method tested. The Novalelle copy number assays are reliable and fast using multiple thermocyclers and function with numerous sample preparation methods.

#### TT043. Confirming Somatic Variant Allele Fractions in Novel Circulating Tumor DNA (ctDNA) Control Material

E. Hughes, M. Al-Turkmani, C. Griffin, G.J. Tsongalis, J. Lefferts  
Dartmouth Hitchcock Medical Center, Lebanon, NH.

**Introduction:** Interest in clinical testing targeting ctDNA has grown rapidly in recent years but few options exist for adequate control material. Low concentrations, low variant allele fraction (VAF) and fragmentation to ~150 bp are some of the characteristics of ctDNA that make it a challenging

analyte in molecular testing. Control DNA with similar characteristics would be valuable in ctDNA testing. We assess a new set of ctDNA controls using droplet digital PCR (ddPCR) to determine how suitable they are for a variety of molecular methods. **Methods:** Purified ctDNA V3.0 controls were obtained from SeraCare Life Sciences (Milford, MA). This set of six controls consist of fractionated human genomic DNA with a set of 19 somatic variants (single-nucleotide variants (SNVs) and insertion/deletions or indels) introduced in ten different cancer genes with a variety of variant allele fractions (VAFs): 0%, 0.1%, 0.5%, 1%, 2.5 % and 5%. The VAF for each control was measured for three representative SNVs (*EGFR* T790M, *BRAF* V600E and *KRAS* G12D) by ddPCR using the QX200 Droplet Digital PCR System (Bio-Rad) using an input of 10 ng per ddPCR reaction. Additional samples previously identified as being positive and negative for these variants were included in each run. Each reaction was performed in duplicate with results averaged after excluding reactions with less than three droplets positive for a variant. An additional ddPCR assay was used to estimate the degree of DNA fragmentation. **Results:** The VAFs for the SeraCare controls correlated well with the expected values for the SeraCare ctDNA controls. The three variant alleles were not detected in the 0% material. The *EGFR* variant was not detected in the 0.1% control but the *BRAF* and *KRAS* variant were detected near the expected values (0.245% and 0.165%, respectively). The average VAFs for all three variants were 0.552%, 1.24%, 2.57% and 5.08%, respectively for the 0.5%, 1%, 2.5% and 5% controls. Additionally, the percentage of DNA in the size range typical of ctDNA was estimated to be greater than 98% of total DNA in the sample, confirming that the control samples are a representative control for ctDNA. **Conclusion:** The control samples tested were found to contain three of the expected variants near the expected VAFs. The amount of input DNA was not expected to reliably detect VAFs of 0.1%. VAFs measured by ddPCR in the remaining controls were remarkably close to the expected values. Together with our measurement of the degree of DNA fragmentation, these data indicate that these control materials are excellent mimics of ctDNA and would be useful in developing, validating and monitoring quality in ctDNA assays.

#### TT044. Baseline ctDNA Levels Predict Patient Outcomes

V. Gupta<sup>1</sup>, A. Shanmugam<sup>1</sup>, S. Sridharan<sup>1</sup>, A.H. Hariharan<sup>1</sup>, S. Katragadda<sup>1</sup>, A. Chaubey<sup>2</sup>, A. Chougule<sup>3</sup>, K. Prabhaskar<sup>3</sup>, U. Bahadur<sup>1</sup>, S. Sankaran<sup>1</sup>, V. Veeramachaneni<sup>1</sup>, P. Kolekar<sup>1</sup>

<sup>1</sup>Strand Life Sciences Private Limited, Bangalore, India; <sup>2</sup>Mazumdar Shaw Medical Foundation, Bangalore, India; <sup>3</sup>Tata Memorial Hospital, Mumbai, India.

**Introduction:** Liquid biopsy is increasingly gaining traction as an alternative to invasive solid tumor biopsies for treatment decisions and disease monitoring. But there is no consensus on the prognostic value of pre-surgical/at biopsy (baseline) ctDNA levels. **Methods:** Matched tumor-plasma samples were collected from 180 patients with varied cancers of different stages. Tumors were profiled using next-generation sequencing (NGS) or quantitative PCR (qPCR), and the mutation status was queried in the matched plasma using digital platforms such as droplet digital PCR (ddCPR) or NGS for concordance. Patient survival outcomes were correlated to pre-surgical ctDNA levels. Wherever follow up samples were available, ctDNA was monitored and matched to disease status. **Results:** Tumor-plasma concordance of 82% and 32% was observed in advanced (Stage IIB and above) and early (Stage I to Stage IIA) stage samples respectively. Baseline ctDNA can stratify patients into 3 categories: high ctDNA (>211 copies per mL plasma) correlated with poor survival outcome, (b) undetectable ctDNA with good outcome and (c) low ctDNA whose outcome was ambiguous. **Conclusion:** ctDNA can be a powerful tool for therapy decisions and patient management in a large number of cancers across a variety of stages. Its levels may help stratify patients into prognostic groups and identify those who may require serial monitoring or aggressive treatment.

#### TT045. Profiling of Tumor Immune Cells by Single-cell RNA-seq

V. Montel, J. Yao, A. So, L. Watson, J.H. Tsai  
Illumina Inc., San Diego, CA.

**Introduction:** Tumors are complex tissues that consist of multiple cell types. Proper profiling of the tumor microenvironment, specifically the

immune content, is highly desired for targeted immunotherapy. Recent advances in single-cell RNA-sequencing (scRNA-seq) allow massively parallel transcriptome inquiry of multiple cells that helps reduce cost and deconvolutes complex biological systems. Here, we evaluate the capability of scRNA-seq to profile the tumor subpopulations in melanoma samples. **Methods:** Dissociated melanoma tumor cells were stained with canonical CD4, CD8, CD45, CD3 and CD19 immune cell markers and either 1) processed through the SureCell WTA 3' RNA-seq assay to generate scRNA-seq data or 2) analyzed by fluorescence-activated cell sorting (FACS) to profile the immune cell population. Unbiased dimensionality reduction-based clustering analysis (t-SNE) was performed on the scRNA-seq data from 1,800 to 4,300 cells per tumor to classify subpopulations. The scRNA-seq immune cluster population was benchmarked with the FACS analysis. Further scRNA-seq gene profile analysis was then performed to annotate the remaining tumor microenvironment subpopulations and to observe intra-tumor gene expression heterogeneity. **Results:** Single-cell RNA-seq data was able to predict the tumor immune content based on the median genes detected per cell ( $R^2 = 0.93$ ), a single-cell assay key metric. The immune subpopulations revealed by the clustering analysis was highly correlated to populations classified by FACS for CD45 ( $R^2 = 0.92$ ), CD3 ( $R^2 = 0.94$ ), CD19 ( $R^2 = 0.96$ ) and CD4 ( $R^2 = 0.81$ ) but at a lesser extend for CD8 ( $R^2 = 0.61$ ). Furthermore, a single sample processed by scRNA-seq was able to profile the entire tumor content based on the transcriptome data. **Conclusions:** Single-cell RNA-seq data is able to accurately identify the tumor immune cell populations in addition to classifying the other tumor subpopulations from a single sample.

#### TT046. The NIH Genetic Testing Registry (GTR): Test Methodologies as a Sensor of the Precision Medicine Environment

A. Malheiro, M.A. Hoepfner, M. Benson, S. Chitipiralla, B. Gu, A. Glodek, W. Jang, K. Katz, C. Liu, Z. Maddipati, D. Maglott, K. McDaniel, M. Ovetsky, R. Villamarin-Salomon, G. Zhou, J.B. Holmes, B.L. Kattman  
National Institutes of Health, Bethesda, MD.

**Introduction:** Eighteen years after the completion of the human genome project, the promise of precision medicine is being realized: the idea that understanding the human genome will lead to insights into the genetic and molecular basis of health and disease and promote the development of therapies to cure or alleviate disease. The Genetic Testing Registry (GTR, <https://www.ncbi.nlm.nih.gov/gtr/>) is a free, searchable, online resource founded in 2012 by the National Institutes of Health as a repository for clinical and research genetic tests, with the goal of providing transparency of genetic testing information and advancing public health and research into the genetic basis of health and disease. As of May 2018, it includes 54,612 genetic tests submitted by 507 laboratories from 40 countries, covering 16,426 genes and 11,208 conditions, including somatic phenotypes, Mendelian disorders, and pharmacogenetic responses. Each GTR test is an orderable unit, with information fields to enable clinicians to find the most appropriate test for the patient. As testing laboratories provide information such as methodologies used, we have analyzed the GTR data to evaluate the spectrum of registered genetic tests. **Methods:** Analysis was performed using the eUtilities eDirect command line program from NCBI, structured query language (SQL) queries of the GTR database, and search options on the GTR website. **Results:** Of the tests in GTR, 99.8% are clinical tests, the remaining are research tests. The tests are categorized by 3 major methods (Tier 1): Molecular Genetics (98.3%), Biochemical Genetics (1%), and Cytogenetics (1%). Some tests use more than one Tier 1 method: 2 tests use 3 methods and 191 tests use 2 methods. A second category tier (Tier 2) is used for each major method; many tests utilize multiple Tier 2 methods. For Molecular Genetics, the most widely used are: Sequence analysis of the entire coding region (55.2%); Deletion/duplication analysis (33.7%); and Targeted variant analysis (7.6%). Top Tier 2 categories for Biochemical Genetics are Analyte (57.4%) followed by Enzyme Assay (36.7%). Fluorescence *in situ* Hybridization (FISH) is used in 80% of Cytogenetic tests. Each Tier 2 category is described by its primary methodology: 'Next-Generation (NGS)/Massively parallel sequencing (MPS)' leads the way with 50.2% of tests in GTR using NGS methodology, including 954 multigene panels

which test for >25 genes, as well as exome and whole genome tests.

**Conclusions:** GTR reflects the current global landscape of genetic tests. NGS is the major method registered in the GTR, consistent with progression in genetic testing to high-throughput, genome-wide methodologies. We will present our analyses of GTR and results, and further discuss the current genetic landscape.

#### TT047. Evaluation of Targeted Lung DNA Panels for Illumina MiSeq and GeneReader Platforms

K. Horvath, A. Ghosal, S. Koo, S. Verma, M. Moore  
ResearchDx, Irvine, CA.

**Introduction:** Next-generation sequencing (NGS) panels that target clinically actionable regions of interest are useful tools for detecting specific mutations that have a known association with a disease or phenotype at a high resolution. Targeted panels can be purchased either off the shelf with preselected content or custom-made for a preferred region of interest. With options available from many vendors, it is critical for a clinical lab to evaluate not just panel content, but also performance characteristics, workflow, cost, ease of use, and hands-on time.

**Methods:** In this study, we have evaluated the workflow and performance characteristics of two Unique Molecular Identifier (UMI)-based targeted DNA panels from Qiagen: QIAseq Targeted Lung DNA panel and GeneRead QIAseq Lung UMI DNA Panel. Multiplex FFPE Reference Standard (HD200, Horizon Inc.) and 10 formalin-fixed, paraffin-embedded (FFPE) lung tissue samples containing clinically actionable mutations were processed using instructions provided with each kit. The QIAseq panel was sequenced on a MiSeq (Illumina Inc.) and analyzed using a web-based pipeline for the QIAseq lung cancer panel. The GeneRead QIAseq panel was sequenced on a GeneReader (Qiagen Inc.) and analyzed with the Qiagen Clinical Insight (QCI) analysis and interpretation software already integrated with the workflow. **Results:** The QIAseq lung panel covers a larger region of interest than GeneRead QIAseq panel (72 vs. 19 genes). Both workflows require similar levels of hands on time (~7 hrs for QIAseq and ~8 hrs for GeneRead). The GeneRead panel provides a streamlined, easy-to-use workflow from sample extraction to data interpretation, with sequencing data automatically analyzed once sequencing is complete. The data analysis is directed towards clinically actionable mutations and allows interactive viewing and interpretation of the variants. In contrast, the QIAseq panel requires user intervention to retrieve sequencing files from the MiSeq and upload them to the web portal. Additionally, a sequencing result from the QIAseq workflow is provided as a VCF file that requires further analysis and interpretation. Both panels displayed >90% sensitivity and specificity for clinically relevant SNPs and insertion/deletions (indels) at 1% or higher VAF. **Conclusions:** Both panels exhibited accurate, reproducible, and a high level of performance in a typical clinical laboratory environment. This high level of performance was attributed to the incorporation of UMI's into each panel. The tools provided for analysis of both panels required no bioinformatics knowledge, and it was easily performed by the laboratory technicians. The clinical reporting of the variants is a key feature of the QCI analysis and interpretation software. The software was intuitive to use, and correctly reported all clinically useful variants.

#### TT048. Suitability of Formalin-2000 Decalcified Paraffin Embedded Tissue for Fluorescence *in situ* Hybridization (FISH) and Next-generation Sequencing (NGS)

D. Lieberman, V. Aikawa, A.R. Oran, J.J. Roth, J.N. Rosenbaum, R.T. Sussman  
University of Pennsylvania, Philadelphia, PA.

**Introduction:** Molecular testing of clinical biopsy specimens often changes therapeutic strategies in oncology. Decalcification of bone specimens improves accessibility of paraffin embedded tissue (PET) sections, but affects the quality of DNA for downstream molecular testing. We investigated the suitability of the decalcifying agent Formalin 2000's use in PET for fluorescence *in situ* hybridization (FISH) and Next-Generation Sequencing (NGS) testing. **Methods:** Formalin 2000 treated bone marrow specimens (Mantle Cell Lymphoma, T-cell Lymphoblastic Lymphoma, non-malignant, and peripheral T-cell Lymphoma) were prepared as both 30um slides for FISH and 10um rolls for NGS. Hybridization was analyzed using CCND1/IGH and BCR/ABL dual color

dual fusion probes as well as the MYC and RET break apart probes. DNA was extracted from the samples and made into genomic libraries using a custom amplicon-based oncology NGS panel. Pre-analytical DNA quality was assessed by calculating the percent of gDNA that was <1,000 bp long (% degraded DNA). **Results:** In FISH testing, all specimens demonstrated probe hybridization. Both dual color dual fusion probes had variable green signal intensities while both break-apart probes resulted in high quality signals. DNA was extracted from four samples with yields between 7ng/μL and 74ng/μL. All samples had >20% degraded DNA, which exceeds the quality cutoff for the NGS oncology panel. Nonetheless, libraries were generated and sequenced from all samples, but only one sample generated sufficient reads to pass sequencing quality control.

**Conclusions:** In our laboratory, tissues decalcified using Formical 2000 are suitable for FISH testing to detect gene rearrangements or copy number alterations, but may be unsuitable for NGS testing for small nucleotide variants, insertions and deletions. These data highlight that different molecular assays have different specimen requirements; in a molecular laboratory offering multiple assays on multiple platforms, changes to specimen procurement and handling must be evaluated for each assay independently. We model an assessment workflow whereby complementary assays are evaluated in parallel from the same specimens. We specifically demonstrate that although testing of Formical 2000-treated specimens may not be feasible by NGS, testing by FISH can offer a viable alternative for the detection of certain alterations.

#### TT049. Optimizing RNA Extraction to Facilitate BCR/ABL1 Quantitative Testing

J. Belman, C. Gentile, J. Lake, J. Roth, C. Watt

Hospital of the University of Pennsylvania, Philadelphia, PA.

**Introduction:** Complete molecular response (CMR) is a clinically significant milestone for patients with chronic myeloid leukemia. Detection of CMR requires a quantitative BCR/ABL1 p210 assay with analytical sensitivity greater than many conventional methods (i.e. detection at MR4.5 or greater). Achieving this sensitivity is dependent on the amount of RNA added to the reaction. During the implementation of a new method capable of CMR monitoring, precision and linear range studies were performed over a wide range of RNA input (250-4,000 ng), demonstrating excellent performance down to MR4.7 when RNA input was ≥1000 ng per reaction. Achieving this RNA input was a challenge because it exceeded the RNA requirements of our institution's antecedent test. A retrospective review of RNA extractions performed over a seven month period showed only 30% of samples yielded sufficient RNA concentration to include ≥1,000 ng RNA per reaction (i.e. 100 ng/μL). The goal of this project was to optimize our RNA extraction method to meet the more stringent RNA concentration needs of the new assay. **Methods:** The estimated theoretical RNA yield from blood for our current extraction method (QIAamp RNA Blood Mini Kit, Qiagen, Valencia, CA) was determined and compared to other common RNA extraction methods. Modifications to the RNA extraction protocol were identified and validated on a sample set (n=21). RNA concentrations were compared seven months before and after modifications were implemented. A subset of the post-modification extractions was also analyzed for additional trends (e.g. time to extraction, elution buffer temperature, etc.) to explore further potential improvements. **Results:** The theoretical RNA yields for the evaluated methods were similar. Further, the QIAamp RNA Blood Mini kit should be capable of providing adequate RNA concentrations for the majority of blood specimens. Minor modifications evaluated on the validation set resulted in 16/21 (76%) samples with RNA concentrations ≥ 100 ng/μL. Post implementation, extracted samples (n=475) had higher RNA concentrations and similar quality compared to the standard lab procedure, with 70% of samples having RNA concentrations ≥100 ng/μL. Further refinements to the extraction process were tested, but have not proven to be reliable or robust. **Conclusion:** Simple workflow changes improved the performance of our column based RNA extraction methodology and greatly increased the number of adequate samples for a highly sensitive quantitative p210 assay. Homogenization of the white cell pellet was determined to be a critical step. Preliminary data suggests further modifications may not greatly alter RNA concentrations; however, additional studies, on a larger sample set will need to be performed to confirm.

#### TT050. Single-cell Analysis of γδ T-cells Reveals Limited TCR Delta Chain Diversity in Mouse Lung Vγ4 γδ T-cells

K.J. Hampel<sup>1</sup>, S.K. Mistri<sup>2</sup>, N. Sidiropoulos<sup>1</sup>, J. Boyson<sup>2</sup>

<sup>1</sup>The University of Vermont Medical Center, Burlington, VT; <sup>2</sup> The University of Vermont Larner College of Medicine, Burlington, VT.

**Introduction:** Immunotherapy is an increasingly utilized therapeutic option for cancer care. γδ T-cells secrete an array of cytokines toxic to malignant cells the understanding of which may inform immunotherapeutic options. Single cell profiling of T-cells repertoires will enhance our understanding of immune responses and guide future research into this important topic. γδ T-cells are unusual innate-like T-cells that act as one of the first responders in our defense against lung pathogens through their rapid production of pro-inflammatory cytokines like IL-17 and IFN-γ. γδ T-cells are known to express TCRs with limited diversity that are often associated with both function and anatomical location. The objective of this study was to identify the major Vγ4 γδ TCR clonotypes in the mouse lung. **Methods:** Lung Vγ4 T-cells were single-cell sorted and their TCR variable regions amplified using nested RT-PCR. A pooled sequencing library was generated for 816 single cell-derived TCRs and sequenced using Illumina MiSeq instrumentation. Clonotypes were identified by bioinformatics analysis of the MiSeq data at the University of Vermont, Larner College of Medicine Bioinformatics Core. **Results:** Single-cell sorting, RNA extraction and T-cell receptor amplification resulted in 816 samples that were sequenced utilizing next generation sequencing methodology. Our sequencing approach generated a significant degree of variability in the number of sequencing reads. Nevertheless, out of these sorted lung C57BL/6 Vγ4 T-cells, we obtained paired TCR γ and δ chain sequences from 472 cells. These data revealed that Vδ5 and Vδ2 chains accounted for 49.80% and 33.07% of the productive TCR δ chain rearrangements, respectively. Of the Vδ5 chains, 56.80% were characterized by a germline-encoded invariant Vδ5Dδ2Jδ1 TCR sequence. **Conclusions:** Our data reveal that the C57BL/6 lung V4 T-cell population is characterized by extremely limited delta chain usage. Since lung Vγ4 T cells are programmed to produce either IFN-γ or IL-17, future studies will determine whether there is a link between lung Vγ4 TCR clonotypes and cytokine production. Single cell profiling of lung γδ T-cell repertoires will inform future work aimed at examining the use of these cells in immunotherapy.

#### TT051. The Use of Native, Amplified and Synthetic ctDNA to Assess Variant Calls from Targeted NGS Panels

M.G. Butler, Y. Konigshofer, J. Dickens, B. Anekella

SeraCare Life Sciences, Gaithersburg, MD.

**Introduction:** The detection of somatic mutations in circulating cell-free DNA (ccfDNA) from plasma samples using next-generation sequencing (NGS) panels is one of the most exciting developments in oncology diagnostics. However, significant obstacles remain before such assays can effectively transition to clinical care. The low relative abundance of circulating tumor DNA (ctDNA) in ccfDNA demands sensitivity and specificity to detect mutant allele frequencies (AF) below 0.1%. The analytical challenges of detecting such low AFs have likely led to poor concordance of results between laboratories. Such challenges include: 1) the number of mutant molecules in different aliquots of a sample will differ based on underlying Poisson distributions, 2) library preparation methodology (e.g., amplicon versus hybrid capture) may alter ctDNA representation, 3) sequencing platform-specific errors, 4) assay-specific errors introduced into libraries, and 5) error correction may mask low AF mutations. Therefore, we evaluated variant calls generated by targeted sequencing NGS panels using different highly multiplex reference ctDNA materials that contain single-nucleotide variants (SNVs), insertion/deletions (indels), structural variants and CNVs at various AFs. **Methods:** Seraseq ctDNA products containing different somatic mutation AFs in genomic DNA derived from the GM24385 cell line as well as native and amplified ccfDNA samples were analyzed with commercially-available kits and several variant callers. From the resulting variant calls, pairwise comparisons, overall distributions and base changes among samples were determined. **Results:** Pairwise comparisons of variant calls from replicate analyses demonstrated that about a third of the total variant calls were shared between duplicates while private variant calls accounted for the remainder. A comparison of the shared variant calls showed that



about 16% (2,240) were present in 6 or more of the duplicates. Of those common shared variants, nearly 90% of the base changes were A>G/T>C transitions. The presence of over 10% of these common variant calls in unrelated ccfDNA samples indicated that some of these variants were likely artifacts introduced by library construction or sequencing. At the same time, most of these apparent variants did not reach the threshold for reporting. **Conclusions:** Understanding common errors introduced either during library construction or next generation sequencing is necessary for interpreting whether variant calls are true positives. Our studies demonstrate that reproducible variants can be produced by library construction and/or sequencing. Consequently, orthogonal methods may be necessary to validate variants observed in ctDNA by targeted NGS panels.

#### TT052. Next-generation Sequencing Aids in the Identification of a Rare *CFTR* Deletion

N.J. Boczek, S. Roellinger, J. Mills, C. Koellner, L. Hasadsri, W. Highsmith, K. Rumilla  
Mayo Clinic, Rochester, MN.

**Introduction:** Mayo Medical Laboratories has a clinically available cystic fibrosis (CF) mutational analysis panel which includes the detection of 106 *CFTR* mutations. A recent sample failed to amplify a variant in exon 2 of *CFTR*. Several follow-up confirmatory methods were utilized to determine the cause of loss of amplification, as a deletion was suspected. Our newly developed custom next generation sequencing (NGS) assay was the ultimate platform that elucidated the exact breakpoints of the deletion. **Methods:** The CF mutational analysis panel is a multiplex PCR-based single base extension assay using the Agena Mass Array platform for variant detection. Standard follow-up confirmatory methods included Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA). In addition, a *CFTR* custom capture based Agilent SureSelect target enrichment followed by 101 basepair paired end reads on an Illumina HiSeq 2500 was utilized. Probes were designed to capture all coding and several noncoding regions where known pathogenic variants have been previously reported. The noncoding regions included probes tiled across intron 1 and intron 3 to help capture exon 2-3 deletions.

**Results:** After a clinical case for our CF mutational analysis panel failed to amplify both wildtype and mutant reactions for a variant located in exon 2, follow-up Sanger sequencing was initiated but unable to amplify any product for exon 2. MLPA showed complete loss of exon 2, while all other exons had a normal copy state. We were confident some portion of exon 2 was homozygously deleted; however, there was significant primer overlap between these three technologies and there are large introns between primers for surrounding exons. Therefore, the size of the deletion could have ranged from ~70bp to 10.7kb. Using our NGS assay, we were able to determine that the deletion was c.54-5811\_164+2186del8108ins182, a rare, previously reported Sardinian founder mutation. **Conclusions:** NGS was able to determine the breakpoints of an exon 2 deletion where previously established laboratory methods could not. This particular variant highlights some of the weaknesses of our other methodologies, as while we were able to visualize the deletion in this case because it was homozygous, a heterozygous exon 2 deletion would be missed by both our mutational analysis panel and Sanger sequencing. MLPA would detect a heterozygous exon 2 deletion, however the breakpoints would be unknown. Our new NGS methodology can identify this exon 2 founder deletion and the breakpoints allowing for a more confident call. Findings such as these are important reminders of some of the limitations of our different methodologies and highlight the utility of NGS platforms in certain situations.

#### TT053. Quality Control of the Oncomine Cancer Panel

D. Thach, M. Ahmad, Q. Phan, R. Iyer  
Inova, Fairfax, VA.

**Introduction:** the Oncomine Comprehensive Cancer Panel interrogates cancer genes via Ion Torrent sequencing of DNA and RNA from formalin-fixed, paraffin-embedded (FFPE) samples. The panel measures 143 genes for hotspot mutations, focal CNV gains, full CDS for DEL mutations, and gene-fusion drivers. Our lab has deployed the assay as a laboratory developed test that is also used for cancer research. Here, we present the results of quality control monitoring. **Methods:** Sections from FFPE blocks

of three cancer types were processed through the Oncomine standard operating procedures. Data were analyzed in Ion Torrent Suite and Ion Reporter. Audit-trail data that tracks all changes in the instrument server were exported for further analyses. Quality control analyses were performed. **Results:** A total of 81 samples were processed over a period of 6 months in 6 batches. The DNA and RNA yields ranged from 2 to 294 ng/ul and from 1 to 3160 ng/ul, respectively. For DNA, the number of mapped reads per sample range was  $5.2 \times 10^5$  to  $3.4 \times 10^7$ , of which 16% to 98% were on target. For RNA, the number of mapped reads per sample range was  $9.3 \times 10^4$  to  $1.6 \times 10^7$  of which 72% to 99% were on target. The lowest outliers of these upstream metrics were associated with less variant calls downstream. Analysis of audit trail data revealed 4 sample identifier entry mismatch, and 2 DNA and RNA sample pairing mismatch. **Conclusions:** These results quantify quality limits for outlier detection, identify error-prone steps for implementation of human double-checks or software improvements, and demonstrate the importance of quality control monitoring for process improvement.

#### TT054. Biocartis Idylla Cartridge-based Microsatellite Instability Assay Shows High Concordance with Immunohistochemical Analysis for Mismatch Repair Status in Colorectal Cancer

N.S. Maloney, D.C. Green, R. Al-Turkmani, X. Liu, G.J. Tsongalis, B. Ren  
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

**Introduction:** The mismatch repair (MMR) status of colorectal cancer (CRC) is essential for identifying patients with Lynch syndrome and is also a potential predictive factor for 5-fluorouracil-based chemotherapy or immune checkpoint inhibitors-based immunotherapy. Current clinical reference methods to detect MMR status include immunohistochemical assay (IHC) for expression of MMR proteins and molecular analysis of microsatellite instability (MSI) markers in the Bethesda panel. The purpose of this study is to investigate whether a novel Biocartis Idylla cartridge-based MSI Research Use Only assay could serve as an alternative method to determine MMR status by comparison with IHC in CRC. **Methods:** Forty-four CRC cases were selected from archived specimens processed at Dartmouth-Hitchcock Medical Center between 2011 and 2018, including 10 MMR-proficient carcinomas determined by IHC with positive/retained nuclear staining of MLH1, PMS2, MSH2, and MSH6 proteins and 34 MMR-deficient carcinomas determined by IHC with loss of one or more MMR proteins. Two 5 µm thick sections of tissue were collected from formalin-fixed paraffin-embedded tissue blocks for each tumor and placed in the Idylla cartridge. The MSI assay was then performed on the Idylla instrument (Biocartis, Belgium). Two cases with discrepant results between the Idylla MSI assay and IHC were further analyzed using the Promega MSI Analysis System v1.2 (Promega Corp.). **Results:** The overall percent agreement was 95% (42/44). The agreement was 100% in the IHC-determined MMR-proficient tumors (10/10) and 94% in the IHC-determined MMR-deficient tumors (32/34). All 10 MMR-proficient tumors by IHC were MSI stable by the Idylla MSI assay. 32 of the 34 MMR-deficient tumors by IHC showed high microsatellite instability by the Idylla MSI assay. Two MMR-deficient tumors by IHC were microsatellite stable by the Idylla MSI assay. These two tumors were further analyzed by Promega MSI Analysis System, which revealed microsatellite stable for both tumors and confirmed the Biocartis Idylla results. **Conclusions:** The novel Biocartis Idylla cartridge-based MSI assay showed high concordance with IHC in determining MMR status in CRC. The findings in our study support the use of the Biocartis Idylla cartridge-based MSI assay as an alternative method to determine MMR status in CRC.

#### TT055. Liquid Biopsy Quality Control: The Importance of Plasma Quality, Sample Preparation, and Library Input for Next-generation Sequencing Analysis

J.L. Whiting, H. Saunders, C.S. Hinahon, A. Cheng  
Thermo Fisher Scientific, Austin, TX.

**Introduction:** Liquid biopsy is emerging as a non-invasive companion to traditional solid tumor biopsies. As next-generation sequencing (NGS) of circulating cell-free nucleic acids (cfNA = cfDNA and cfRNA) becomes common, it's important to understand the impact of sample preparation on quality, specificity, and sensitivity of liquid biopsy tests. Plasma samples are often limited, and may have undesirable characteristics such as

lipemia or hemolysis that contribute unwanted genomic DNA (gDNA) to the sample. Low cfDNA concentration can also limit the amount available for NGS library prep. In this study, we explore the effects of suboptimal plasma and low library input on liquid biopsy NGS. **Methods:** K2-EDTA plasma was collected from four healthy donors, including one normal control, one lipemic, and two hemolyzed samples (100 mg/dL). All samples were spun to cell-free. Replicate cell-free nucleic acid isolations were performed with or without a reference standard cfDNA spike-in using the MagMAX Cell-free Total Nucleic Acid Isolation Kit. Yield and quality of isolated cfDNA were assessed via Qubit, 2100 Bioanalyzer, 4200 TapeStation, and qPCR, and gDNA contamination was visualized on the 2100 Bioanalyzer. NGS libraries were prepared at 5, 10 and 20 ng nucleic acid input and sequenced with the Ion Chef and Ion GeneStudio S5 System to compare NGS metrics including molecular coverage, allelic frequency, and false positives. **Results:** Replicate isolations had high reproducibility (Avg SD =  $\pm 0.2$  ng/ $\mu$ l) regardless of hemolysis or lipemia, and mean cfDNA fragment length was equivalent (cfDNA peak = 173-176bp for all). In the normal donor, the percentage of gDNA to cfDNA was 13%, which increased 6- to 9-fold in the lipemic and hemolyzed samples (lipemic = 125%, hemolyzed = 86%, 97%). Decreasing library input from 20ng increased short NGS byproducts, resulting in mean read lengths that were ~11% and ~26% shorter for matched libraries at 5ng and 10ng input, respectively. As expected, molecular coverage reduced for low input libraries, leading to less sensitive limits of detection. False positives were detected in hemolyzed plasma, suggesting poor plasma quality impacts NGS results. **Conclusions:** This study used analytical methods to assess the quality of liquid biopsy NGS samples from initial sample preparation through NGS analysis. The results demonstrate that reduced sample input into NGS libraries increases small byproducts and reduces molecular coverage. In addition, lipemic and hemolyzed K2-EDTA plasma had higher gDNA contamination, reducing the percentage of cfDNA in the NGS library prep. Together, this data indicates plasma quality, nucleic acid quantity, and the size profile of isolated nucleic acids are important quality control metrics for liquid biopsy NGS.

#### TT056. Detection of EGFR Exon 19 Deletions in Liquid Biopsy Samples Using Targeted Next-generation Sequencing and Droplet Digital PCR

L. Jackson, H. Mellert, G. Pestano  
Biodesix, Inc., Boulder, CO.

**Introduction:** Blood-based methods for profiling of tumor-sourced nucleic acids have become increasingly important in the clinical workup for cancer patients, particularly in non-small cell lung cancer (NSCLC). Blood-based assays address several limitations of tissue-based testing approaches such as extended time to results, inability to result poor quality specimens, and invasive specimen collection procedures. Targeted droplet digital PCR (ddPCR) and broad-profiling next-generation sequencing (NGS) methods have been used to detect rare variants in blood samples. However, accurate detection by NGS can be more challenging in blood due to the read depths required to make accurate variant calls. To address this issue, targeted NGS panels are being developed to increase depth of coverage in regions of interest without requiring additional sample input or reduction in throughput. This is particularly beneficial in the context of liquid biopsy where sample quantity is often limited. This study focused on the development of a custom targeted NGS test for detection of actionable mutations in liquid biopsies. **Methods:** An NGS panel was designed to target 8 regions of clinical relevance in NSCLC using the TruSeq Custom Amplicon chemistry. Thirty-two cell-free DNA samples were evaluated using the NGS test, representing a range of input quantities from approximately 20-40ng. These samples were assessed in parallel using a validated, non-discriminating multiplexed ddPCR method which tests for at least 15 EGFR Exon 19 deletion (del19) variants in a single reaction well. **Results:** Of the 32 EGFR del19 variants detected by ddPCR, 27 variants were detected by NGS at minor allele frequencies as low as 2.3%. Forty-four percent of the positive samples were positive for the most prevalent del19 variant, p.E746\_A750delELREA (COSM6223), and six additional variants were detected at varying frequencies in the test population. **Conclusion:** This study adds to the growing body of knowledge supporting the usefulness of molecular methods for somatic variant mutation detection in liquid biopsy. While ddPCR remained the

more sensitive of the two assays in this study, the targeted NGS assay successfully identified 84% of the EGFR exon 19 deletion variants detected by ddPCR and provided variant specific information.

#### TT057. Enzymatic Methyl-seq: A Novel Approach to Determine the Methylome at Single-base Resolution

L. Williams, V. Ponnaluri, B. Sexton, L. Saleh, K. Marks, M. Samaranayake, L. Ettwiller, S. Guan, H. Church, N. Dai, E. Tamanaha, E. Yigit, Z. Sun, B. Langhorst, T.C. Evans, R. Vaisvila, E. Dimalanta, T.B. Davis

New England Biolabs, Ipswich, MA.

**Introduction:** Discriminating between cytosine (C) and its methylated forms, 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) is important in understanding gene regulation. Bisulfite sequencing (BS) is the gold standard for detecting methylated C's however, DNA is commonly damaged and degraded by the chemical bisulfite reaction. Whole genome bisulfite sequencing (WGBS) demonstrates high GC-bias and enriched methylated regions. Here we describe NEBNext Enzymatic Methyl-seq (EM-seq), an enzymatic approach to detect methylated C's. EM-seq overcomes many of the disadvantages associated with BS as the DNA remains intact, enabling longer sequencing reads and reduced GC bias which in turn results in more even genome coverage. **Methods:** EM-seq and WGBS libraries were prepared using 10 ng, 50 ng or 200 ng NA12878 genomic DNA, spiked with control DNA's that were fragmented to 200-300 bp. DNA was end repaired and corresponding adapters were ligated. For EM-seq, oxidation of 5mC's and 5hmC's and the deamination of C's was followed by PCR. This generated Illumina sequencable libraries. WGBS libraries were made using Zymo EZ DNA methylation Gold kit. All libraries were sequenced using Illumina's NovaSeq 6000 and data were analyzed using a standard Bismark workflow. GC bias and insert size distribution were determined using Picard 2.17.2. MethylKit was used for correlation analysis. **Results:** EM-seq libraries have longer inserts, lower duplication rates, a higher percentage of mapped reads and less GC bias compared to WGBS libraries. Global methylation levels are similar between EM-seq and WGBS libraries while CpG correlation plots demonstrate that EM-seq libraries are superior. EM-seq libraries identify ~2 million more CpG's than WGBS libraries and have a higher percentage of CpG's covered for all minimum coverage depths examined (up to 20X). Methylation profiles across genomic features (TSS, CpG island, etc.) were similar, however, normalized coverage was much higher for EM-seq compared to WGBS. Dinucleotide analysis showed EM-seq did not suffer from depletion of C containing dinucleotides nor enrichment of A/T containing dinucleotides as seen in WGBS. **Conclusion:** EM-seq is superior compared to WGBS for detecting 5mC's and 5hmC's. It is robust and works over a wide range of inputs with better sequencing metrics. More CpG's were detected over a wide range of genomic features using the EM-seq method. CpG correlation plots also highlighted the robustness of EM-seq libraries. This method will be useful for clinical samples as it does not degrade DNA and provides accurate methylome information even at lower sequencing levels and lower DNA inputs.

#### TT058. Assessment of the Oncomine Cell-free DNA Lung Assay to Detect Low-frequency Mutations Using Reference Material

L. Cong, J. Baum, P. Ruggiero, J. Siple, Q. Pan, H. Rennert  
Weill Cornell Medicine and New York-Presbyterian Hospital, New York, NY.

**Introduction:** Plasma cell-free tumor DNA (cfDNA) from liquid biopsy is a potential source of tumor genetic material, which in the absence of tissue biopsy, can assist in early-stage diagnosis of lung cancer and monitoring treatment response. However, circulating levels of cfDNA in blood vary significantly among patients and are generally low in early-stage disease requiring high-sensitivity assays. In this study we evaluated the impact of DNA input using standardized reference material on the performance of the Oncomine Lung cfDNA Assay to detect low-frequency somatic mutations. **Methods:** To evaluate the quality metrics, sensitivity and specificity of variant detection, we used the Seraseq ctDNA reference DNA containing 0.1%, 1% or 2% variant allele frequency (VAF) of well characterized variants (n=11) in six principal genes (EGFR, KRAS, BRAF, TP53, PIK3CA and ERBB2) covered by the assay. cfDNA was evaluated at 1ng, 5ng, 10ng and 20ng of input DNA in triplicate for each VAF (n=36).

Libraries preps were templated with Ion Chef and sequenced with the Ion S5 Sequencing instrument (Thermo Fisher Scientific). Data analysis was performed using the variantCaller plugin and the Torrent Suite Software v5.4. **Results:** Mean depth of coverage across all targets on the panel ranged from 22,806x to 89,886x for input DNA of 1ng to 20ng, respectively. The median read coverage ranged from 18,909x to 72,577x and the median molecular coverage ranged from 197x to 2,691x for 1ng and 20ng, respectively. The precision and average alternate allele molecular coverage varied considerably by DNA input (5 reads versus 31 reads for DNA input  $\leq 5$ ng and  $\geq 10$ ng, respectively), regardless of the allelic frequency (AF) of the variants analyzed. Observed mean AFs and coefficient of variations (CVs) were 0.6% (154%), 1.1% (63%) and 2.1% (38%) for the 0.1%, 1% and 2% VAF levels tested. We achieved >99% sensitivity with 20ng and >95% sensitivity with 10ng input DNA for the panel mutations at frequency 0.1% in the sample, but no variants at 0.1% AF were detected at DNA input  $\leq 5$ ng. False calls of variants in hotspot positions were identified in numerous samples and were typically associated with low coverage ( $< 4$  reads) and low AF ( $< 0.2\%$ ) regardless of DNA input. **Conclusions:** This study demonstrates that targeted ultra-deep sequencing of cfDNA reference standard using the OncoPrint Lung cfDNA assay is greatly impacted by DNA input. With higher DNA input ( $\geq 10$ ng) we observed better quality metrics and sensitivity at all VAF levels. The higher the panel coverage depth, the lower the specificity, thus pushing the limits of sensitivity  $< 1\%$  VAF warrants thorough validation. Overall, this assay is reliable and reproducible, enabling sensitive detection of specific mutations in plasma cfDNA.

#### TT059. Cell-free DNA Allograft Rejection Monitoring Using Low-coverage Whole Genome Sequencing

N. Krumm, M. Beightol, E.Q. Konnick, C.M. Lockwood, S. Salipante, K. Smith, C. Pritchard  
University of Washington, Seattle, WA.

**Introduction:** Quantitation of donor-derived cell-free DNA (dd-cfDNA) has been shown to be a sensitive, non-invasive marker of acute allograft rejection for heart and kidney transplants. Existing methods are based on high-density SNP microarrays, high coverage whole-genome sequencing (WGS), and/or targeted amplification and sequencing of polymorphic SNPs to determine the fraction of dd-cfDNA present. In a clinical setting, these methods may be costly and difficult to implement, prompting us to investigate if existing low-coverage WGS used for cell-free DNA prenatal aneuploidy screening could be utilized for dd-cfDNA quantification.

**Methods:** Six unrelated HapMap samples were sequenced using our clinically validated cell-free DNA prenatal aneuploidy WGS assay (15-48 million 36bp paired-end reads per sample). Using sequencing data, 111 *in-silico* mixtures were generated to simulate dd-cfDNA content from 0.1% to 20%. Three different dd-cfDNA quantification strategies were tested: Strategy 1, using low-density genotype information of donor and recipient (based on the CytoSNP-850K BeadChip), in which reads corresponding to donor DNA were counted and used to estimate dd-cfDNA fraction; Strategy 2, an extension of (1) using imputed genotypes from low-density data; and Strategy 3, using reads aligning to copy-number polymorphisms (CNPs). **Results:** Excellent concordance was present between expected and observed dd-cfDNA fractions ( $R^2=0.997$ ) in the *in silico* mixtures from our baseline low-density genotype and low pass sequencing strategy (strategy 1). However, strategy 1 was associated with an increased coefficient of variation (CV) as dd-cfDNA content decreased (CV of 56% at 0.1% fraction). Expanding analysis to all imputed SNPs with strategy 2 improved the CV to 17.2% at the lowest dd-cfDNA fraction of 0.1%. The CNP-based quantification of strategy 3 was accurate for donor fractions above ~1%, but lacked sufficient power to resolve dd-cfDNA mixtures  $< 1\%$ . **Conclusions:** We demonstrate that low coverage sequencing paired with modest genotyping density (strategy 1) is suitable for quantification of dd-cfDNA fractions as low as 0.01%. Imputation of additional SNPs increases precision and decreases assay CV (strategy 2), although it introduces a new imputation bias. Closely related or poorly represented populations in reference databases may be more challenging for this imputation-based approach. Finally, using CNP-aligned reads (strategy 3) is only accurate at dd-cfDNA fractions  $> 1\%$ . Increasing the number of CNPs may increase the power of this approach. Taken together, low-coverage sequencing and genotyping has the potential to

quantify dd-cfDNA at clinically relevant fractions for allograft rejection monitoring in a cost-effective and workflow-friendly manner.

#### TT060. Stat EGFR Mutation Detection in Fresh Lung Cancer Tissue Specimens Using Touch Preparation and the Idylla System

M. Al-Turkmani, E.J. Rizzo, S.N. Schutz, G.J. Tsongalis  
Dartmouth-Hitchcock Medical Center and Geisel School of Medicine, Lebanon, NH.

**Introduction:** Accurate and timely genomic assessment of tumor tissues has become imperative for optimal therapeutic strategies for cancer patients. The Idylla system (Biocartis, Mechelen, Belgium) is a fully integrated, cartridge-based platform that provides automated sample processing (deparaffinization, tissue digestion and DNA extraction) and real-time PCR-based mutation detection with all reagents included in a single-use cartridge. Here we describe an approach for rapid somatic mutation detection in lung cancer cases using touch preparation and the Idylla system. **Methods:** Touch preparation samples were obtained from fifteen lung cancer tissue specimens in the pathology gross room shortly after resection. This involved making a single incision into the tumor body at room temperature using a scalpel blade and touching one 10 mm filter paper on each of the two sides of the inner tumor surface and holding it in position for approximately 3 seconds. The two filter papers were placed in an Idylla EGFR Mutation Assay cartridge (Research Use Only) and the cartridge was subsequently placed in the Idylla instrument for automated EGFR mutation analysis. The tumor tissue specimen was subsequently processed using standard pathology protocols for fixation, embedding and sectioning. Idylla results were compared against those obtained by subsequent somatic mutation analysis by next-generation sequencing (NGS) using the Ion AmpliSeq 50-gene Cancer Hotspot Panel v2 (Thermo Fisher Scientific). **Results:** Idylla testing gave valid results for all 15 samples tested and revealed an EGFR L858R mutation in one sample and a 15-base pair exon 19 deletion in another sample. The remaining 13 samples showed no mutations in EGFR. Subsequent NGS analysis of eight of the 15 cases that met the institutional criteria for NGS testing showed complete concordance with Idylla results and confirmed the mutated or wild type status of EGFR as determined by the Idylla, with no other clinically actionable mutations detected. The total time for performing touch preparation and subsequent analysis by the Idylla was less than 3 hours for all samples, compared to an average turnaround time of 20.75 days (range: 14 - 30 days) for NGS testing.

**Conclusions:** Combining touch preparation with the simplicity and sensitivity of the Idylla instrument provides a simple means to rapidly detect actionable somatic mutations in lung cancer tissue specimens after resection while preserving tumor tissue for subsequent processing and analysis. This approach provides fast turnaround times that allow timely management decisions for time-sensitive cancer cases while awaiting more comprehensive tumor genome profiling.

#### TT061. Simultaneous Detection of Single Nucleotide Variants and Copy Number Variations in Expanded Carrier Screen Using Next-generation Sequencing

P. Hetterich, M. Ta, T. Lewis, D. Taylor, A. Patrick, M. Saniepay, A. Barry, J. Quan, A. Jeffers-Brown, B. Sutton, G. Smith, W. Xu  
True Health Diagnostics, Richmond, VA.

**Introduction:** Expanded carrier screening (ECS) is a testing approach that involves screening for many genetic conditions without regard to ethnicity. With recent advances in next-generation sequencing (NGS) technology and bioinformatics techniques, ECS has become a feasible and cost-effective approach for evaluating the carrier status of couples considering pregnancy. We established a custom ECS panel of 220 genes that is capable of detecting not only single nucleotide variants (SNVs) and small insertions/deletions (indels), but also copy number variations (CNVs) using NGS coupled with an advanced bioinformatics pipeline and tools developed in-house. **Methods:** DNA was isolated from whole blood and saliva samples and then enriched for targeted regions using PCR with specific primer sets to create DNA libraries. A custom QIAGEN QIAseq Targeted DNA Panel was used for the library preparation and Hamilton Microlab STAR instruments were used to automate the process. Prepared libraries were then loaded on an Illumina NextSeq 500 and sequenced at 2 x 150 bp reads. Data generated by the sequencer was processed using



a custom bioinformatics pipeline developed at True Health for molecular tagged sequencing reads. SNVs and small indels were called using FreeBayes and CNV analysis was performed using BioDiscovery Nexus Copy Number software. **Results:** Our ECS panel of 220 genes was designed based on ACMG and ACOG guidelines. The panel covers the exonic regions and common hotspots associated with genetic conditions in the sequenced genes. Characterized specimens (n=82) were interrogated for known variants reported in literature or identified through sequencing assays performed by CLIA-certified reference laboratories. Minimum mean sequencing coverage for the panel is 400X and base positions were evaluated for sequencing variants if they achieved the required 50X minimum coverage for variant calling. High coverage uniformity was observed across the targeted bases. Accuracy was found to be 99.99% for SNVs and small indels and 100% for CNVs. Analytical specificity was determined by sequencing the NIST GIAB reference standards NA12878 and NA24385 and specimens with positive CNVs previously confirmed by an independent reference laboratory. Specificity was found to be 100% for SNVs and small indels and 99.65% for CNVs. Precision was found to be 100% for SNVs, small indels, and CNVs. The sensitivity/limit of detection study showed variant detectability down to 10% allele frequency. **Conclusions:** Based on the results of this study, the custom ECS panel was validated for patient testing in our CLIA-certified laboratory. This ECS panel has the advantage of being able to detect SNVs, indels, and CNVs with a single NGS assay.

**TT062. The SureMASTR Tumor Hotspot Plus Assay Combined with MASTR Reporter Analysis Provides an Accurate and Precise Workflow for SNV and Indel Detection in FFPE Derived DNA**

A. Rotthier, J. Vermeire, R. De Smet, N. Remmerie, O. Herd, L. Standaert, J. Crappé, J. Del Favero  
Agilent Technologies, Niel, Belgium.

**Introduction:** Identification of cancer-associated mutations has become standard of care for cancer treatment. The determination of oncogenic mutations is important to decide the treatment options for patients with cancer. The Tumor hotspot (THS) assay identifies single-nucleotide variants (SNVs) and insertion/deletions (indels) in frequently mutated cancer-associated genes, focusing on 26 relevant genes and amplifying only the hotspot target regions (full exon coverage for PTEN and STK11 genes). The assay is optimized to detect mutations from formalin-fixed, paraffin-embedded (FFPE) tumor samples but is also compatible with FFT or blood derived DNA. **Methods:** The THS assay is comprised of 4 plexes and contains 252 amplicons with a size ranging from 88 to 205 bp. The analytical performance of the assay was evaluated by an external laboratory on 37 FFPE derived samples, previously characterized by Sanger and High-Resolution Melting analysis, and 6 reference samples. The 6 reference samples included the FFPE derived Quantitative Multiplex Reference standard and KRAS Gene Specific reference standard (Horizon) and Acrometrix' Multmix H samples. Illumina's MiSeq (v3 chemistry), MiniSeq and NextSeq were evaluated as sequencing platforms. Data analysis of these samples, including data quality control and variant calling of SNPs and indels, was performed using MASTR Reporter. **Results:** The THS assay reached high sequencing specifications, as 1) 99% of all amplicons mapped to the target region and 2) 96% of the amplicons reach a coverage of 20% of the mean coverage, indicating a high uniformity of amplification. Both specifications are crucial for optimal routine use of the sequencing capacity. While the 37 FFPE derived samples mainly comprised mutations in *NRAS*, *KRAS* and *BRAF*, the reference samples carried mutations over 14 different genes with variant allele frequencies (VAF) as low as 1%. As the limit of detection is determined at 5% for all platforms, only variants with a VAF of 5% or higher were considered for determination of the sensitivity and specificity. Considering all annotated variants in the clinical samples and three commercial reference samples, an observed sensitivity of 99% was obtained for Illumina MiSeq (v3 chemistry) and MiniSeq and 100% for Illumina NextSeq. Specificity was calculated based on the three Genome in a Bottle reference samples and reached 100% for both MiSeq and MiniSeq and 99.99% for NextSeq. **Conclusions:** We have established a targeted and cost-effective next generation sequencing (NGS) workflow for the accurate detection of single-nucleotide variants (SNVs) and indels

in FFPE, FFT or blood derived DNA samples for a set of frequently mutated cancer-associated genes that can be implemented routinely.

**TT063. Analyzing Copy Number Variation Inheritance with dNTP Limited PCR and High-resolution Melting Analysis**

L. Zhou<sup>1</sup>, R. Toydemir<sup>2</sup>, C. Wittwer<sup>1</sup>

<sup>1</sup>University of Utah, Salt Lake City, UT; <sup>2</sup>ARUP Laboratories, Salt Lake City, UT.

**Introduction:** A variety of genetic disorders are associated with DNA copy number variation (CNV). Most of them are the result of loss or gain of large segments of DNA. We seek to reliably and rapidly confirm CNVs using limited dNTPs during PCR. Using this technique, we are able to preserve initial target to reference copy number ratios otherwise lost during primer limited plateaus in standard PCR. We are able to visualize CNV by limited dNTP and high-resolution melting analysis (HRMA).

**Methods:** CNV detection results on 50 people by microarray and fluorescence *in situ* hybridization (FISH) were confirmed by limited dNTP PCR and HRMA. Duplex PCR consisting of a reference and a target was carried out with limited dNTP concentration of only 6.25 uM. Target PCR amplicons were designed within the CNVs located by microarray. Both reference and target amplicons were short (54 to 96 bp) with unique genomic sequences, no identifiable SNPs and single melting domains. Software uMelt was used to predict the T<sub>m</sub> of amplicon and the domain of the melting peak. The T<sub>m</sub> between reference and target was around 3 to 10°C. UCSC human genome database was used to check for amplicon uniqueness and verify SNP status in the target sequence. Software MeltingWizard 6.2 was used to analyze duplex amplicon melting peaks and the ratio of reference to target. **Results:** Limited dNTP-PCR and HRMA on 50 people in 18 families confirmed 100% of CNVs detected by microarray and FISH in a blind test. One family had an affected proband with 7 people in 3 generations. The proband and one sibling had a deletion on chromosome 9 *KANK1* gene. The deletion was inherited from mother. The maternal grandfather was normal and the maternal grandmother's DNA sample was unavailable, thus the mother's deletion status was unknown. Additionally, 17 affected proband CNVs initially detected by microarray and FISH were confirmed using limited dNTPs and HRMA. In these 17 families, 11 of the CNV were inherited from parent (4 deletion, 7 duplication), 5 of the CNV were sporadic (5 deletion). One family's abnormal fetal had a duplication. The mother was normal but father's DNA sample was not available. This fetal's inheritance was unknown. The CNV detection by limited dNTP and HRMA was 100% correlated with microarray and FISH. **Conclusion:** Once the CNV located by microarray of proband or fetal in genome, using dNTP limited PCR and HRMA to test inheritance from parents is rapid and inexpensive. The entire process including primer design, overnight delivery of primers after synthesis, PCR, and analysis was routinely performed in less than 24 hours.

**TT064. Developing a Customizable Panel of Real-time qPCR Assays on a Microfluidic Device for Respiratory Tract Pathogen Detection**

K. Li<sup>1</sup>, J. Li<sup>1</sup>, N. Fantin<sup>1</sup>, S. Patel<sup>1</sup>, B. Moy<sup>1</sup>, T. Gulham<sup>1</sup>, B. Hunag<sup>1</sup>, H. Ji<sup>1</sup>, I. Pagan<sup>1</sup>, E. Zeringer<sup>2</sup>, T. Hartshorne<sup>1</sup>, L. Nguyen<sup>1</sup>, N. Puri<sup>1</sup>, K. Varma<sup>1</sup>

<sup>1</sup>Thermo Fisher Scientific, San Francisco, CA; <sup>2</sup>Thermo Fisher Scientific, Austin, TX

**Introduction:** Respiratory tract infection is the most common infection in the world, presenting serious health issues to individuals and communities. In the US alone, uncomplicated respiratory infections account for over 25 million physician visits each year. A wide variety of bacteria and viruses are capable of causing upper and lower respiratory tract infections. Panel based testing using molecular methods to identify these pathogens has clear advantages over non-molecular or single target molecular testing. However, many current methods are limited to either fixed panels or low target throughput. To address these unmet needs, we have developed a large collection of TaqMan assays for respiratory tract pathogens and leveraged TaqMan Array Card (TAC) technologies. The combination presents a robust application for respiratory pathogen detection and allows customization of both the size and content of the test panel. **Methods:** A novel set of TaqMan qPCR assays was developed to over 40 distinct respiratory pathogen targets, including bacteria, DNA

viruses, RNA viruses, and fungi. We identified species-specific gene targets and designed TaqMan assays using the proprietary assay design pipeline. These FAM dye-labeled assays were pre-loaded and lyophilized onto wells on TaqMan Array Card (TAC), a microfluidic device that enables 8 samples tested for up to 48 targets simultaneously. These assays were evaluated with synthetic DNA or RNA templates and ATCC genomic DNA or RNA controls for sensitivity and specificity on TAC platform. PCR efficiency for each assay was evaluated and calculated based on their slopes from serial dilution studies. **Results:** We report test data for sensitivity, specificity, accuracy and reproducibility of these respiratory tract pathogen assays on the microfluidic TAC. Analytical sensitivity and linearity was demonstrated with at least 5 log linear dynamic range (with  $R^2 > 0.99$ ), limit of detection (LOD) down to 1-10 copies/ $\mu$ l on TaqMan Array Cards, and high PCR efficiency. For specificity, each assay was tested against all the rest of plasmids and ATCC gDNA/gRNA controls and no significant cross-reactivity was observed. A novel protocol was developed to increase assay sensitivity without sacrificing its specificity. **Conclusions:** We have developed a new application of a customizable qPCR assay panel for respiratory tract pathogens, in which any subsets of the targets can be built on the microfluidic TAC. We also have demonstrated excellent analytical performance of these assays on TAC. The application enables researchers to study a large number of respiratory tract pathogens in a single reaction with a simple workflow, fast turnaround time, and high throughput yet flexible sample/target combinations.

#### TT065. The Genomic Oncology Academic Laboratory (GOAL)

##### Consortium: A Collaborative Approach to NGS Development

D.L. Aisner<sup>1</sup>, S. Kadri<sup>2</sup>, J.T. Auman<sup>3</sup>, M.D. Ewalt<sup>1</sup>, C.D. Gocke<sup>4</sup>, K.J. Hampel<sup>5</sup>, D. Jones<sup>6</sup>, F.C. Kuo<sup>7</sup>, N.I. Lindeman<sup>8</sup>, C.M. Lockwood<sup>9</sup>, J. Longtine<sup>10</sup>, A.C. Mackinnon<sup>11</sup>, M.M. Mansukhani<sup>12</sup>, J.D. Morrisette<sup>13</sup>, L.L. Ritterhouse<sup>2</sup>, L.J. Tafe<sup>14</sup>, J.A. Thorson<sup>15</sup>, J. Van Ziffle<sup>16</sup>, E. K. Wakeland<sup>17</sup>, R. Xian<sup>4</sup>, J.P. Segal<sup>2</sup>

<sup>1</sup>University of Colorado Denver, Denver, CO; <sup>2</sup>University of Chicago, Chicago, IL; <sup>3</sup>University of North Carolina, Chapel Hill, NC; <sup>4</sup>Johns Hopkins University, Baltimore, MD; <sup>5</sup>University of Vermont, Burlington, VT; <sup>6</sup>The Ohio State University, Columbus, OH; <sup>7</sup>University of California, Los Angeles, CA; <sup>8</sup>Brigham and Women's Hospital, Boston, MA; <sup>9</sup>University of Washington, Seattle, WA; <sup>10</sup>Yale University, New Haven, CT; <sup>11</sup>Medical College of Wisconsin, Milwaukee, WI; <sup>12</sup>Columbia University, New York, NY; <sup>13</sup>University of Pennsylvania, Philadelphia, PA; <sup>14</sup>Dartmouth Hitchcock Medical Center, Hanover, NH; <sup>15</sup>University of California, San Diego, CA; <sup>16</sup>University of California, San Francisco, CA; <sup>17</sup>UT Southwestern, Dallas, TX.

**Introduction:** Next generation sequencing (NGS) based assays for assessment of somatic alterations are becoming standard-of-care for determination of prognosis and selection of appropriate therapy for patients with a variety of malignancies. As these assays grow in complexity, laboratories are facing increasing wet-lab and bioinformatics development burdens. This is exacerbated by highly divergent assay chemistry and design across academic medical centers (AMCs), which hinder collaborations geared towards minimizing costs and redundant effort. To address these issues, we assembled a group of 17 AMC laboratories across the U.S. to design and purchase a large-scale modular capture reagent set, in order to reduce assay costs and lay the groundwork for future collaborative efforts. **Methods:** The AMC laboratory group was assembled with an initial focus on designing and ordering a large-scale capture bait set (xGen Lockdown Probes, IDT) to provide substantial capture assay cost reduction. The gene list was developed through a nomination process, resulting in a list of 2,640 genes, 1,275 of which were shared by at least 2 institutions, and 715 of which were shared by at least 4 institutions. Other nominated features included tumorigenic viruses, SNPs for copy number and identity testing, and short tandem repeats for microsatellite instability (MSI) analysis. A one-gene-per-tube pooling approach was selected, allowing for highly flexible assay customization at each site. **Results:** Probe tiling and coverage strategies were determined based on pilot studies, using previously run clinical sample libraries to assess depth uniformity and variant detection concordance, including large insertion/deletions (indels) and structural variants. Final design specifications included primarily 1X tiling with a two

probe per exon minimum and 2X tiling of fusion intron territory. All sites have now received the full set of probes, and preliminary studies to date indicate excellent performance, including within regions of very high G-C content which are problematic to analyze via most assay types including the majority of commercial custom capture platforms. **Conclusions:** AMC laboratories are likely to benefit from collaborative approaches to NGS assay design, implementation and bioinformatics analysis. We anticipate that the GOAL consortium will be able to leverage group expertise to contribute to standardization of assays and facilitate continued demonstration of high-level inter-laboratory concordance. Further efforts are underway for standardization of core gene content and protocols, as well as exploration of shared bioinformatics resources to jointly address genome-wide metrics such as tumor mutation burden (TMB) and MSI calculations, and data on these efforts will be presented.

#### TT066. Effect of Plasma Separation Time after Blood Collection on the Concentration and Integrity of Circulating Cell-free DNA

M. Al-Turkmani, T.L. Gallagher, M.A. Cervinski, G.J. Tsongalis, J.A. Lefferts

Dartmouth-Hitchcock Medical Center and Geisel School of Medicine, Lebanon, NH.

**Introduction:** Liquid Biopsy is emerging as a powerful means for assessing molecular alterations in patients with solid tumors using tumor-derived cell free DNA (cfDNA). Pre-analytical factors can significantly affect the yield and quality of cfDNA. One of these factors is the variable contribution of higher molecular weight genomic DNA (gDNA) resulting from lysis of peripheral blood cells during blood storage and processing. In this study, we assessed the effect of plasma separation time on cfDNA yield and integrity using blood samples from healthy donors and droplet digital PCR (ddPCR). **Methods:** Six blood samples were obtained from each of 6 healthy volunteers, 3 males and 3 females, in 10 mL K2-EDTA tubes (Becton Dickinson, NJ). Blood was stored at room temperature and plasma was separated at baseline (< 30 minutes), 2, 4, 8, 12 and 24 hours after collection by double centrifugation at 1600 g for 10 minutes at 4°C. Cell-free DNA was extracted from a 3-mL plasma aliquot using the QIAamp Circulating Nucleic Acid Kit (Qiagen, CA) and kept at -20°C until further analysis. Quantification and size distribution of DNA was determined by ddPCR (Bio-Rad Laboratories, CA) analysis using two primer sets targeting a specific gene locus to amplify 41-bp and 305-bp fragments that correspond to total DNA and gDNA, respectively. Cell-free DNA concentration was determined by the difference between total and gDNA concentrations and cfDNA fraction was determined as percent of total DNA. **Results:** Average plasma cfDNA concentrations were 9.58 ng/mL (range 3.1- 24.2) at baseline, 9.54 ng/mL (range 2.78 - 25.72) at 2 hours, 8.19 ng/mL (range 3.51- 16.31) at 4 hours, 8.25 ng/mL (range 4.24 -16.69) at 8 hours, 8.66 ng/mL (range 3.21-15.81) at 12 hours, and 7.45 ng/mL (range 4.33 -15.66) at 24 hours. Average gDNA increased from 1.26 ng/mL at baseline to 1.87 ng/mL at 24 hours and that was accompanied by a decrease in average cfDNA fraction of total compared to baseline by 2%, 1%, 2%, 3% and 6% at 2, 4, 8, 12 and 24 hours, respectively. The differences in cfDNA concentration, gDNA concentration, and cfDNA fraction between baseline and various time points up to 24 hours until centrifugation were not significant as determined by the Student's *t*-test. **Conclusions:** Gradual decrease in cfDNA fraction due to contamination by gDNA was observed with increased time to centrifugation after blood collection, with greatest decrease observed at 24 hours. Such effects must be taken into consideration during downstream cfDNA analysis as this contamination by gDNA can potentially result in false negative results for somatic mutations.

**TT067. Novel Spatial Multiplex Screening of Uropathogens Associated with Urinary Tract Microbiota Research Using the Nanofluidic qPCR Platform**

S. Patel<sup>1</sup>, K. Li<sup>2</sup>, J. Li<sup>2</sup>, B. Moy<sup>2</sup>, B. Huang<sup>2</sup>, I. Pagani<sup>2</sup>, E. Zeringer<sup>2</sup>, N. Fantin<sup>2</sup>, N. Puri<sup>2</sup>, E. Diamond<sup>2</sup>, L. Nguyen<sup>2</sup>, K. Varma<sup>2</sup>

<sup>1</sup>Thermo Fisher Scientific, Austin, TX; <sup>2</sup>Thermo Fisher Scientific, South San Francisco, CA.

**Introduction:** Accurate identification of uropathogens in a timely manner is important to correctly understand urinary tract infections (UTI's), which affects nearly 150 million people each year. The current standard approach for detecting the UTI pathogens is culture based. This method is time consuming, has low throughput, and can lack sensitivity and/or specificity. In addition, not all the uropathogens grow equally well in a standard culture condition which may increase failure to detect the species. To address these gaps, we have developed a unique workflow from sample preparation to target identification using the nanofluidic OpenArray platform for spatial multiplexing of target specific assays. In this study, we tested pre-determined blinded research samples and compared the results with the culture results. **Methods:** The in-house solution allows for the detection of 17 uropathogens including 16 bacterial and 1 fungal target. All assays have been verified with different sample types such as synthetic plasmid control and ATCC gDNA samples for sensitivity and specificity testing. More than 120 pre-determined blinded samples from relevant sources were processed using MagMAX DNA Multi-Sample Ultra Kit and screened on nanofluidic qPCR Platform using target specific TaqMan pathogen detection assays. **Results:** We observed more than 87% concordance in the results when compared to the culture data. All the positive samples identified by culture method are confirmed positive by our assays and nanofluidic platform. However, we have seen some discordance where we have positive results in the negative culture samples. This discordance data was further investigated by orthogonal testing using capillary electrophoresis DNA sequencing. The discordance of the results is due to lack of testing particular pathogens in culture based method. The research samples were tested at two different sites, and the results were highly reproducible with more than 98% concordance. **Conclusions:** The results from the research samples concludes that our application was highly concordant and more sensitive when compared to culture results for the same sample set. In summary, we have developed highly efficient, cost-effective research application for urinary tract microbiota pathogen profiling using high performance validated assay for each microorganism.

**TT068. Reproducible Exome Capture of RNA-seq Libraries from Low Input and Formalin-Fixed, Paraffin-Embedded (FFPE) Samples**

A.H. Potts, K. Lai, K. Giorda

Integrated DNA Technologies, Redwood City, CA.

**Introduction:** Next generation sequencing is firmly established as a tool to identify single nucleotide variants (SNVs), insertion/deletions (indels), and copy number variations associated with disease states. However, it is becoming increasingly clear that DNA alone does not provide a full picture of molecular signatures associated with disease. RNA-seq can identify important changes in gene expression, RNA structural variations, and SNVs that can inform treatment and prognosis. However, such analyses can be problematic due to loss of signal from poorly expressed genes and low frequency structural variants. In addition, RNA samples from tissues are often limiting and degraded, and thus require extensive amplification before sequencing. Here we show that target capture of sequencing libraries tagged with unique molecular identifiers (UMIs) can overcome these obstacles. **Methods:** Stranded RNA-seq libraries were constructed from total or mRNA enriched Universal Human Reference (UHR) RNA and RNA extracted from formalin-fixed paraffin embedded tissue of varying qualities. The adapters used in library preparation added sample indexes and 9 base degenerate UMIs. RNA-seq libraries were used as input into target capture with Integrated DNA Technologies (IDT) xGen Exome Research Panel, which targets the coding portion of the human genome. Libraries were sequenced on an Illumina platform, and the data was analyzed using publicly available bioinformatics tools. **Results:** Normalized expression was highly correlated (> 85%) between captured and uncaptured UHR RNA samples across a wide range of expression levels. In addition, normalized expression of captured RNA libraries was

high correlated among total RNA and mRNA enriched samples (> 90%). Captured samples also had a greater depth of coverage across exons with over 90% on target bases. This made it possible to more reproducibly identify poorly expressed genes, allowing us to reliably detect over 300,000 additional exons in captured samples. In addition, enhanced coverage combined with the ability to identify PCR duplicates with UMIs allowed us to reproducibly measure expression over a wide range of RNA inputs (5-400 ng). Similar results were obtained with degraded RNA, making it possible to avoid mRNA enrichment steps that lead to sample loss. **Conclusions:** We show that target capture of RNA-seq libraries reliably maintains expression information present in uncaptured libraries while increasing coverage for poorly expressed genes and low frequency fusions. This would greatly enhance the power of differential expression analysis and sensitivity of fusion finding algorithms. The addition of UMIs to differentiate between PCR duplicates and unique starting molecules also makes it possible to reliably analyze even highly amplified libraries.

**TT069. Comparison of Redundant Coding versus Binary Coding Scheme for Increased qPCR Multiplexing Levels and Improved Test Performance with ChromaCode's HDPCR Platform**

C. MacDonald, D. Yurk, S. Powell, A. Rajagopal

ChromaCode, Carlsbad, CA.

**Introduction:** HDPCR is a novel chemistry and coding approach to increase multiplexing levels on existing qPCR instrumentation without requiring hardware changes. ChromaCode previously published HDPCR data with a proof-of-principle 12-plex respiratory viral panel (RVP) that utilized a binary coding scheme to achieve 3 targets per color channel multiplexing. In an attempt to increase multiplexing levels further while maintaining or improving sensitivity and specificity, this study describes the performance of a new redundant coding scheme compared to the binary coding scheme with the same proof-of-principle RVP assay. **Methods:** Binary and Redundant Coding Schemes: Multiplexing with traditional qPCR relies on differentiation of targets by color and is limited by the number of color channels available. The binary coding HDPCR scheme differs from this approach in that the probes for multiple targets in a single color channel use the same color fluorophore, but are differentiated by signal intensity (brightness). Like binary coding, redundant coding HDPCR differentiates targets in a single color channel by signal intensity. However, the redundant coding scheme differs from the binary coding scheme in that targets are coded simultaneously across multiple color channels and are read out with a different call algorithm to determine target or targets present. The redundant coding scheme has the potential to cover 16 different targets across 4 color channels. RVP Designs: With the binary coding RVP, there are 3 unique targets per color channel across 4 color channels for a total of 12 targets. With the redundant coding RVP, the 12 different targets are present across multiple channels for each target to better account for expected prevalence and possible co-infections. RVP targets include adenovirus, human metapneumovirus, influenza A, Influenza A/H1, influenza A/H3, influenza B, parainfluenza 1, 2, and 3, rhinovirus, RSV A, and RSV B. Testing and Analysis: Synthetic single-stranded DNA templates of the 12 RVP targets at high and low concentrations down to 10 copies in single and dual presence combinations were tested on a ViiA7 and QuantStudio 7. **Results:** A total of 480 synthetic samples have been tested to date with both the binary and redundant coding RVP tests. Call accuracy increased to 98% from 94% using the redundant coding method while maintaining the same level of multiplexing. **Conclusions:** ChromaCode's HDPCR multiplexing technology can be enhanced from a performance and potentially multiplexing level using a redundant coding scheme versus a binary coding scheme.

**TT070. Universal Design and Rapid PCR for Genotyping by High Resolution Melting**

J. Houskeeper, Z. Dwight, L. Narramore, E. Larkin, C. Wittwer

University of Utah, Salt Lake City, UT.

**Introduction:** A vast amount of genomic variation can be attributed to single nucleotide variants (SNVs). Small insertions and deletions in the genome further contribute to variation. While many methods exist to assess these genomic differences, the use of inexpensive PCR based assays followed by high resolution melting is simple, inexpensive, and



fast. Based on this premise, user-friendly software has been created to further ease SNV assay design using small amplicons by outputting a single thermocycling protocol and primer sequences to detect the target of interest. **Methods:** Automated scripts were designed to obtain targets from NCBI's ClinVar with a reported minor allele frequency of  $\geq 0.45$ . Following target selection, a SNV web tool known as uVariants (<https://www.dna.utah.edu/variants/>) was customized to automate universal primer selection. With a rs# as input, the software displays the first primers with a  $T_m \geq 60^\circ\text{C}$  as close to the target as possible excluding a flanking base on either side of the SNV of interest. All targets were thermocycled on a LightScanner 32 (BioFire). Two-step amplification was achieved using an annealing temperature of  $65^\circ\text{C}$  and a denaturation temperature  $\leq 3^\circ\text{C}$  above the product melting temperature. The annealing temperature was held for 1 second and denaturation had no hold time. All samples had an initial denaturation at  $95^\circ\text{C}$  for 5 seconds to denature genomic DNA. Following thermocycling, high resolution melting was performed on each sample for genotyping. Resulting melting curves were normalized and background fluorescent signal removed by exponential subtraction. Normalized and derivative melting curves were assessed by their ability to distinguish genotypes, typically associated with the appearance of distinct homozygous and heterozygous peaks. Gel electrophoresis and amplification plots were also assessed to ensure specific amplification and avoidance of non-templated additions (primer-dimers). Over 30 metrics related to primers and PCR target were recorded to improve and optimize the software. **Results:** Over 100 primer sets of SNVs acquired from ClinVar were assessed using a modified uVariants primer design and thermocycling protocol. Each primer set included one no-template control and 11 different DNA samples to reveal multiple genotypes. Successful amplification and clear genotyping was apparent in a majority of the primer sets. Cases where failure of amplification occurred were used to further study primer design and the effect close neighboring SNVs. **Conclusion:** A simple primer design algorithm was paired with a universal PCR protocol to simplify design for uVariant users to achieve amplification and detection of SNVs.

#### TT071. A Robust, Streamlined, Enzyme-based DNA Library Preparation Method Amenable to a Wide Range of DNA Inputs

L. Apone, K. Duggan, V. Panchapakesa, B. Langhorst, T. Shtatland, C. Song, P. Liu, C. Sumner, C. Rozzi, M. Arn, D. Munafo, F. Stewart, E. Dimailanta, T. Davis

New England Biolabs Inc., Ipswich, MA.

**Introduction:** In order for Precision Medicine to be widely implemented, robust, accurate, methods to process and interrogate large numbers of human samples are required. While advances in Next Generation sequencing (NGS) technologies have positioned it at the forefront of the Precision Medicine Initiative, it is ideally suited for deep molecular analysis of a single sample, rather than the processing and interrogation of thousands. In order to overcome this limitation, we have developed a robust, streamlined library construction method that integrates enzyme-based DNA fragmentation with end repair and dA-tailing. This method utilizes a single protocol for a wide range of DNA input amounts, as well as cell lysates. In addition, it eliminates the need for expensive equipment to fragment DNA and numerous cleanup and liquid transfer steps, reducing time, cost and errors associated with library construction.

**Method:** Genomic DNA (100pg – 500ng) and human cells were used to construct Illumina libraries using the NEBNext Ultra II FS DNA Library Prep Kit. Purified gDNA and gDNA in cell lysates were fragmented, end repaired and dA-tailed in a single step followed by adaptor ligation in the same tube. PCR amplified libraries were sequenced, reads aligned to the appropriate reference genome, and quality metrics generated using Picard tools. To confirm that FS does not introduce bias into library construction, DNA reference standards containing multiple common cancer mutations (Horizon Discovery, Inc.) were spiked into gDNA. Target enrichment and deep sequencing of a panel of cancer genes were performed. Variant frequency of input DNA and enriched FS libraries were quantified using digital droplet PCR. **Results:** Libraries constructed from intact gDNA using Ultra II FS produced substantially higher yields than those generated using standard mechanically fragmented DNA. The greatest differences were observed with the lowest DNA inputs and most challenging samples. Sequencing quality of libraries generated with purified gDNA, as well as

from cellular lysates, show similar coverage uniformity, GC bias and fragment size distribution. Correlation coefficient  $> 0.9$  was obtained in variant frequencies of the input and target enriched FS libraries, suggesting that FS library construction maintains mutation abundance in samples. **Conclusion:** Ultra II FS enables high-quality, high throughput, library construction from a broad range of DNA quantities and qualities, including human cells. Sequencing metrics of FS libraries are similar or superior to those produced with mechanically sheared DNA. Finally, FS maintains mutation abundance in samples without introducing bias. These improvements will help advance the widespread implementation of the Precision Medicine Initiative.

#### TT072. Development of an Automated Nucleic Acids Co-extraction Platform for Clinical FFPE Samples

M. Yang, J. Vega, M. Bolisetty, S. Pant  
Bristol-Myers Squibb, Pennington, NJ.

**Introduction:** Clinical samples preserved using formalin fixation and paraffin embedding (FFPE) are an important resource for genomic studies and clinical diagnostics due to the prevalence of FFPE matrix biopsies. The limited quantity of FFPE material has spurred the growth of techniques for co-extraction of DNA and RNA from the same sample. Currently available co-extraction kits are largely feasible for manual operation only, while automated high throughput DNA/RNA co-extraction remains challenging. To this end, we have developed and are optimizing a magnetic purification-based automation platform that enables sequential isolation of DNA and RNA from the same FFPE sample. **Methods:** We employed the KingFisher Presto nucleic acid extraction system from ThermoFisher as part of an automated purification workflow. We optimized the ThermoFisher MagMAX FFPE DNA-RNA Ultra Kit for DNA and RNA co-extraction. The yield and the integrity of KingFisher extracted FFPE DNA/RNA were compared with that manually extracted using the same kit, as well as with that from the Qiagen AllPrep DNA/RNA FFPE Kit. To control the quality of input materials, we used adjacent sections from the same FFPE tumor blocks. DNA and RNA were quantified using Qubit Fluorometer and quality checked using Agilent TapeStation and Bioanalyzer for DNA and RNA integrity, respectively. **Results:** With the automated co-extraction system, we were able to isolate sufficient DNA and RNA with acceptable quality using as few as 1 FFPE slide (85-90 mm<sup>2</sup> X 4  $\mu\text{m}$ ). The DNA and RNA were recovered in separate eluates. The yields and integrities of the DNA and RNA extracted on the automation system were comparable with those from the manual extractions. **Conclusions:** We have optimized automation of FFPE DNA RNA co-extraction on the KingFisher Presto nucleic acids extraction system. This high throughput platform is capable of isolating DNA and RNA from one single FFPE slide, with acceptable yield and integrity. We plan to further investigate the quality of the extracted nucleic acids using next-generation sequencing techniques.

#### TT073. Stabilization of Complement Proteins in Biological Samples

J. Desharnais, J.M. Vasquez, W.A. Coty, M.R. Reshatoff  
Biomatrix, Inc., San Diego, CA.

**Introduction:** Complement activation is a key feature of the immune system's response to infection, tissue injury, and antigen-antibody complexes. Complement analyte measurements are often plagued by inconsistency due to ex vivo autoactivation, particularly during pre-analytic handling/ processing. The complement cascade can also be activated by common materials used in sample collection. As a result, clinical complement testing is subject to restrictive sample handling requirements, namely processing of plasma or serum at the draw site within 2 hours. If these strict procedures are not followed, interpretation of laboratory results can be altered dramatically. Reliable sample handling methods can improve complement test results in two major areas: chronic disease monitoring and drug development/clinical trials. **Methods:** This study evaluates formulations designed to stabilize the levels of complement proteins iC3b (single protein) and sC5b-9 (multi-protein complex) in biological samples, including whole blood and plasma. Performance of the formulations was assessed using healthy donor samples and samples from patients with Lupus and Lupus Nephritis. Protein levels were assessed either by commercially available enzyme-linked immunosorbent assay (ELISA) or via lateral-flow assay (LFA). Complement protein levels

in the presence of the stabilizing formulations were determined for fresh samples and for sample stored for up to two days at 2-8°C, 25°C, and 37°C and compared with non-stabilized samples. **Results:** We show preservation of protein concentration levels of iC3b and sC5b-9 for up to two days post-blood draw for samples stabilized in novel formulations but not for non-stabilized samples stored in EDTA blood tubes. Additionally, we show preservation of iC3b and sC5b-9 concentration levels in Lupus and Lupus Nephritis blood plasma samples for two days when mixed with formulations even when stored at elevated temperatures. **Conclusions:** These novel formulations provide stabilization of key complement analytes (iC3b and sC5b-9) that is superior to standard EDTA blood tubes under a variety of environmental conditions, which allows removal of the restriction that samples be processed immediately to achieve accurate results.

#### TT074. Multi-patient Longitudinal Monitoring of Cancer Mutations from Circulating DNA of using Personalized Single Color Digital PCR Assays

C.M. Wood-Bouwens<sup>1</sup>, D. Haslem<sup>2</sup>, B.T. Lau<sup>1</sup>, A. Almeda<sup>1</sup>, B. Moulton<sup>2</sup>, R. Romero<sup>2</sup>, L. Nadauld<sup>2</sup>, H.P. Ji<sup>1</sup>

<sup>1</sup>Stanford University, Stanford, CA; <sup>2</sup>Intermountain Healthcare, St. George, UT.

**Introduction:** With the emergence of precision cancer medicine, there is an increasing need for new longitudinal diagnostic tests to evaluate patients at the initial diagnosis, during treatment, and for routine monitoring. As a high performance and flexible solution, we developed a single-color digital PCR (sc-dPCR) assay that detects and quantifies circulating DNA somatic cancer mutations collected from the plasma of cancer patients. The assay has a sensitivity of 0.1% mutation allelic fraction and can be designed for nearly any cancer mutation. Given its design flexibility, low cost, and robust performance, sc-dPCR offers many advantages for longitudinal monitoring of cancer patients during treatment.

**Methods:** We selected seven patients diagnosed with metastatic cancer of various types, whose tumors had diagnostic genotyping information available. We then developed personalized sc-dPCR assays for one or two clinically relevant mutations identified for each patient in essential cancer drivers such as *BRAF*, *KRAS* and *PIK3CA*. We collected longitudinal blood samples at each cycle of treatment and extracted circulating cell free DNA (ccfDNA) from 1mL of plasma. We then tested the ccfDNA at each time-point for the presence of somatic mutation bearing circulating tumor DNA (ctDNA) molecules. **Results:** We successfully detected ccfDNA from plasma in all seven patients tested. In four patients we were able to identify ctDNA molecules bearing the specific mutation targeted. In addition to the ctDNA mutation molecule count, we evaluated the clinical information as well as outcome and survival. These include circulating tumor marker levels, CEA, CA-19-9, and CA-15-3, as well as PET/CT scan images at various time points. Using our sc-dPCR ctDNA molecule count, we validated trends among these patients who were receiving active treatment with chemotherapy or targeted agents. For example, one patient demonstrated low or undetectable *BRAF* V600E molecule count across all time points, thus potentially indicating the patient's disease burden was under adequate control on *BRAF*-targeted treatment. In another patient under active treatment, we detected increasing quantities of ctDNA molecules over time suggestive of recurrence. This hypothesis was corroborated by increases in tumor marker levels and subsequent imaging of the tumor.

**Conclusions:** With our sc-dPCR technology, we identified personalized mutation bearing ctDNA molecules from individual cancer patients and identified trends in ccfDNA matching the clinical responses that were observed. Overall, our study provided longitudinal information specific to an individual patient's response to treatment, suggesting that our assay technology is a valuable tool for precision medicine and monitoring of cancer patients.

#### TT075. Application of Next-generation Sequencing in Pleural Effusion Molecular Profiling Using Oncomine Lung Cell-free Total Nucleic Acid Research Assay

C. Xiang, R. Zhao, S. Ma, L. Guo, Y. Han

Shanghai Chest Hospital/Shanghai Jiao Tong University, Shanghai, China.

**Introduction:** Pleural effusion is commonly observed in patients with advanced lung cancer and its molecular profiling has not been fully investigated. Cell-free (cf) DNA or RNA isolated from cancer patient's plasma has allowed noninvasive tumor genome analysis, however, there are few study of detection and characterization of cfDNA or cfRNA in pleural effusions and its application in cancer patient treatment. Here we reported the utilization of cell free total nucleic acid (cfTNA) isolated from pleural fluid of late stage lung cancer patient with a robust Ion Torrent Next-generation sequencing (NGS) liquid biopsy assay-Oncomine Lung cfTNA Assay. The assay simultaneously query about 168 hotspot across *ALK*, *BRAF*, *EGFR*, *ERBB2*, *KRAS*, *MAP2K1*, *MET*, *NRAS*, *PIK3CA*, *ROS1*, and *TP53*, *CNV* (*MET*), gene fusions (*ALK*, *RET*, *ROS1*) as well as *MET* exon 14 skipping which have been frequently identified in NSCLC. A comprehensive study of these mutations provides information for therapy decision, disease monitor as well as new drug development.

**Methods:** Twenty paired cfTNA were isolated from pleural fluid of Non-Small Cell Lung Cancer (NSCLC) subjects (MagMAX Cell-Free DNA Isolation Kit, Thermo Fisher Scientific), and their matched tumor TNA were extracted from cell pellet collected from same pleural fluid or formalin-fixed paraffin-embedded (FFPE) tissue. Libraries were prepared according to manufacture user guide using Oncomine Lung Cell-Free Total Nucleic Acid Research Assay (Thermo Fisher Scientific), then purified with Agencourt Ampure XP beads (Beckman Coulter) and quantified with qPCR (Ion Library Quantitation Kit, Thermo Fisher Scientific). Six plex libraries were templated with Ion Chef automated templating instrument (Thermo Fisher Scientific) and sequenced with Ion S5 Sequencing instrument (Thermo Fisher Scientific). Data were analyzed using Ion Reporter 5.6. **Results:** All cfTNA have been isolated from twenty patient pleural fluid (yield range is 15-786ng from 4ml fluid). Bioanalyzer profile of these cfTNA verified the fragmentation pattern, which is similar to cfTNA isolated from plasma. 20ng of cfTNA were tested with Oncomine cfDNA Lung assay test, matched somatic variants were detected at a frequency between 1.3% and 54.5% in cfDNA and between 0.0% and 67.6% in corresponding DNA isolated from FFPE. **Conclusions:** Oncomine Lung cfTNA research assay provided an easy, quick and reliable solution in detecting single-nucleotide variants (SNVs), *CNV*, fusion and *MET* Exon14 skipping using cfTNA isolated from pleural fluid. Molecular analysis of pleural fluid can be used in clinical practice for treatment of late stage cancer patients with pleural fluid.