Genetics

G01. Development and Validation of a High-Throughput Next-Generation Sequencing Assay from Buccal Cell DNA as a Cost-Effective Screening Method for Celiac Genetic Risk

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Introduction: An estimated 1% of the global population has been diagnosed with celiac disease (CD), an autoimmune condition triggered by dietary gluten in individuals who carry HLA-DQ2 and/or HLA-DQ8 (DQ2/DQ8) celiac risk alleles. Clinical diagnosis of active CD is based on symptoms, serologic tissue transglutaminase (tTG) antibody testing, and characteristic histopathologic changes identified by small intestinal tissue biopsy. The presence of DQ2/DQ8 genes is necessary but not sufficient for development of CD, and the negative predictive value (NPV) of negative DQ2/DQ8 test results is >90%. Approximately 30% to 40% of the general population carries specific allelic combinations for DQ2/DQ8 that would place them on a spectrum of risk for CD. However, despite the high NPV of DQ2/DQ8 testing, routine screening of celiac family members and asymptomatic individuals has traditionally been performed by tTG serologic testing and/or small intestinal biopsy. Disadvantages to these approaches include false negative results in individuals who are not consuming dietary gluten, and unnecessary healthcare costs associated with repeated testing of asymptomatic individuals. In the current study we developed and validated a low-cost, non-invasive, buccal cell DNA-based next-generation sequencing (NGS) population screening method for the DQ2/DQ8 alleles. Methods: Buccal cell DNA was collected from 98 healthy individuals (including multi-generation family members) and analyzed using a next-generation sequencing technology to amplify and sequence across HLA-tagging celiac risk SNPs for DQ2.5, DQ8, DQ2.2, and DQ7 at a minimum of 40X coverage. Sequencing data were analyzed in Galaxy, where the fasta files were aligned, variants called, and SNPs identified. Results: All possible heterozygous and homozygous combinations of celiac risk alleles were identified in the study population including non-celiac genetics (NCG), DQ2(2s), DQ2(trans) DQ8, and DQ7. Allelic inheritance could be clearly traced through multiple generations of families. A subset of 20 samples with known DQ2/DQ8 status performed by an outside CAP/CLIA-certified clinical laboratory was used for qualitative analytical validation of the assay which showed 100% concordance with known DQ2/DQ8 results. Conclusions: DQ2/DQ8 typing by NGS is a cost-effective screening method for celiac genetic risk in individuals, families, and populations. The assay identifies those with high-risk genetics who would benefit from annual serologic and/or small intestinal biopsy testing while allowing NCG low-risk, asymptomatic individuals to forgo further screening for celiac disease.

G02. Copy Number Variant Analysis Improves the Diagnostic Yield in a Cohort of Pediatric Patients with Previously Negative Constitutional Exome Sequencing Results

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Introduction: Exome sequencing (ES) is the current standard for the diagnosis of Mendelian genetic disorders. The reported rate for identification of the causative genetic abnormality across all phenotypes using ES analysis ranges from 25% to 45%. Typical ES is not validated for gene level copy number variant (CNV) detection or identification of regions of homozygosity. CNVs ranging in size from intragenic deletions or duplications to cytogenomic microdeletions and duplications are known to cause a subset of genetic disorders. The goal of our study was to evaluate the feasibility and diagnostic yield of CNV analysis of ES data from undiagnosed cases in the clinical diagnostic setting. Methods: A total of 290 cases with variable clinical phenotypes referred for ES or focused exome sequencing (FES) from September, 2019 to May, 2020 were evaluated. Cases with pathogenic or likely pathogenic sequence variants that could explain the patients’ reported phenotypes were excluded, leaving 201 cases that were negative or had variants of unknown significance (VUS). This included 46 ES cases, consisting of trios, duos, and singletons, and 155 singleton FES cases. CNV analysis and assessment of heterozygosity were performed using NexClinical (Biodiscovery). Genes related to patients’ phenotypes were generated using Human Phenotype Ontology terms and used to filter for analysis. Parallel CNV analysis was performed using an in-house algorithm, LUBA. Uniparental disomy (UPD) and microdeletions/duplications were confirmed by CytoScan (Thermo Fisher), and intragenic CNVs that were considered disease-causing were confirmed using the Kox array (Thermo Fisher). Results: Clinically significant CNVs, classified as pathogenic or likely pathogenic, fully explaining the reported phenotypes, were identified in 19 cases, ranging in size from a single exon deletion to an 8.7 Mb deletion. Diagnostic categories included small exonic deletions (n = 11), larger microdeletion syndromes (n = 6), and absence of heterozygosity (n = 2) including a case with genome-wide UPD. Three cases showed a combination of sequence variants and CNVs that accounted for autosomal recessive disorders. Additionally, CNVs were classified as VUS in 10 cases. Conclusions: Retrospective CNV analysis yielded novel sequenced-based diagnoses in 9.45% of previously unsolved cases. Our study demonstrates the feasibility and significantly increased yield of CNV analysis of ES data in the clinical setting. The bioinformatic and confirmatory wet-lab approaches described above are being incorporated into our prospective ES-based studies. As additional causative CNVs are discovered, the overall diagnostic yield of ES analyses will continue to improve.

G03. A Retrospective Study of Products of Conception with More Than 44,000 Specimens in 27 Years at a National Cytogenetic Reference Laboratory

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Introduction: Many studies have assessed the frequency and types of chromosome abnormalities in products of conception (POCs). However, these studies were relatively small, with the largest being <5,600 specimens. Larger populations provide more accurate frequency estimates, especially if conditions are rare. We examined results from >44,000 specimens to reassess the frequency and types of chromosome abnormalities in POCs. Methods: This retrospective study included 44,316 POC specimens submitted to a national reference laboratory for karyotype. Specimens were submitted from 1993 to 2019. Results: In the total of 44,316 specimens, 375 had only maternal decidua. In the remaining 43,941 specimens, 11,501 (26.2%) failed cell culture due to no countable metaphases and/or culture contamination. Fifty-six had uncertain findings such as 46,XX with 92,XXX in some cells (46 cases) and incomplete karyotype due to poor morphology (10 cases). A total of 32,384 specimens had reportable results with fetal component based on visual inspection before culture, in which 55.2% (17,880) had normal karyotype. The ratio of normal female to normal male was 5.3. The abnormal rate was 44.8% (14,503). In 14,503 abnormal cases, 1,987 (13.7%) had sex chromosome abnormalities (SEX), 9,179 (63.3%) had trisomies including the ones with at least 2 (≥2) trisomies, and 97 (0.7%) had monosomies. Trisomy (3n) and tetrasomy (4n) contributed to 15.0% (2,182 cases) and 2.8% (403) of the abnormal cases, respectively. Structural abnormalities only contributed to 4.5% (654) of the abnormal cases, including balanced Robertsonian translocations (ROBs), and other balanced and unbalanced structural alterations. Trisomy 16 (+16) and Turner syndrome (TS) were the most common abnormalities, accounting for 13.8% and 12.8% of abnormal karyotypes, respectively, followed by +21 (8.4%), +15 (6.7%), +13 (4.1%), and +18 (3.6%). Trisomies, especially results with ≥2 trisomies, showed a trend to be associated with advanced maternal age (AMA). TS, 3n, and 4n were not associated with AMA. Conclusions: We confirmed that +16 and TS are the most common genetic abnormalities in POCs. Trisomies are related to AMA, but TS, 3n, and 4n are not. Double trisomies are associated with AMA more strongly than are single trisomies, following the trend seen in previous studies.
Introduction: Examination of aneuploides in amniotic fluid cells using fluorescence in situ hybridization (FISH) is critical for prenatal screening and diagnosis. However, the traditional FISH method is time consuming and complicated; therefore, updated solutions and optimized strategies are necessary for clinical practice. As a flexible toolbox, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas9) system provides versatile application outside the realm of gene editing, and has facilitated rapid and efficient in situ labeling of highly repetitive elements in the genome. To this end, we developed a CRISPR/Cas9-based strategy for in situ detection of trisomy 13 and reported its performance using an aneuploid chromosome cell line. Methods: We employed the GM02948 cell line (47,XY,+13) as a model. Cultured cells were fixed at −20°C for 20 mins in prechilled methanol and acetic acid (1:1) and stored at 4°C until use. We synthesized sgRNAs against the −1,200 bp of the target (5'-GGTAAAGCATTGAGACCTTCT-3') on chromosome 13. The sgRNA was incubated with AF488-labeled dCas9 protein for 10 mins at room temperature, and the resulting dCas9/sgRNA complex was applied to fixed cells and incubated at 37°C for 5 mins. The reaction was terminated by removing dCas9/sgRNA complexes and rinsing fixed cells in PBS 3 times. Images were collected using a Nikon Eclipse 50i microscope with a CF100× immersion objective (1.45 NA, oil). Results: We counted 300 cells and observed that 96% nuclei showed bright signal spots. Of these cells with fluorescent signal, 4% nuclei exhibited 1 spot, 5% nuclei exhibited 2 spots, 86% nuclei exhibited 3 spots, 4% nuclei exhibited 4 spots, 1% nuclei exhibited 5 spots. There is an average of 2.93 signal spots in each nucleus, which is consistent with the 3 copies of chromosome 13 in interphase GM02948 cells. Aneuploid of the cell line has been verified by karyotypic analysis. Conclusions: The CRISPR/Cas9-based in situ hybridization enabled rapid and efficient labeling of trisomy 13, relying on specific recognition and binding of dCas9/sgRNA complexes with highly repetitive sequences on chromosome 13. The hybridization step of this method can be completed within 5 mins under mild conditions by eliminating prolonged heat treatment required in FISH, which not only saves time but also preserves cell morphology. Therefore, this strategy potentially provided a superior alternative to traditional FISH. It is worth noting that this method is remarkably limited to highly repetitive sequence labeling. The labeling of trisomy 21/18 requires further study due to the lack of specific repetitive sequences with sufficient copy numbers.

G05. Reevaluation of Genomic Test Results for Germline Disorders: A Framework of Critical Considerations on Behalf of CLSI Document Development Committee (DDC) on Nucleic Acid Sequencing (MM09)

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Introduction: Clinical exome sequencing is a routine diagnostic tool for rare Mendelian disorders; however, more than 50% of patients remain undiagnosed. Previous studies have demonstrated the added value of exome reanalysis to increase the diagnostic yield. While challenges remain, it is important for both ordering physicians and clinical laboratories to recognize the need of exome reanalysis as a routine clinical practice given the evolving nature of the genomic field. The American College of Medical Genetics and Genomics (ACMG) recently provided guidance in the reevaluation and reanalysis of genomic test results at various levels; however, a practical, ready-to-use, and concrete guidance is needed for physicians ordering reanalysis and for the clinical diagnostic laboratories.

Methods: To address the need for more detailed guidance, the Clinical and Laboratory Standards Institute (CLSI), with representation from the Association for Molecular Pathology (AMP), assembled an interpretation working group within the CLSI Document Development Committee (DDC) on nucleic acid sequencing (MM09) to create a practical resource that provides step-by-step guidance for implementing interpretation of next-generation sequencing (NGS) data for clinical laboratories that includes considerations of reanalysis of genomic data and variant reclassification. The committee consisted of experts in NGS, and quality management and accreditation requirements from academic and commercial entities.

Results: The interpretation working group developed practical, ready-to-use worksheets that will guide a laboratory through critical decision paths for exome reanalysis and reporting. In addition, detailed considerations for two distinct but complementary approaches, physician-initiated patient-level reanalysis and laboratory-initiated cohort-level reanalysis, are established. We describe advantages and limitations for both approaches and provide worksheets for the clinical laboratory to use as a reference.

Conclusions: As exome analysis continues to be a common practice in genetics, we expect that exome reanalysis would be critical in increasing the diagnostic yield in clinical practice. The worksheets on exome reanalysis will be publicly available and updated with user and expert feedback as the landscape continues to evolve.
G07. Detection of Allelic Dropout in a Mass Array HFE Genotyping Assay
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Introduction: Hereditary hemochromatosis (HH), an autosomal recessive disorder of iron metabolism with a carrier frequency of approximately 1 in 10 in Northern European ancestry, is characterized by an accelerated rate of intestinal iron absorption. Almost 94% of the mutations associated with HH can be detected by DNA genotyping assay which identifies C282Y, H63D, and S65C mutations in the HFE gene. The most common mutation in the HFE gene is C282Y (exon 4, c.845G >A, gnomAD Exomes European 3.5% to 5.7%), which is associated with a more severe clinical presentation, whereas the H63D (exon 2, c.187C >G, gnomAD Exomes European 10.4% to 14.4%) and S65C variants (exon 2, c.193A>T, -2%) are associated with milder phenotypes. This study describes the identification of HFE allelic dropout in patients compound heterozygous for c.187C >G and c.193A>T mutations that were genotyped by Mass Array.
Methods: Clinical and CAP specimens previously tested by Mass Array were selected for testing on an HFE qPCR assay. The Mass Array procedure involved multiplex PCR followed by multiplexed primer extension for mass-modified base labeling. For the HFE qPCR assay, each allele was individually genotyped using TaqMan probe sets and the 7500 FAST platform. Among these samples, 14 were homozygous for c.187G and 10 were heterozygous for c.187C>G. Twelve specimens were heterozygous for c.193A>T, including 4 samples genotyped as c.187G homozygous. Sanger sequencing was used to resolve discordant sample results. Results: Four samples genotyped by Mass Array as homozygous c.187G heterozygous c.193A>T were tested as heterozygous c.187C>G/heterozygous c.193A>T by qPCR. The heterozygous c.187C>G/heterozygous c.193A>T result was confirmed by Sanger sequencing. Analysis of the c.187 extension primer sequence revealed overlap with c.193. This likely contributed to dropout of the c.187C allele in the presence of c.193A>T mutations. Allelic dropout was not detected during Mass Array validation due to the admix of several commercial controls for specificity testing. Conclusions: Compound heterozygous HFE c.187C>T and c.193A mutations are an uncommon occurrence (6/870 patient samples, 0.7%, tested at Geisinger). Assay design and a lack of suitable compound heterozygous test samples contributed to the failure to detect allelic dropout in the Mass Array assay during validation.

G08. Result Interpretation for Clinical Exome and Genome Sequencing: On Behalf of CLSI Document Development Committee (DDC) on Nucleic Acid Sequencing (MM09)
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Introduction: Next-generation sequencing (NGS) of gene panels, exomes, and genomes has become an essential tool in the clinical care of patients with suspected germline disorders. Whereas professional guidelines for developing and validating NGS-based genotyping tests exist, these remain limited to technical aspects of sequencing and bioinformatics analysis. However, implementation of a comprehensive NGS test can include numerous additional steps after bioinformatics analysis, including development of a robust approach for variant filtration and prioritization; processing and interpreting a large number of variants for potential reportability based on clinical significance; and a strategy for reclassification or reanalysis when results are uninformative. For assays such as clinical exome and genome sequencing (WES/WG), these processes are critically important, yet laboratory guidance continues to be limited. As a consequence, how laboratories process, interpret, and report variants remains variable. Methods: The Clinical and Laboratory Standards Institute (CLSI), with representation from the Association for Molecular Pathology (AMP), assembled an interpretation working group within the CLSI Document Development Committee (DDC) on nucleic acid sequencing (MM09) to address the need for recommendations and resources in the areas of interpretation and reporting of NGS-based tests for germline conditions. The committee consisted of experts in NGS, bioinformatics, quality management, and accreditation requirements from academic and commercial entities. This working group developed a series of practical, ready-to-use worksheets that guide laboratories through critical decision paths for interpretation and reporting. Results: The CLSI interpretation workgroup developed worksheets with flexibility to accommodate a range of clinical applications; however, WES/WGS were given additional considerations due to the increased complexity of interpreting and reporting these tests. Prioritization of variants most likely to be disease associated, assessment for potential overlap with a tested individual’s features, and consistent reporting decisions when encountering a large number of diverse, potentially relevant variants are critical components of broad, phenotype-agnostic tests like WES/WG that were addressed by the worksheets. Conclusions: Clinical evaluation of WES/WGS data involves many additional processes compared to gene panels and other NGS-based test applications. This resource assists clinical laboratories with the complexities of interpreting large NGS-based tests and has additional considerations for specific aspects of WES/WGs. Worksheets facilitate internal laboratory organization, leading to greater standardization of NGS-based testing within a highly dynamic environment, and therefore improve patient care.

G09. Incidental Diagnosis of NR5A1-Related 46,XY Disorder of Testicular Development in Neonate with Mosaic Partial Trisomy 2q
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Introduction: Identification of congenital anomalies in a newborn warrants clinical genetics evaluation and diagnostic testing including SNP-based chromosomal microarray analysis (CMA). This testing detects clinically significant duplications or deletions of genetic material that may result in congenital anomalies as well as other medical and/or developmental differences. In addition to the detection of chromosome copy number variants relevant to the indication for testing, clinically significant incidental findings can be identified. Methods: SNP-based CMA was ordered in a full-term large-for-gestational-age newborn presenting with truncus arteriosus and macrocephaly. Molecular analysis was completed internally to verify sample identity, including identity testing of the blood sample and DNA extracted from the stored fetal placenta. Once identity was verified, the preliminary report documenting the discordant sex findings by CMA was finalized. Genetic counseling was provided and follow-up diagnostic testing was coordinated, including karyotype and sequencing using a disorder of sexual development (DDSD) panel. Results: CMA results revealed discordant sex chromosome findings consistent with an unexpected XY karyotype [arr (X)x1, (Y)x1] in a phenotypic female. CMA also showed an increase in its log2 ratio from chromosome region 2q11.2q37.3 suggestive of a mosaic gain of 142 Mbp. This gain was below the reliable limit of detection by this assay at a copy number state of ~2.15. Giemsa-banded karyotype analysis confirmed the 46,XY genotypic male in all cells analyzed. Locus-specific metaphase fluorescence in situ hybridization (FISH) to SRY was positive, confirming the presence of the sex-determining region on the Y. Interphase FISH for the 2q subtelomeric region confirmed the mosaic gain from within 2q in 29 of 200 interphase cells examined (14.5% of cells). Further next-generation sequencing (NGS) using the DSD panel detected a likely pathogenic, heterozygous novel nonsense variant in NR5A1 (c.1096C>T, p.Gln366*). Conclusions: The initial discordant sex chromosome results and low-level mosaicism in this case required a number of follow-up molecular and cytogenetic analyses to ensure the appropriate diagnoses were identified and diagnosis-specific management and genetic counseling could be provided for the proband and her family. Interestingly, the mosaic gain appears to be unrelated to the XY complement. Although genotype-phenotype correlation for this gain is challenging due to gene content and mosaic state, it may contribute to the patient’s congenital heart defect and macrocephaly. The early detection of a nonsyndromic disorder of testicular development with apparent complete gonadal dysgenesis will ensure provision of important healthcare for this patient.
G10. Optimization and Validation of a Sanger Sequencing Clinical Assay for Germline BRCA1/2 Gene Mutation Detection at King Hussein Cancer Center

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Introduction: Breast cancer is the most common cancer in Jordan and worldwide. Published data had shown that 5% to 10% of breast cancer cases are hereditary and mostly related to BRCA1/2 gene mutations. Efforts to identify such mutations are extremely important given the high penetrance rates among its carriers. Carriers of pathogenic mutated BRCA1/2 genes may escalate their lifetime risk of breast cancer up to 90% and to 40% for ovarian cancers. Risk-reduction interventions, like bilateral mastectomies and oophorectomies, are highly recommended in such patients. In this study, we designed and validated a genetic testing protocol to screen for BRCA1/2 mutations in Jordanian women via Sanger sequencing. Methods: A validation cohort was chosen from 550 breast cancer patients being part of a genetic counseling program. Nearly 13% (70 patients) tested positive for a pathogenic mutation in BRCA1/2 as reported by a reference laboratory. From the 70 patients, we selected 45 samples harboring BRCA1 and two negative controls for the validation study. Only exons with mutations were validated. SNVs and INDELs were validated separately for each gene using GA3500 and SeqStudio instruments. The validation study consisted of: 1) an accuracy test to calculate concordance and analytical specificity/sensitivity, and 2) a precision test to assess intra-/inter-run variations and quality of sequencing traces. Hitherto, we executed the validated sequencing protocol on 5 patients and 5 asymptomatic family members to predict and/or confirm presence or absence of BRCA1/2 aberrations. Results: All primer pairs used yielded the anticipated amplions and high-quality traces. During accuracy assessment, sequencing quality for all samples was very good, as reflected by a percentage of QV20+ and trace scores (>90% and >40%, respectively). All mutations were accurately called in all 45 samples. Our results correlated 100% with the reference laboratory results. All accuracy and precision measurements reached 100%. With this test, we confirmed presence of BRCA1/2 pathogenic mutations in the 5 patients and 2 family members tested so far. Conclusions: We have successfully developed and validated a reliable in-house genetic test to screen for BRCA1/2 mutations via Sanger sequencing. To our knowledge, this is the first validated in-house BRCA mutation testing protocol in Jordan. Clinical reports generated from this test would efficiently steer clinical and prophylactic decisions for breast cancer patients and their family members. We foresee this test as a cornerstone for a national breast cancer screening program to provide policymakers and health care professionals with data for early detection and risk-reduction intervention.

G11. Mosaicism in Cancer Susceptibility Genes in Unselected Cancer Patients

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Introduction: Germline pathogenic variants affecting cancer susceptibility genes (CSGs) result in an increased risk of cancer. Most often, these germline variants are inherited from one parent. However, mutagenesis may also occur during embryogenesis, resulting in mosaicism where a variant is present only in a subset of an individual’s cells. Mosaicism affecting CSGs has been reported in individuals meeting the clinical criteria of syndromes such as tuberous sclerosis and neurofibromatosis. Whether mosaicism affecting CSGs may result in an increased risk of apparently sporadic cancers has yet to be determined. Methods: To identify candidate patients harboring mosaic variants affecting CSGs, we applied a set of filtering criteria to the matched tumor/blood sequencing data for 35,511 consecutive cancer patients undergoing testing using MSK-IMPACT. Sixty-two CSGs that show an autosomal dominant mode of inheritance and have moderate to high penetrance for cancer susceptibility were included in the analysis. In brief, possible mosaic variants were defined as those present in peripheral blood leukocytes with variant allele fractions (VAF) ≥1.5% and <25%, VAF ≥10% in the tumor specimen; tumor/blood VAF ratio ≥2, and ≥6 reads of the alternate allele in the blood. To distinguish mosaicism from circulating tumor cells, we excluded cases with >1 variant meeting these criteria. To confirm mosaicism, tissue samples of different embryologic lineages from each patient were micro-dissected and sequenced for the candidate mosaic variant and other variants present in tumor tissue. Results: Thirty-four pathogenic variants (25 loss of function, 9 missense) in CSGs were identified by our filtering criteria as candidate mosaic variants. The most frequent CSG with mosaic variants were TP53 (N = 13), RB1 (N = 5), BRCA2 (N = 3), and APC (N = 3). A total of 21/235 candidate cases underwent documented genetic testing for the gene with the identified mosaic variant. The 5 RB1 variants were reported as mosaic, but the other 16 cases had negative genetic testing. Tissue samples from 10 cases with the candidate mosaic variant displaying VAFs in blood ranging from 1.7% to 21% (mean VAF = 6.8%) were subjected to microdissection, with at least 3 tissue types from different embryonic lineages tested for each case. In all cases, the mosaic variant was identified in multiple cell lineages, whereas none of the somatic mutations identified in the tumor were detected in these cells, confirming the presence of mosaicism. Conclusions: Using tumor:normal sequencing data, mosaicism confering cancer susceptibility was detected in ~1/1,000 unselected cancer patients. Most of these individuals had prior negative genetic testing, highlighting the increased sensitivity of tumor/normal sequencing for detecting these clinically important mosaic variants.


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Introduction: There are three main strategies for tumor testing by next-generation sequencing (NGS): tumor-only testing, tumor-normal pairs with germline subtraction, and tumor-normal pairs with enrichment for a set of disease predisposition genes. In our laboratory, we perform tumor-only sequencing. Any presumed germline pathogenic variants (PGVs) are flagged in the interpretation section of the report with a disclaimer that tumor-only testing is performed and the variant should be confirmed. We also recommend referral to our Familial Cancer Program (FCP) for genetic counseling and follow-up testing. Here we report our experience with PGVs since implementing an expanded NGS panel in February 2020. Methods: DNA and RNA were isolated from formalin-fixed, paraffin-embedded (FFPE) tissues with the Qiagen AllPrep DNA/RNA FFPE Kit Protocol on the QIAcube instrument. DNA was sheared using the Covaris ME220 Focused-ultrasonicator. Illumina TruSight Tumor 170 libraries were prepared using the Biomek NX (Beckman Coulter) and sequenced using the Illumina NextSeq 500 System. Following sequencing, the TST170 Local App v1.0.1 (Illumina), housed in the Clinical Genomics Workspace (CGW; Pierian Dx), was used to perform alignment and variant calling. Variants detected from paired DNA and RNA samples were combined into a single sample output and report in CGW. Variants were classified as IA to III according to the AMP/ASCO/CAP Guidelines. Results: To date, we tested 255 samples from 248 patients. Nine patients (3.6%) were identified with PGVs: three in mismatch repair genes (MSH2) and MSH6[1], six in homologous repair deficiency genes (BRCA1[3], BRCA2[2], and RAD51C[1]). Conclusions: PGVs were identified in 3.6% of our patients using a tumor-only sequencing assay. Special consideration and a coordinated effort should be taken to identify PGVs, and get appropriate referrals and subsequent confirmatory testing.
G13. Comparison of Universal versus Traditional Genetic Testing Models for Cancer Patients


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Introduction: Genetic testing for hereditary cancer predisposition is traditionally performed in a guideline-dependent manner, with patients being tested for specific genes based on their tumor type, age of onset, and/or family histories according to established criteria from professional organizations. Recently, universal genetic testing has been shown to uncover hereditary cancer predisposition in patients who do not meet current testing criteria. We characterized the yield of genetic testing through the traditional guideline-based method versus universal testing of a broad cancer patient population in a 5-year period at Memorial Sloan Kettering Cancer Center (MSKCC).

Methods: The rate of pathogenic/likely pathogenic (P/LP) variants in a core set of genes known to confer susceptibility for each given cancer diagnosis was compared between two cohorts of patients with breast, ovarian, colorectal, and pancreatic cancer: 1) 4,113 patients who had guideline-based genetic testing ordered by a clinical genetics service, and 2) 7,235 cancer patients unselected for current genetic testing criteria who had testing for 76 or 88 cancer predisposition genes through MSK-IMPACT between 7/2015-4/2020. In patients who had MSK-IMPACT, clinical implications of additional findings in genes outside the core gene set were analyzed.

Results: In the cohort who had traditional guideline-based testing after counseling through a clinical genetics service, 7.5% (95% CI 6.6% to 8.3%) of 3,341 breast, 17.4% (14% to 21.6%) of 3,341 ovarian, 13.5% (9.8% to 18%) of 252 colorectal, and 8.8% (5.1% to 14.8%) of 136 pancreatic cancer patients had P/LP variants. In the cohort who had broad genetic testing via MSK-IMPACT, 9.6% (8.4% to 10.9%) of 2,249 breast, 14.4% (12.4% to 16.6%) of 1,126 ovarian, 7.6% (6.5% to 8.9%) of 2,060 colorectal, and 9.6% (8.3% to 11.1%) of 1,800 pancreatic cancer patients had P/LP variants in genes routinely tested for their cancer types. Additionally, 626 patients (9%) with broad genetic testing had P/LP variants outside the clinically indicated core gene set routinely tested for their cancers, with 303 of them (48%) having medically actionable findings leading to recommendations for prophylactic surgery (56 patients, 9%), or early surveillance (247 patients, 39%) for future disease. Conclusions: At our institution, universal testing of a broad patient population has yielded comparable actionable rates of positive results to traditional guideline-based testing, particularly in breast, ovarian and pancreatic cancer patients over the course of the past 5 years. Universal genetic testing has also uncovered additional findings in genes not routinely tested for the patient’s diagnosis, which are likely to have implications for future disease surveillance and prevention in the patients and their family members.


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Introduction: The National Cancer Institute (NCI) estimates that 5% to 10% of cancers are hereditary. Next-generation sequencing (NGS) has enabled multi-gen testing to help identify carriers of DNA changes that potentially increase the risk of developing certain cancers. The detection of relevant pathogenic variants may help physicians make more informed clinical decisions in patient management strategies.

Methods: We validated an NGS-based inherited cancer screening assay. Genomic DNA (buccal swab) was enriched for regions known to be associated with the development of cancer. Each sample was sequenced using the Ion Torrent technology. Patients were tested for variants in 33 genes associated with the most common hereditary cancer syndromes: APC, ATM, BAP1, BARD1, BMPR1A, BRCA1, BRCA2, BRIP1, CDH1, CDK4, CDRN2A, CHEK2, COL1A1, EPCAM, FB1N, GREN1, MIF2, MLH1, MSH2, MSH6, MUTYH, NBN, NF1, PALB2, PM2, POLQ1, POLY1, POLE, PTEN, RAD51C, RAD51D, SMAD4, STK11, T5P3. Variants were evaluated according the American College of Medical Genetics (ACMG)/Association for Molecular Pathology (AMP) classification guidelines.

Results: We evaluated 4,853 patients (ages 7 to 101). The results were evaluated in comparison to national cancer statistics. We found that 57% of patients (2,800) had no detectable pathogenic or likely pathogenic variants; 37% (1,805) had a variant of undetermined significance (VUS); and 5% (249) had at least one pathogenically significant variant detected. A total of 7,206 variants were detected: 1,147 VUS (15.9%) and 116 (1.6%) pathogenic/likely pathogenic variants. A majority of variants were detected in four genes: ATM, APC, BRCA2, and MUTYH, which comprised 40% of all variants.

Conclusions: Pathogenic/likely pathogenic and VUS variants exhibited different distributions by gene, ATM exhibited the highest number of VUS (17.8%), and MUTYH exhibited the most pathogenically significant mutations (29.5%). A total of 40% of all pathogenic/likely pathogenic variants or VUS were found in the 70 to 79 age range. Pathogenic/likely pathogenic variants and VUS were evenly distributed among all other age groups and genders.


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Introduction: Spinal muscular atrophy (SMA) is an autosomal recessive disorder predominately caused by bi-allelic loss of the survival motor neuron 1 gene (SMN1). It is characterized by dysfunction and then loss of the alpha motor neurons in the spinal cord that causes progressive muscle atrophy and weakness. The functional loss of SMN1 results in a deficiency of the SMN protein; however, the protein is not completely absent in affected patients due to the presence of a low-functioning, nearly identical parafunctional gene, SMN2, which is located centromeric to SMN1. Numerous studies have shown that an increased SMN2 copy number is associated with an increase in the SMN protein and a less severe phenotype. Clinical laboratories must measure SMN1 and SMN2 copy number accurately to identify SMA carriers and patients who are likely to benefit from therapeutic interventions. Having publicly available and appropriately characterized reference materials (RM) with various combinations of SMN1 and SMN2 copy number variants is critical to assure accurate SMA clinical testing, which also has public health significance as SMA has been increasingly included in state-based newborn screening programs.

Methods: To address this need, the Division of Laboratory Systems, Centers for Disease Control and Prevention-based Genetic Testing Reference Material Coordination Program (Get-RM), in collaboration with members of the genetic testing community and the Coriell Institute for Medical Research, has characterized 15 SMA DNA RMs derived from Coriell cell lines. DNA samples and live cell cultures were distributed to 4 volunteer testing laboratories for genotyping using a variety of commercially available and laboratory-developed methods.

Results: The characterized samples had a wide variety of copy numbers for each of the genes, ranging from 0 to 4 copies of SMN1 and 0 to 5 copies of SMN2. The samples also contained a variety of allele combinations, ranging from 2 copies of each gene, to those with 1 or no copies of SMN1, and 3, 4, or 5 copies of SMN2. Also included were several samples that tested positive for the g.27134T>G variant (rs143838139) that is part of a haplotype associated with SMN1 duplication in silent carriers (2 copies of SMN1 on one chromosome and 0 to 5 copies of SMN2 on another)
no copies on the other) in some populations. **Conclusions:** The publicly available and renewable SMA RMAs developed during this study can be used by laboratories to design and validate assays as well as for quality control and proficiency testing. These and other reference materials characterized by the GeT-RM are available from the Coriell Institute for Medical Research.

### G16. A Rare Single Nucleotide Variant Causing a False-Negative HTT CAG Repeat Expansion Result in the Evaluation of a Patient for Huntington Disease

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**Introduction:** Huntington disease (HD) is an autosomal dominant, neurodegenerative disease resulting from the expansion of a CAG trinucleotide repeat tract in exon 1 of HTT. The length of the normal CAG repeat is 26 or fewer, whereas symptomatic individuals usually have repeat lengths of 36 or greater. Repeat-primed PCR is the most commonly used method to test for CAG expansions in HD. We present the case of a 58-year-old man with symptoms and imaging findings typical of HD. Genetic testing for HTT CAG repeat expansion at an outside laboratory demonstrated homozygous, non-pathogenic allele size of 15 repeats. His father had similar clinical symptoms and HTT test results, and autopsy findings were consistent with HD. Given high clinical suspicion of HD and the patient’s family history, HTT testing was repeated at our institution with a different assay. **Methods:** The number of CAG repeats in exon 1 of HTT was evaluated by the AmpliDex PCR/CE HTT kit (Auragen Inc.) using genomic DNA isolated from blood. A two-primer, anchor-primed PCR was performed, and the products analyzed by capillary electrophoresis. Peaks were compared to the ROX 1000 size standard and converted from size in base pairs to number of CAG repeats using the AmpliDex PCR/CE HTT Macro. The true alleles were distinguished as the highest fragment peaks. The PCR products were analyzed by Sanger sequencing. Identified sequencing variants were interrogated using publicly available databases. A literature review was conducted to identify other cases of false negative HTT testing. **Results:** One HTT allele of 15 repeats in the normal size range and one allele of 38 CAG repeats in the reduced penetrance range (36 to 39 repeats) were identified. Sanger sequencing identified a C to G single nucleotide variant (SNV) in the expanded allele immediately upstream of the first CAG repeat (HTT c.51C >G, p.Phe17Leu). This variant is not reported in publicly available databases (gnomAD or ClinVar) and has been reported once in the ENCODE database in the context of an aberrantly homozygous allele. **Conclusions:** We present a case of a patient with a high clinical suspicion of HD and initially normal HTT CAG testing. Repeat testing identified a 38-repeat allele which could explain the patient’s symptoms. A sample from the deceased father will be tested to confirm the finding. The failure of the first assay to amplify the expanded repeat is likely explained by the SNV interfering with primer annealing, resulting in amplification of only the normal allele. Although potentially rare, this case highlights the importance of orthogonal confirmation of HTT test results in the setting of high clinical suspicion and apparent homozygous sizing of the HTT CAG tract.

### G17. Amplification-Free Targeted Enrichment Powered by CRISPR-Cas9 and Long-Read Single Molecule Real-Time Sequencing Can Efficiently and Accurately Sequence Challenging Repeat Expansion Disorders

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**Introduction:** Genomic regions with extreme base composition bias and repetitive sequences have long proven challenging for targeted enrichment methods, as they rely upon some form of amplification. Similarly, most DNA sequencing technologies struggle to faithfully sequence regions of low complexity. This has been especially trying for repeat expansion disorders such as fragile X disease, Huntington disease, and various ataxias, where the repetitive elements range from several hundreds of bases to tens of kilobases. **Methods:** We have developed a robust, amplification-free targeted enrichment technique, called No-Amp Targeted Sequencing that employs the CRISPR-Cas9 system. In conjunction with SMRT Sequencing, which delivers long reads spanning the entire repeat expansion, high consensus accuracy, and uniform coverage, these previously inaccessible regions are now accessible. This method is completely amplification-free, therefore removing any PCR errors and biases from the experiment. Furthermore, this technique also preserves native DNA molecules, allowing for direct detection and characterization of epigenetic signatures. The No-Amp method is a two-day protocol that is compatible with multiplexing of multiple targets and multiple samples in a single reaction, using as little as 1 µg of genomic DNA input per sample. **Results:** We have successfully targeted a number of repeat expansion disorder loci including HTT, FMR1, C8orf72 as well as built an ataxia panel which consists of 15 different disease-causing repeat expansion regions. Using the No-Amp method we have isolated hundreds of individual on-target molecules, allowing for reliable repeat size estimation, mosaicism detection, and identification of interruption sequences with alleles as long as >2,700 repeat units (>13 kb). In addition to multiplexing several targets, we have also multiplexed at least 20 samples in one experiment, making the No-Amp Targeted Sequencing method a cost-effective option. **Conclusions:** Combining the CRISPR-Cas9 enrichment method with single molecule, real-time sequencing provided us with base-level resolution of previously inaccessible regions of the genome, like disease-causing repeat expansions. No-Amp Targeted Sequencing captures, in one experiment, many aspects of repeat expansion disorders which are important for better understanding the underlying disease mechanisms.

### G18. A Single-Assay Diagnostic Workflow for Genotyping and Phasing SNPs with Repeat Expansions for Allele-Selective Therapy in Huntington Disease

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**Introduction:** Huntington disease (HD) is a neurodegenerative disorder that affects 30,000 people in the US and is caused by expansions of >35 CAG repeats in exon 1 of the HTT gene. Wave Life Sciences has developed stereopure antisense oligonucleotides (ASOs) designed to selectively bind pathogenic mutant HTT (mHTT) transcripts, and preserve wild-type allele expression that may be neuroprotective and needed for normal brain functioning, by targeting SNPs such as rs362307 (SNP1) and rs362331 (SNP2) on the mHTT allele. Up to 90% of HD patients have SNP1 and/or SNP2 only on the mHTT allele which supports clinical trials such as WVE-120101 and WVE-120102 for allele-selective ASO therapies. Identifying patients with the target SNP on the mHTT transcript can be complex, however, because it requires phasing assays that can bridge the >7.5 kb gap between SNPs and the CAG repeat. We describe an accurate and streamlined HTT RT-PCR assay that genotypes SNP1 and SNP2, quantifies the CAG repeat tract, and links these sequences on the same allelic transcript. **Methods:** Blood was collected in PaxGene RNA blood tubes, and RNA was extracted and reverse transcribed. Phased SNP/CAG repeat amplicons were generated in successive PCR reactions and analyzed by capillary electrophoresis (CE) on an ABI 3500 Genetic Analyzer. SNP zygosity, CAG repeat length, and SNP/repeat phasing were determined from the CE electropherogram with a software algorithm developed using machine learning. The assay was evaluated with 78 blood samples from presumed healthy individuals, HD patient-derived cell-line samples, and 13 HD patient whole blood samples. **Results:** The concentration of blood RNA recovered from HD patients (mean = 83.3 ng/µL, SD = 41.7) was significantly lower (p = 0.013) than from presumed healthy individuals (mean = 127 ng/µL, SD = 70.7 ng/µL). RNA integrity was not significantly different (p = 0.44). All 8 HD cell-line samples and 12 of 13 clinical HD patient samples met PCR/CE assay signal criteria. Each sample was correctly phased and genotyped compared to reference results procured from a combination of HTT CAG repeat PCR, and Sanger and long-read sequencing analysis. One HD patient sample had low RNA concentration and yield, and failed to
produce an assay signal trace. **Conclusions:** Current methods for SNP genotyping and phasing with the HTT repeat tract rely on multiple workstreams and/or exploratory technologies. We describe an accurate, unified PCR-based workflow on an IVD-ready CE platform using automated genotyping capabilities with potential to expedite patient selection and improve the efficiency of clinical trials. The assay also has implications for diagnostics, including companion diagnostic kits, for other allele-selective, repeat expansion therapies.


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**Introduction:** Expansions of simple sequence repeats are responsible for more than 40 human diseases, with an increasing number of repeats resulting in more severe phenotypes. Repeat ranges are well documented for the classic repeat disorders and include normal, intermediate, reduced penetrance or pre-mutation and full penetrance or full mutation. It is important to accurately determine the number of repeats, especially for alleles that border categories. The repeat number of shorter amplicons can be determined using the internal lane standard (ILS) and size calling from genotyping software. However, amplicons containing repeats migrate faster than amplicons of equal length in the ILS, which is most pronounced with longer amplicons. Consequently, the number of repeats calculated for larger repeat amplicons via correlation with an ILS can result in underestimation of the true repeat number. **Methods:** Four independent methods for calculating the number of repeats were compared to using the ILS alone to calculate repeat size. Method one used a single control sample containing 4 or more fragments of known repeat size to generate a standard curve (peak size in base pairs versus known number of repeats), which was then used to determine the repeat size. The second method was similar to the first but used multiple calibrator samples that contained fragments of known repeat size. The third method used control samples of known repeat size that were merged in silico for calculation of the correction and mobility factors. The data analysis for methods 1 and 2 was performed using the repeat expansion analysis (REA) or the GeneMarker software, whereas the data for method 3 was analyzed independent of the REA. Finally, samples for fragile X syndrome testing were sized relying on the ILS. Samples with larger or absent amplicons were reflex tested with a size calibrated control. **Results:** Methods 1 and 2 were initially used to calculate the number of repeats for each sample. In a cohort of previously characterized Huntington disease patients, the ILS method was used to re-calculate the number of repeats. The preliminary data demonstrated that repeats in the normal range were consistently 3 bp apart, whereas alleles in the intermediate, inconclusive penetrance and full penetrance categories were consistently underestimated by the ILS method. **Conclusions:** Consistently, large repeats were underestimated using ILS sizing methods alone. Methods 1 and 2 are useful for characterizing diseases where the migration of fragments in the pre-mutation and full-mutation range vary significantly from the standard curve of known diseases where the migration of fragments in the pre-mutation and full-sizing methods alone. Methods 1 and 2 are useful for characterizing pharmacogenomics impact of the ADH5 genotypes in asthma is under investigation as part of the PrecISE (Precision Interventions for Severe and/or Exacerbation-Prone Asthma) Network Study. Therefore, we validated a clinical laboratory-developed assay to test for two variants in ADH5. **Methods:** DNA samples were obtained from Coriell Cell Repositories. Genotyping of ADH5 was performed using TaqMan SNP Genotyping Assays using probes detecting two different variants in the gene; rs7669660 (c.966A >G) and rs11547772 (c.775T >G). **Results:** A total of 183 DNA samples used as reference materials for pharmacogenomics clinical testing were run for genotyping of the two variants. The assay showed complete concordance with reported consensus results for the alleles tested with sequencing data available (n = 72; https://www.internationalgenome.org/1000-genomes-browsers/) for 100% accuracy. The analytical sensitivity is 100% (95% CI; 93 to 100), and the analytical specificity is 100% (95% CI; 98 to 100). The assay further identified genotypes of ADH5 for the remaining 111 samples that were not previously characterized. **Conclusions:** We validated a laboratory-developed test for genotyping two variants in ADH5, and further characterized the variants of the gene for the additional 111 samples. Characterized DNA reference materials are publicly available for assay development, validation, proficiency testing, and quality assurance, and will help study the implications of the ADH5 genotypes in the pharmacogenomics of asthma.

G21. CYP2D6 Guided Methadone Dosing in a Multi-Ethnic Popula


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**Introduction:** Methadone is used for heroin and opioid detoxification. Methadone treatment has grown substantially. There has also been an increase in methadone-related adverse events. Standard protocols use low initial dosing with slow adjustments. Dosing too slowly could promote relapse. Methadone is primarily metabolized via cytochromes 3A4 and 2D6. Polymorphisms in these subunits can change the enzyme activity and metabolism of methadone, changing its effects. Genotypic testing of relevant CYP subunits may allow more efficient dosing of methadone, reducing adverse events and time to adequate symptom management. **Methods:** After receiving approved consent, we assisted with buccal swabs from 30 methadone maintenance patients treated at Ku Aloha Ola Mao, a methadone clinic based in Honolulu, Hawaii. Patients’ ethnicities ranged from Caucasians, Hawaiians, Japanese, Filipinos, and predominately mixed ethnicities of Chinese, Japanese, Filipino, and Alaskan natives. DNA was extracted and run on a multitarget genotypic assay of SNPs in CYP3A4, 3A5, 2C9, 2C19, and 2D6. Analysis of CYP2D6 included assessments of copy number and rearrangement. Specific alleles and associated enzyme activity were determined. Enzyme activity was correlated with methadone maintenance dose. **Results:** Genotypic frequencies ranged from poor metabolizers to ultrarapid metabolizers in CYP2D6 and CYP2C19. CYP2D6 deletions (3) and duplications (3) were identified. A statistically significant difference in methadone dose was present between CYP2D6 metabolizers, with the highest dosages of 210 and 240 mg QD among CYP2D6 URMs (p = 0.0380). There were no significant differences in dosage among the other CYP subunits. CYP2D6 intermediate metabolizers trended toward longer time on methadone therapy. **Conclusions:** Pharmacogenomic testing may achieve a better approximation of the daily methadone dose. This testing could help achieve a faster serum steady state and, more importantly, symptom relief. This is a pilot study. This positive correlation between cytochrome P450 polymorphisms and methadone final dosage supports a larger-scale study that may permit effective guidelines in methadone therapy. A guided methadone treatment plan can identify ultra-rapid metabolizer patients to expeditiously get them to therapeutic dose and direct them to additional and alternative therapeutic options.
G22. Developing DPYD Genotyping Method for Personalized 5-fluorouracil Therapy
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Introduction: 5-fluorouracil (5-FU) is widely used for patients with solid tumors, and is the chemotherapy backbone in colorectal and other gastrointestinal chemotherapy regimens. It has been reported that as many as 10% to 30% of patients treated with 5-FU-based drugs experience severe toxicity. The DPYD gene encodes the rate-limiting enzyme dihydropyrimidine dehydrogenase (DPD) responsible for 5-FU catabolism. The US Food and Drug Administration (FDA) added warning statements to the drug labels for 5-FU against its use in patients with DPD deficiency. Since 2017, the Clinical Pharmacogenetics Implementation Consortium (CPIC) has updated its practice guideline for DPYD genotype and fluoropyrimidine dosing. This study aims to develop a robustness, reliability, and accuracy of the assay met the requisite clinical laboratory quality standards. It can be used for screening for variants and optimizing assay conditions. Proficiency testing samples with known genotypes were analyzed for test validation. All variants detected were confirmed by Sanger sequencing.

Methods: Based on the CPIC guideline, we developed an allele-specific polymerase chain reaction (PCR)-based DPYD genotyping panel for the detection of five variants: c.1905+1G >A (*2A, rs3918290), c.1679T >G (*13, rs66970783), c.2846A >T (rs67370738), c.1129-5923C >G (rs75017162), and c.557A >G (*13, rs55886062). Healthy population samples were used for screening for variants and optimizing assay conditions. In healthy population samples, five samples were found to be heterozygous for the c.557A >G variant (out of 122 African samples screened), two were heterozygous for c.1129-5923C >G (out of 49 Caucasian samples), and one was heterozygous for c.2846A >T (out of 96 Caucasian samples). There were no positive samples found for the c.1679T >G and c.1905+1G >A variants in 384 Caucasian samples screened. All proficiency samples tested (n = 20) were concordant with the assigned genotype results, including wild-type (n = 13), c.1679T >G (n = 3), c.1905+1G >A (n = 2), and c.2846A >T (n = 2) genotypes.

Conclusions: Our DPYD genotyping method has been developed for the detection of the common variants recommended by the CPIC guideline. The robustness, reliability, and accuracy of the assay met the requisite clinical laboratory quality standards. It can be used for screening DPYD variants to identify patients with increased risk of toxicity when prescribed 5-FU based therapy.

G23. High-Throughput Fetal-Fraction Amplification Increases Analytical Performance of Noninvasive Prenatal Screening
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Introduction: Since its introduction into clinical care nearly a decade ago, noninvasive prenatal screening (NIPS) based on cell-free DNA (cfDNA) has provided millions of pregnant women with information about their risk for fetal chromosomal abnormalities. A primary driver of NIPS sensitivity and specificity for aneuploidy in a given maternal plasma sample is the fetal fraction (FF), which describes the proportion of cfDNA fragments that originate from the placenta. For the majority of samples, FF values are between 4% and 30%, and many laboratories fail samples with FF <4% to diminish the risk of issuing false-negative reports. On certain NIPS platforms, however, FF values of women with high body mass index (and >5% overall) receive a test failure due to low FF (44%). Methods: A scalable fetal-fraction amplification (FFA) technology that is routinely applied to all samples undergoing whole-genome sequencing (WGS)-based NIPS in our laboratory was analytically validated on 1,264 samples tested with and without FFA. Results: Zero samples had FF <4% when screened with FFA, whereas 1 in 25 of these same patients had FF <4% without FFA. The average increase in FF was 2.9-fold for samples with low FF (2.3-fold overall), and 99.8% had higher FF with FFA. For all abnormalities screened on NIPS, z-scores increased 2.2-fold on average in positive samples and remained unchanged in negative samples, powering an increase in NIPS sensitivity and specificity. With FFA, the combined sensitivity for five common microdeletions is 97.2% with a specificity of 99.8%. For DiGeorge syndrome in particular, FFA has an expected analytical sensitivity of 95.6% with an analytical specificity of 99.5%. Finally, FFA enables better distinction of male and female fetuses, reducing the chance of sex miscalls due to borderline FF values by an estimated 318x. Conclusions: FFA transforms low-FF samples into high-FF samples. By combining FFA with WGS-based NIPS, a single round of NIPS can provide nearly all women with confident results about the broad range of potential fetal chromosomal abnormalities across the genome.

G24. A Software Tool That Prevents Incorrect Estimations of Gestational Age and Maternal Age at Estimated Date of Delivery
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Introduction: The recent College of American Pathologists (CAP) noninvasive prenatal testing (NIPT) educational exercises revealed that a significant number of laboratories incorrectly estimated the gestational age (GA) at the time of blood draw and the maternal age at the estimated date of delivery (EDD). Based on the CAP NIPT Participant Summary Report (PSR), the incorrect estimation was likely due to using the ultrasound (US) date for the calculation when the draw date and US date were different. In other cases, maternal age at draw date was calculated, but not at EDD. Clearly the discrepancy of some of the decimal weeks/years was due to implementing an incorrect formula. The primary concern is that the inaccurate estimations of the GA at the draw date may affect the proper evaluation of whether a sample is acceptable for testing in a lab, and that the wrong maternal age at EDD may impact the reporting of the corresponding positive predictive value (PPV) based on maternal age at EDD for positive NIPT screening results. To address these issues, we created a graphical user interface (GUI) Java program to calculate the GA, EDD, and maternal age at EDD for NIPT testing to eliminate the errors presented in the CAP surveys. Methods: To make our software tool useful to most of the laboratories who perform NIPT tests and doctors who order those tests, we designed a program based on information needed from requisition forms by major NIPT testing labs. Our program uses the built-in Java system date utility to ensure that accurate dates were used in the calculations of the GA at the time of draw date, EDD, and maternal age at EDD. To ensure the accuracy of the calculations, we conducted blind tests using the datasets from the 2018 and 2019 CAP educational exercises, and compared our results with the intended solutions provided in the CAP PSRs. Results: Our tool correctly generated GA, and maternal age at EDD for all samples from the 2018 and 2019 CAP educational exercises, by either performing calculations of each sample individually through manual entry or a batch calculation of all the samples together from an Excel spreadsheet. Our program can be used on its own, through the web, and as a library for other programs. Conclusions: Our software tool is able to correctly estimate GA at the draw date, EDD, and maternal age at EDD for NIPT testing, regardless of the information provided at the time of test request. In addition, our tool allows for batch data processing and individual calculations as well as integration into a lab’s software program. The ability to output other calculated values, such as EDD, can help ordering physicians fill in NIPT requisition forms according to the testing lab’s requirements.

G25. Genetic Insights and Incidental Findings from Maternal Cell Contamination Testing
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Introduction: Prenatal specimens such as amniocytes, chorionic villus tissue, and products of conception have the potential to contain maternal cells in addition to cells derived from the fetus. The presence of maternal cell contamination (MCC) can confound the interpretation of prenatal tests; therefore, these specimens are often separately assessed using methods which are designed to detect the presence of any maternal-specific alleles in the fetal specimen. Methods: MCC testing is routinely performed in our laboratory using capillary electrophoresis followed by fragment analysis of the PCR products from 24 polymorphic short tandem
repeats from the gestational carrier’s blood and the fetal specimen. The results of the MCC test are reported separately but in conjunction with the results of the prenatal test such as a chromosomal microarray or an exome. The MCC test is specifically validated to assess whether an admixture of maternal cells exists among the fetal cells in a prenatal specimen. Results: We present three cases where the MCC result provided confirmatory and additional information about the genetic mechanism and relationship of the gestational carrier and fetal sample. Case 1 was diagnosed as trisomy 21 from a microarray performed on the products of conception (direct fetal tissue). The MCC confirmed the results and provided supportive evidence that the extra chromosome 21 came from the father. Case 2 was diagnosed as triploidy with a 49, XXX karyotype from a microarray performed on the products of conception (direct fetal tissue). The MCC confirmed both of these results and provided sequence variation, which did not correlate with the chromosome copy number. Case 3 was from a female who had undergone IVF and had her own fertilized egg and donor fertilized eggs implanted. The MCC test revealed that the fetus was from the gamete donor. Conclusions: MCC testing is mainly used for quality assurance of the fetal sample. Any additional allelic information obtained from the MCC result is not typically reported; however, it can corroborate assurance of the fetal sample. Any additional allelic information obtained from the MCC test revealed that the fetus was from the gamete donor.

G27. Two-Site Evaluation of a Rapid and Simple CFTR PCR/CE Assay and Software Targeting Mutations across Diverse Ethnic Groups
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Introduction: Cystic fibrosis is a progressive, hereditary disease characterized by the accumulation of viscous mucus in multiple organs and caused by mutations in the CFTR gene. Although more than 2,000 genetic mutations have been identified to date, >250 of these are pathogenic, and their prevalence is dependent on ethnicity. Recent American College of Medical Genetics (ACMG) guidelines, a 23-variant panel is minimally recommended for targeted testing. Here, we describe a targeted assay that addresses >92% mutant prevalence represented in different databases and across diverse populations (CFTR2 database, gnomAD, Beaugachamp, et al., 2019) and detects >85 pathogenic variants, including the ACMG-23. We report a two-site evaluation of a prototype of this PCR/capillary electrophoresis (CE)-based assay using independently genotyped, residual clinical samples. Methods: Seventy-four previously genotyped residual clinical samples and eight controls were assayed by two laboratories. Samples were amplified in a two-tube assay using Amplitude PCR reagents, and resolved by CE on the Applied Biosystems 3500xl. One site also analyzed the samples on 3130xl, 3730xl, and SeqStudio Genetic Analyzers. Genotypes were determined by prototype modules in GeneMapper 5.0, and compared using automated peak calling and classification software at one site. Each sample batch was processed within five hours from PCR setup through CE and peak calling. Results: Using the CFTR PCR/CE assay, both sites accurately genotyped 74 clinical samples, including 53 with CFTR variants (48 heterozygous and five homozygous, harboring 21 distinct variants) and controls; all genotypes at both sites agreed with reference results. IVS-8 poly-T and TG repeats were also accurately sized between the two sites associated with 21q22 and CFTR. Conclusions: In this work, we report the assessment of targeted testing for 23 pathogenic variants across multiple ethnic groups and individuals across two laboratories. Both sites achieved high concordance using an automated CE peak calling and classification software. The current assay may be adapted to any CE system via peak calling and classification software. This assay is likely more cost-effective than a targeted next-generation sequencing approach.

G28. The Relationship between Variant Type and Phenotype among Diseases Screened by the Foresight Expanded Carrier Screen
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Introduction: Using a high-throughput approach to expanded carrier screening in healthy adults, Myriad Genetics has identified more than 793,000 sequence variants across >175 genes associated with autosomal recessive and X-linked diseases. Among these variants, more than 37,000 variants have been classified as disease causing. To gain greater insight into how different sequence alterations (SNVs, small INDELs, CNVs) and their location within a gene (exonic, intronic, splice site, UTR, initiation codon, stop loss) contribute to disease, we analyzed total and gene-specific distributions of pathogenic variation by variant type. To our knowledge, this work represents the most comprehensive summary of gene-specific pathogenic variant distribution across the diseases we tested. These data provide confirmatory and additional information about the genetic implications for addressing health disparities.

G29. SMN1 and SMN2 Copy Number Distribution in 733 Clinical Cases of Carrier Screening for Spinal Muscular Atrophy
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Introduction: Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by degeneration of alpha motor neurons, leading to muscular weakness and atrophy. SMA is usually caused by a homozygous deletion of the SMN1 gene, including at least exon 7. In patients with SMA, disease severity correlates with copy number of the SMN1 paralog, SMN2. SMA carrier screening is now recommended to all women considering pregnancy or currently pregnant, leading to significant increases in screening volumes in the clinical lab. The pan-ethnic population carrier frequency of SMA is approximately 1 in 50, and as high as 1 in 35 in white/Caucasian ethnic populations. This study reviews the results from the first 733 carrier screening cases and the distribution of SMN1 and SMN2 copy numbers across our patient population. Methods: Extracted DNA from whole blood was obtained from 733 patients undergoing SMA carrier screening. We validated the droplet
digital PCR (ddPCR; BioRad) assay and currently utilize it to clinically assess copy number variation of SMN1 and SMN2. A DNA input of 40 ng is used for the ddPCR protocol, which targets exon 7 of the SMN1 and SMN2 genes, as well as a reference gene. Allele, carrier, and presumed silent carrier frequencies for the first 733 cases are reviewed. **Results:**

The first 733 clinical carrier screening cases processed in our lab resulted in the following SMN1 copy number distribution: 23/3 (14%) with 1 copy, 650 (84.6%) with 2 copies, 59 (8.05%) with 3 copies, and 1 (0.14%) with 4 copies. The SMA carrier frequency identified in our population is approximately 1 in 32 (23/733 have one copy of SMN1 only), which is similar to or slightly higher than the Caucasian population frequency estimate of 1 in 35. The allele frequency of a zero-copy allele of SMN1 is at least 23/1,466 (>1.5%). In addition, if we assume that individuals with three or four copies of SMN1 have two copies of SMN1 in cis, then 61/466 alleles in our population have duplicated SMN1 copies on the same allele. Combining this frequency with the allele frequency of zero copies of SMN1 (23/1,466) gives a 0.065% or 1 in 1,538 chance of being a silent (2+0) carrier in our population. Given our SMA carrier frequency of 1 in 32, these results indicate that approximately 1 in 48 carriers are silent carriers (0.08%), which is similar to the estimated 2% of carriers suggested by the literature. **Conclusions:** Using ddPCR for the first 733 carrier screening cases showed an interesting SMN1/SMN2 copy number distribution, and suggests an expected or slightly higher SMA carrier rate in our rural population of New Hampshire and Vermont. This analysis of our SMA ddPCR assay is useful as a quality assurance metric, demonstrating ongoing high-quality assay performance.

**G29. Proof-of-Concept for Single-Platform Trio Carrier Screening of FMR1, SMN1/2, and CFTR Variants Using PCR and Capillary Electrophoresis with Consolidated Workflows**


**Asuragen, Inc., Austin, TX.**

**Introduction:** Carrier screening can provide valuable information for couples to help guide their reproductive decision-making. Screening studies have shown that as many as 1 in 20 individuals is a carrier for at least one of the conditions: spinal muscular atrophy, fragile X syndrome, and cystic fibrosis. The genes causing these disorders each present a unique technical challenge, and each usually requires a distinct molecular diagnostic method and analysis platform. As a result, a simple, cost-effective, and unified screening system is not yet available for this trio of carrier genes. Here we demonstrate the feasibility of analyzing combinations of PCR products from these genes on a single capillary electrophoresis instrument using a co-injection strategy. **Methods:** DNA samples were PCR amplified using AmpliDx PCR/CE FMR1 and SMN1/2 Plus kits (Asuragen) and electrophoresed on the Applied Biosystems 3700 and 3730 Series Genetic Analyzers (Thermo Fisher Scientific). FMR1 amplicons (labeled with FAM) were combined with SMN1/2 products (labeled with HEX) to create samples with different genotype combinations. Twelve different cell-line DNA samples representing zero to 4 copies of SMN1 or SMN2, and FMR1 genotypes of normal, premutation, intermediate, or full mutation were used to generate 86 sample combinations. Data were processed using AmpliDx Reporter Software. **Results:**

- **Co-injection of CFTR and SMN1/2 amplicons was demonstrated on a 3500 CE instrument using 94 combinations of CFTR amplicons (created from prototype reagents) and SMN1/2 amplicons of different genotypes.**

- **Excluding controls for each assay, the overall percent agreement between the genotype calls of the co-injected PCR amplicons and their individually injected counterparts was 100% for SMN1/2 and FMR1 on the 3500 instrument for 62 samples.** For the 3730, the concordance was 100% and 96.5% for SMN1/2 and FMR1, respectively. The co-injection of FMR1 and SMN1/2 products did not alter the genotype category or copy number calls when compared to their respective, individually assessed results. Similarly, the co-injection of CFTR and SMN1/2 amplicons did not affect variant or copy-number calls compared to the analysis of single-assay amplicons. **Conclusions:** The co-injection of PCR products of FMR1 and SMN1/2 genes can be achieved using commonly available CE instruments. This approach consolidates existing workflows and improves run and cost efficiencies. Our study demonstrates the potential for a unified single-instrument analysis of FMR1, SMN1/2, and CFTR using a simple, scalable, and cost-effective system to meet the increasing needs of carrier screening.

**G30. The Single-Tube SLIMamp NGS Assay for Detection of Mutations Associated with Thalassemia Is both Rapid and Robust**


**Asuragen, Inc., Austin, TX.**

**Introduction:** Next-generation sequencing (NGS) is widely used clinically for carrier screening tests (CSTs) for monogenic disorders. However, variant detection, especially of structural variants (SVs), remains a challenge in highly homologous genes such as HBA1 and HBA2 for alpha-thalassemia. Beta-thalassemia is caused by HBB mutations, which are mostly SNVs and small indels (sindels). Most CSTs require two assays for deletion detection and SNVs/sindels. To enable the simultaneous detection of SNVs, sindels, and SVs, we developed a single-tube SLIMamp multiplex-PCR assay, the INHERIT/Reveal Thalassemia Panel, to cover the extended regions of HBA1, HBA2, and HBB. Also, we developed a proprietary CNV caller in our NGS analysis tool (PiVAT). Additionally, known SVs are also detected with gap-PCR NGS in the same pool, which enables the precise identification of the breakpoints. **Methods:** The panel contains 131 amplicons ranging from 130 bp to 174 bp. The CNV caller integrates various approaches to minimize coverage variations caused by different reagent lots and different batches of sample prep. A set of 102 samples with 57 known positive SVs in the HBA region and 45 SV-normal samples was used for CNV caller development. A different set of 20 blinded samples with 15 HBA CNVs was used as an external validation. Of the 122 samples, 45 samples had pathogenic SNVs in the three genes. All mutations were confirmed by Sanger sequencing and conventional gap-PCR assays. To assess assay performance, representative samples were tested with different input amounts and run in three batches. All libraries were sequenced with the 2x150bp protocol on Illumina MiSeq or NextSeq and the data were analyzed by PiVAT 2020. **Results:** The assay performed well with a mapping rate of 99.7% ± 0.1%, on-target rate of 98.5% ± 0.1%, and coverage uniformity >0.2x the mean of 98.5% ± 2.2%. The CNV caller detected all 57 HBA SVs in the training set and all 15 HBA SVs in the validation set. All the detected SVs were correctly identified and annotated with the named CNV types (e.g., SEA, Thai). All 69 confirmed SNVs and sindels were detected. In a 3-run reproducibility study with as little as 5 ng of input DNA, all 7 HBA-CNV types were correctly identified, except for one case of alpha-3.7dup. An average coverage depth of 400 reads was sufficient for the variant calls, suggesting high sample multiplex levels can be achieved per sequencing run. **Conclusions:** The INHERIT/Reveal Thalassemia Panel provides robust detection of CNVs, SNVs, and sindels from a rapid, high-throughput NGS assay. Furthermore, the PiVAT software is optimized to detect multiple variant types, and identifies and annotates conventional HBA SVs (e.g., alpha-3.7).

**G31. Exploring Mosaic Mutations in Megalencephaly and Other Growth Disorders by Next-Generation Sequencing**

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**Introduction:** This is a retrospective study aimed at analyzing genetic variants and levels of mosaicism identified in a cohort of patients clinically tested for brain and body overgrowth phenotypes between 2014 and 2019 through the Megapixel multi-gene panel offered at the University of Washington. We analyzed the megapixel data to further characterize the molecular basis of overgrowth phenotypes and to optimize future interpretation and analysis of this panel. **Methods:** In this study, we examined samples from 173 clinical patients diagnosed with brain and body overgrowth disorders. An additional 27 samples were collected from parents to determine the inheritance of compelling variants. The panel consists of 37 genes known to be associated with brain and body overgrowth disorders. DNA samples were extracted from blood in 56% of the samples, from tissues in 36%, and from other tissue sources including saliva, bone and amniocytes in 8% of samples. Capture-based Next-
Generation sequencing (NGS) was performed using custom-designed Sure Selecte probe libraries and analyzed using short read sequencing on Illumina HiSeq 2000 or MiSeq sequencers. Identified mutations were confirmed and analyzed using a custom in-house bioinformatics pipeline. 

Results: Of the 200 samples tested, 64 (32%) and 17 (8%) had pathogenic or likely pathogenic mosaic mutations, respectively. Most of these variants were in PIK3CA (N = 50, 25%), and PTEN (N = 6, 3%). There were no pathogenic or likely pathogenic mutations reported in 91 cases (46%). In this study, variants of uncertain significance were reported in 28 (14%) of cases, 7 of which carried unique de novo mutations. DNA from affected tissue samples was more likely to yield mosaic findings than DNA extracted from blood. 

Conclusions: Ultra-deep NGS can efficiently identify mosaic mutations in megalenopahy and overgrowth disorders including: loss of 13q14 and/or loss of TP53 in 9% of nuclei, and was negative for all other markers tested for patients with Noonan syndrome. 

Hybridization (FISH) analyses identified trisomy 12 in 77% of nuclei and 80% of cells. Concurrent molecular studies detected a p.V157F mutation, and the presence of the p190, is 0.0016 and it is <0.1, so the efficiency of the two targets’ amplification was approximately equal. The assigned percentage of BCR-ABL1/IgH International Scale values of the 4 levels of the secondary material panels were 0.0013, 0.0154, 1.3808 and 19.4266, respectively. In validation trials, 82.8% (24/29) of laboratories obtained valid conversion factors for the p190 BCR-ABL1 t assay. 

Conclusions: We developed an innovative, easy, and effective approach to achieving standardization for the quantification of p190 BCR-ABL1 FG transcripts. Our study could facilitate the time-consuming and expensive process of standardization, and might contribute to studies of the management of p190-positive leukemia. This strategy can also be applied to the standardization of other fusion genes and other types of reference materials.

Hematopathology

H03. Limitation in Confirming Low Allele Frequency Calls from Sensitive Cancer Assays: MSK Experience with the LiquidPlex cDNA Panel on Hematologic Samples


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Introduction: To address the need for next-generation sequencing (NGS) assays with high sensitivity, quick turnaround time (TAT), and low DNA input requirements, we assessed the viability of using a sensitive 28 gene cDNA LiquidPlex panel on hematologic samples processed in parallel with our 94 cancer gene MSK-Ampliseq NGS assay. Although these two amplicon-based assays have similar TAT, the cDNA LiquidPlex assay is more sensitive. 

Screened LiquidPlex variants below the limit of detection (LOD) of the MSK-Ampliseq assay were confirmed with a longer TAT MSK-IMPACT Heme NGS assay or an ultrasensitive dPCR assay. 

Uncertainty remains for LiquidPlex calls under the LOD of the available clinical assays. 

Methods: Library construction of 111 heme samples, including 31 blood and 80 bone marrow samples, was performed on 40 ng of DNA by multiplexed amplification of 238 LiquidPlex and 2,126 Ampliseq amplicons, followed by sequencing on Illumina NexiSeq500 or Ion Torrent SS Sequencer, respectively. Due to unique molecular barcodes ligated to the sample original fragments, sequencing errors are filtered out by the Archer LiquidPlex analysis detecting real variants at 0.2% variant frequency (VF). SNVs were filtered at 5% and 1% VF by the AmpliSeq and IMPACT custom pipelines, respectively, and 0.1% VF for the
H04. Somatic Mutation Testing for Pediatric Patients with Known or Suspected Inherited Bone Marrow Failure Syndromes

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Introduction: Inherited bone marrow failure syndromes (IBMFS) are a clinically, morphologically, and genetically heterogeneous group of disorders that typically manifest in childhood. Patients with IBMFS are at increased risk for developing myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). In the context of worsening cytopenias and/or morphologic dysplasia, targeted next-generation sequencing (NGS) to assess for pathogenic somatic mutations may herald neoplastic transformation and guide clinical management. Methods: Forty-three patients over a 12-month period with known or suspected IBMFS underwent somatic NGS testing (PLD-NGS) of bone marrow aspirate DNA. Additional clinical and laboratory data and germline sequencing results were collected from retrospective chart review. Barcoded libraries were constructed with the KAPA Biosystems HyperPlus kit followed by capture hybridization-based target enrichment using a custom-designed Roche NimbleGen SeqCap Target Enrichment probe set enriched for 152 genes mutated in hematologic malignancies. After sequencing on the Illumina MiSeq System, FASTQ files were aligned to the GRCh37 (hg19) reference human genome using Burrows-Wheeler Aligner and NextGEn v2.4.1.2. Variant calling was performed using NextGEn v2.4.1.2 and Platypus v0.8.1, and variants were annotated with Variant Effect Predictor. Results: Germline sequencing data were available for 32 of 43 patients (22 male, 21 female; median age: 11.2 years [4 months to 19.5 years]). Sixteen patients (37.2%) harbored pathogenic/likely pathogenic germline variant(s) in SBDS (n = 5), ELANE (n = 4), DKC1, FANCA, FANCG, RAD50, RPL11, SAMD9, or SAMD9L. PLD-NGS detected a U2AF1 p.S34Y mutation in a patient with Shwachman-Diamond syndrome and mild megalakaryocytic hypoplasia. In an AML patient with features suggestive of a germline GATA2 deficiency, PLD-NGS detected GATA2, NFI, NPM1, and TET2 mutations. Variants of confirmed somatic origin. PLD-NGS also unmasked germline variants in two patients. One patient with evolving MDS and monosomy 7 harbored TP53N1, SETB1, and ASXL1 somatic mutations as well as a SAMD9 p.F437C germline variant at a variant allele fraction of 7.3%, consistent with adaptation by aneuploidy. In another patient with persistent thrombocytopenia and no known genetic etiology, an ET6V p.T1390A mutation was detected and familial thrombocytopenia was diagnosed after germline confirmation. Conclusions: Somatic mutation testing proved clinically useful in our pediatric cohort by revealing clinically significant mutations consistent with evolving neoplastic processes, confirming the somatic origin of potential germline mutations, and also unmasking previously unrecognized germline variants. Both germline and somatic mutation testing should be considered in pediatric patients with known or suspected IBMFS.

H05. A Highly Reproducible Single-Day FISH Assay for Detection of t(11;14) in Multiple Myeloma Patient Samples

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Introduction: Multiple myeloma (MM) is a malignancy of clonal plasma cells (PC) characterized by well-defined cytogenetic abnormalities, including the t(11;14) translocation. Recently, the targeted BCL-2 inhibitor venetoclax demonstrated a 40% overall response rate as a monotherapy in patients with the t(11;14) relapsed/refractory MM, improving to 60% in combination with dexamethasone. Herein, we report comparability and precision of a novel single-day single-color FISH assay using only (UIO) t(11;14) fluorescence in situ hybridization (FISH) assay. Methods: The t(11;14) status was determined with the Vysis CCND1/IGH XT FISH Probe premixed in Vysis IntelliFISH Hybridization Buffer in a 2 to 4 hour FISH assay. Assay reproducibility was evaluated across 3 testing sites, 3 reagent lots, multiple days, instruments, and operators. The 14-specimen reproducibility panel included 4 negative, 4 near cutoff, 4 low positive, and 2 high positive specimens. PCs from MM patient bone marrow aspirates were immunomagnetically selected using EasySep CD138 reagents (STEMCELL Technologies). The endpoint for reproducibility analyses was the qualitative assay result: abnormal (positive) or normal (negative). Overall agreement (OA), positive percent agreement (PPA), and negative percent agreement (NPA) between expected result (determined for each specimen by a majority call method) and FISH test result were calculated. OA, average positive agreement (APA), and average negative agreement (ANA) were calculated for site-to-site, lot-to-lot, and day-to-day pairwise comparisons. The precision analysis endpoint was the mean CCND1/IGH enumeration results (percentage of abnormal nuclei exceeding cutoffs). Results: For all 14 panel members combined, the OA, APA, and NPA between expected result and FISH test result were 99.7% (95% confidence interval CI), 99.9% (95% CI), 99.9% (95% CI), and 100% (95% CI), respectively. Precision comparative analyses for each panel member: OA, APA, and ANA for site-to-site, lot-to-lot, and day-to-day ranged from 89% to 100%, 94% to 100%, and 100%, respectively. For precision analysis, the percent coefficient of variation (%) ranged from 0.3% (high positive panel member) to 34.1% (near cutoff panel member). For all negative panel members, the percentage of abnormal nuclei exceeding cutoffs was zero; therefore, percentage of CI could not be calculated. Conclusions: The Vysis IntelliFISH CCND1/IGH XT FISH Probe assay is highly reproducible for assessment of t(11;14) status in MM patients. The single-day FISH assay facilitates rapid testing turnaround time, which is particularly important for MM patients who may qualify for ongoing clinical trials with novel biomarker-directed therapeutics.
HiSeq 2500 instrument (Illumina, San Diego, CA). Samples were then separately processed with the KAPA HyperPrep library protocol (Roche, Pleasanton, CA), and captured independently with DNA-based xGen Lockdown Probe Pool (IDT, Coralville, IA) and Twist Oligo Pool (Twist Biosciences, San Francisco, CA) baits. Two samples were individually captured with each DNA reagent set, and the other 6 samples were co-captured in single plate wells, before sequencing on a MiSeq instrument (Illumina, San Diego, CA).

**Results:** A total of 210 single nucleotide variants and indels were identified in the set of 8 samples by the current SureSelect method. Results were concordant for 205/210 variants (97.2%) with both IDT and Twist data sets, with the discordant results attributed to chemistry and informatics pipeline differences. On-target read averages were similar between SureSelect (48%) and Twist (50%), with IDT performing most efficiently at 63%. Single versus co-captured samples had negligible differences in on-target read metrics in Twist data, but single-capture IDT data was within two standard deviations lower than the co-capture set. The MiSeq-based capture protocols required 13.5 hours of laboratory time from sample receipt to final library generation, compared to 34 hours for SureSelect.

**Conclusions:** This focused comparison of capture-based NGS protocols revealed very close performance in variant call accuracy and on-target metrics, despite differences in library preparation and sequencer platform. These data suggest that high performance can be expected when using different capture reagents, with choices based largely on considerations of cost, speed, and efficiency (i.e., co-capture capability).

H08. **Genomic Landscape of Primary Breast Lymphoma Diffuse Large B-Cell Lymphoma (PB-DLBCL)**

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**Introduction:** Primary breast diffuse large B-cell lymphoma is a rare subtype of non-Hodgkin lymphomas (NHL). Less than 1% of NHL and 3% of extranodal lymphoma cases were PB-DLBCL. A previous study revealed that MYD88 L265P and CD79B mutations were frequently detected in PB-DLBCL, and that they might play an important role in PB-DLBCL lymphomagenesis. Here, we further profiled the molecular landscape of PB-DLBCL patients by next-generation sequencing (NGS).

**Methods:** Formalin-fixed, paraffin-embedded (FFPE) tissue samples from 14 PB-DLBCL patients were retrospectively collected between January 2009 and December 2019 from The Forth Hospital of Hebei Medical University and sequenced by target-panel of 112 genes related with lymphoma. The genomic pattern of this PB-DLBCL cohort was then compared to a public dataset of DLBCL from NCBI, comprising 1,001 patients, including 74.9% (n = 750) intranodal DLBCL, 3.49% (n = 35) primary testicular DLBCL (PTL), and 2.39% (n = 24) primary central nervous system DLBCL (PCNSL).

**Results:** In total, we identified 203 mutations spanning 36 genes from PB-DLBCL patients. The most frequently mutated genes included PIM1 (70%), MYD88 (64%), CD79B (36%), DTX1 (36%), TIPKB (29%), KMT2D (29%), PRDM1 (21%), and CD58 (21%). Significantly higher mutation rates of PIM1 (78.6% versus 15.3%; P < 0.01), MYD88 (64.3% versus 14.9%; P < 0.01), CD79B (35.7% versus 3.4%; P < 0.01), CD58 (21.4% versus 2.3%; P < 0.01), and PRDM1 (21.4% versus 2.8%; P < 0.01) among PB-DLBCL patients were detected, compared to the patients with intranodal DLBCL. In addition, mutation rates of PIM1 (78.6% versus 45.7%; P = 0.056), MYD88 (64% versus 21.4%; P = 0.01), and CD79B (35.7% versus 2.3%; P = 0.015) were higher in PB-DLBCL than PTL, although they did not reach statistical significance because of a small sample size. Meanwhile, PB-DLBCL harbored a significantly higher likelihood to mutate in PIM1 (78.5% versus 20.8%; P = 0.01) compared to PCNSL.

**Conclusions:** With 112-gene panel target-sequencing, ours is the first study that depicts the genomic landscape of PB-DLBCL, which shows distinct gene pattern compared with other types of DLBCL.
AMP Abstracts

manual Integrative Genomics Viewer review included six ASXL1 variants, six UTR/intronic/splice site, and six miscalls. Additionally, two variants were detected with this assay but not on the Illumina platform. **Conclusions:** The 40 genes in this panel have been described as having clinical relevance in myeloid neoplasms. A few of the genes in this panel are also known to have diagnostic or clinical utility in lymphoid neoplasms. The Ion Torrent Oncomine Myeloid Research Assay, when implemented on the Ion Chef System, relinquishes some flexibility in return for a reliable user-friendly library prep workflow. We found several discrepancies between the two assays, mostly of ASXL1 and intronic variants, but most could be rectified with targeted manual gene review.

H10. Proteomics-Based Biomarkers in Squamous Cell Carcinoma: A Pilot Study Correlating Proteomic Profiles and Tumor Differentiation

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**Introduction:** Squamous cell carcinoma (SCC) is a tumor seen in the sun-exposed parts of the skin, upper aero-digestive tract, and ano-genital mucosa, among others. Actinic injury often predisposes to cutaneous SCC, whereas some of the mucosal neoplasms have a viral basis. The 5-year survival rate is better in well differentiated tumors that are detected early, but is lower in those that are poorly differentiated, or present late with metastases. In this context, novel tumor biomarkers can be helpful if they can predict prognosis, and assist in the planning of treatment protocols. While optimizing a pilot project on biomarker discovery in SCC, we examined the relationship between tumor differentiation and proteomic profiles to ascertain whether distinct patterns might emerge between low and high grade tumors. **Methods:** Tissue micro-dissected from archived paraffin-embedded SCC tumor and normal tissues from 14 patients with ten years of clinical follow-up were available for this study. The tumors were categorized into well-differentiated (WD), moderately differentiated (MD), and poorly differentiated (PD) carcinomas based on their histologic characteristics. Protein extractions were performed with commercial and in-house reagents, which liberated peptides after trypsin digestion. Mass spectrometric analysis (Orbitrap) was performed as per established protocols documented earlier. **Results:** Data were entered as spectral quantitative counts derived from the Mascot/Scaffold/X-Tandem program. More than 3,500 proteins were generated from most cases. For comparison of proteins identified in tumor versus surrounding normal tissue, p values and fold changes were calculated and sorted to generate a list of unique proteins that were significantly expressed in tumors but not in normal tissue. A total of 634 unique proteins were common to all 3 tumor types. WD tumors had 297 unique proteins, MD had 268 proteins, and PD had 43 proteins that were exclusively expressed in those groups alone. These potential biomarker proteins are currently being evaluated and correlated with clinical and therapeutic parameters to establish whether useful prognostic information can be obtained to predict outcomes. **Conclusions:** Bioinformatic analysis of mass spectrometry-based proteomic data can reveal distinct profiles that are unique to morphologically defined subsets in squamous cell carcinoma. Only a few proteins overlapped among tumor subsets in this pilot study. Expanding the search to a larger series of cases will improve the statistical validation of our findings.

H11. Detection of Low-Frequency Variants for Minimal Residual Disease (MRD) Monitoring of Acute Myeloid Leukemia


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**Introduction:** Monitoring of minimal residual disease (MRD) plays a critical role in the evaluation of patients diagnosed with acute myeloid leukemia (AML). Initially, elevated cell burdens produce significant negative consequences, and are easily characterized by multiple technologies. After treatment, the remaining leukemic cells can be small in number, and present little to no physical symptoms. The discovery of these cells through traditional morphological methods has proven difficult. Alternatively, next-generation sequencing (NGS) provides increased sensitivity through deep sequencing of the patient’s genome, providing early detection of relapse due to treatment resistance or non-targeted leukemic cells. Herein, we evaluated modifications to a custom ArcherDX assay workflow to permit somatic variant detection down to 0.1% variant allele frequency (VAF). **Methods:** DNA isolated from peripheral blood of healthy Myeloid donors was mixed with samples containing variants of known frequency to generate mixtures at 0.1%. VAF. Libraries were constructed using a modified ArcherDx protocol designed to improve molecular retention and decrease the total error rate of the preparation. Libraries were quantified using the KAPA qPCR method, followed by normalization, pooling, and sequencing on the Illumina NextSeq system. Data processing was performed using a collection of in-house bioinformatic resources and the Archer Analysis software package. **Results:** The modifications to the protocol produced higher library yields for normalization and pooling compared to the standard workflow. Furthermore, the changes resulted in increased total unique fragments identified during sequencing, although the overall de-duplication of fragments was slightly reduced due to more consistent de-duplication across the entire ROI. Combined, these effects allowed robust detection of mutations down to 0.1% VAF in the tested samples. Similarly, false positive calls were reduced with the workflow changes introduced after variant filtering utilizing in-house derived key parameters. **Conclusions:** Based on the samples tested, MRD detection down to 0.1% mutation level was possible. The changes implemented in the wet-lab increased overall sensitivity of the assay, enhancing the capture of low frequency mutations and reducing noise levels. By employing key parameters for filtering, false positives were minimized and specifically improved. Overall, these results indicate that MRD detection through NGS assays can aid in early detection of re-emergent disease and assist in clinical management.

H12. A Next-Generation DNA Sequencing Assay for Detection of SNVs, Insertions, Deletions, and Copy Number Variants in 25 Lymphoma Genes in Samples

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**Introduction:** Lymphomas, including Hodgkin lymphoma, diffuse large B-cell lymphoma (DLBCL), and others, are clinically heterogeneous: Certain individuals respond well to therapy, but many succumb to the disease. The literature suggests that much of this variability in response reflects molecular heterogeneity in the tumors. Defining somatic variants including SNVs, insertions, deletions, and copy number variations (CNVs) is important in characterizing these samples. Further, it is highly desirable to be able to detect variants using fine needle aspirate (FNA) samples, low abundance DNA, and formalin-fixed, paraffin-embedded (FFPE) samples. **Methods:** We describe a next-generation sequencing (NGS) panel with 25 genes, the Oncomine Lymphoma Research Panel, including ARID1A, ATM, B2M, BCL2, BCL6, BRAF, BTK, CARD11, CD79B, CDKN2A, CREBBP, EZH2, GNA13, HIST1H1E, KMT2D, MTOR, MYC, MYD88, PIM1, SF3B1, SOCS1, TNAIP3, TNFRSF14, TP53, and XPO1, that requires only 20 ng input DNA. The PCR primers for these genes were optimized, and performance tested on control and representative clinical research samples. Another 48 genes relevant to lymphoma are available for addition to the panel. A comprehensive bioinformatics analysis solution was developed to detect SNPs, indels, and CNVs; to perform filtering for the most relevant variants; to annotate these variants with a wide variety of bioinformatics databases; and to report on the interpretation of the selected variants. **Results:** We tested this panel on cell line controls and FFPE samples. Uniformity of coverage, on target mapping, reproducibility, and sensitivity to detect variants were high in all cases and above established quality criteria (>90% or >95%). Examples of our integrated analysis pipeline are shown, including variant calling, CNV detection, functional annotation, population MAF, predicted protein effects, and relevant (e.g., ClinVar, COSMIC) annotations. **Conclusions:** An NGS assay with a comprehensive data analysis approach was developed that is capable of detecting both small mutations and CNVs simultaneously with high sensitivity in FFPE samples, providing an important tool for...
lymphoma translational research. For research use only. Not for use in diagnostic procedures.

H13. High Throughput TRG Sequencing in a Clinical Laboratory: Analysis of Equivocal Results
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Introduction: T-cell receptor (TCR) gene rearrangement studies are an important tool in distinguishing between reactive and neoplastic T-cell processes. Currently, most clinical diagnostic assays involve multiplex PCR with BIOMED2 primers followed by fragment analysis, but interpretation can be subjective, and this method is limited in sensitivity and specificity. More recently the development of next-generation sequencing (NGS) methods has led to increased sensitivity and specificity for detecting clonal TRG or TRB rearrangements as well as the ability to track specific clones over time. Furthermore, the quantitative nature of NGS assays promotes more objective result interpretation. In this study, we investigate the clinical, technical, and specimen-related factors that lead to an equivocal result for T-cell clonality by NGS.

Methods: All clinical NGS-based and PCR-based TRG sequencing assays performed at our institution between 6/2016 and 3/2020 were identified, and relevant data were abstracted from the electronic medical record. Results: Twenty-five percent (173/688) PCR-based tests were equivocal for T-cell clonality (resulted as either “indeterminate” or “oligoclonal”). In comparison, only 12% (90/768) of NGS-based tests were equivocal for T-cell clonality. Of the NGS-based equivocal results, specimen types included blood (41/90, 46%), formalin-fixed, paraffin-embedded (FFPE) (26/90, 29%), bone marrow (13/90, 14%), and fresh tissue (10/90, 11%). Sixty-four unique patients were identified with adequate clinical follow-up. Clinical diagnoses included T-cell neoplasms (59/64, 56%), non-T-cell malignancies (17/64, 26%), autoimmune disease (5/64, 8%), immunodeficiency (3/64, 5%), and reactive etiologies (3/64, 5%). The T-cell neoplasms included mycosis fungoides (18/36, 50%), T-cell large granular lymphocytic leukemia (7/36, 19%), angioimmunoblastic T-cell lymphoma (4/36, 11%), peripheral T-cell lymphoma not otherwise specified (3/36, 8%), lymphomatoid papulosis (2/36, 6%), primary cutaneous anaplastic large cell lymphoma (2/36, 6%), and hypopigmented T-cell dyscrasia (1/36, 3%). Refex TRB clonality testing resulted in a definitive positive or negative result in 52% (30/59) of these cases.

Conclusions: In our clinical molecular diagnostic laboratory, the implementation of an NGS-based T-cell clonality assay reduced the rate of equivocal results by 50% compared to the previous PCR-based assay. Both specimen type and the biology of the clinical process influence the rate of equivocal results by the NGS-based TRG sequencing assay. Selectivity was demonstrated perfect reference concordance when the V-gene pileup sequence, FR, and sample levels, respectively. Specificity was 0.99/0.97/0.80, and accuracy was 0.98/0.98/0.93, respectively. In “live” mode, these parameters yielded sensitivities of 0.89/0.93/0.94, specificities of 0.98/0.96/0.92, and accuracies of 0.97/0.96/0.93, respectively, in the entire cohort. Out of 56 samples, CR missed 1 clone sample, and over-called 3 negative samples. Conclusions: We developed an automated program to analyze Ig NGS that leverages gold-standard pathologist review to determine optimal parameters for clone calling. In our cohort, CR derived parameters that identified clones with high accuracy. Future studies will include evaluation of CR in a separate validation cohort of 50 samples targeting both Ig and TCR genes. Once validated, “live” mode of CR can rapidly screen large datasets to reduce the effort of manual data review. Since sensitivity is of utmost importance, “live” mode can accommodate additional grey-zone categories to rescue potential false negative calls. CR will be publicly available, and can serve as an out-of-the-box solution for labs interested in decreasing the bioinformatics burden of Ig and TCR NGS analysis.

H14. CloneRetriever: An Automated Algorithm to Identify Clonal Immunoglobulin Gene Rearrangements by Next-Generation Sequencing
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Introduction: Clonal immunoglobulin detection by next-generation sequencing (NGS) has been implemented clinically for minimal residual disease (MRD) monitoring. Given high complexity and cost, it is not routinely used for diagnosis. Yet the advantages afforded by NGS warrant further development, particularly for primary diagnosis. We aimed to address the bioinformatics challenges of Ig, and TCR, NGS by designing a novel tool, CloneRetriever (CR), which pairs gold-standard determination of clonality with a flexible and fast software-based algorithm to train and identify clones.

Methods: Fifty-six mixed-type specimens were sequenced using LymSnpTrack IGH targeting framework regions (FR) 1-3. Data were aligned using vendor software. Each sequence, FR, and sample was manually determined to be clonal, or not, by 2 molecular pathologists. The cohort was then separated in 2 groups (training/testing), and the annotated data files were provided to CR. CR operates in 2 modes: validation for determining optimal parameters, and live for prospective clone calling. In validation mode, CR randomly splits the training cohort, and derives an optimal model for clonality based on 3 variables (repeatability, clonal dominance, percent read) using a weighted logistic regression classifier that prioritizes sensitivity. This model is then applied to the test cohort to assess model performance. Results: Seventy-two clonal sequences, 33 clonal FRs, 24 clonal samples, and 32 non-clonal samples underwent parallel pathologist review and CR analysis. Training by CR showed that ≥5x clonal dominance and repeatability were required for clonality, but not percent read. Applied to the test cohort, these parameters yielded sensitivities of 0.9/1/1 on the sequence, FR, and sample levels, respectively. Specificity was 0.99/0.97/0.80, and accuracy was 0.98/0.98/0.93, respectively. In “live” mode, these parameters yielded sensitivities of 0.89/0.93/0.94, specificities of 0.98/0.96/0.92, and accuracies of 0.97/0.96/0.93, respectively, in the entire cohort. Out of 56 samples, CR missed 1 clone sample, and over-called 3 negative samples. Conclusions: We developed an automated program to analyze Ig NGS that leverages gold-standard pathologist review to determine optimal parameters for clone calling. In our cohort, CR derived parameters that identified clones with high accuracy. Future studies will include evaluation of CR in a separate validation cohort of 50 samples targeting both Ig and TCR genes. Once validated, “live” mode of CR can rapidly screen large datasets to reduce the effort of manual data review. Since sensitivity is of utmost importance, “live” mode can accommodate additional grey-zone categories to rescue potential false negative calls. CR will be publicly available, and can serve as an out-of-the-box solution for labs interested in decreasing the bioinformatics burden of Ig and TCR NGS analysis.

H15. IGH V-Gene Somatic Hypermutation Assessment by Hybrid-Capture
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Introduction: The identification of somatic hypermutation can be prognostically actionable in chronic lymphocytic leukemia (CLL). IGH V-gene mutation status is typically assessed using amplicon-based methods, with either Sanger or next-generation sequencing (NGS)-based methods employed to compare query rearranged V-gene sequence(s) to reference data. We have previously described an approach to IGH locus assessment using hybrid-capture for library enrichment. In our previous proof-of-concept, we were able to distinguish between monoclonal and polyclonal specimens by way of our IGH feature set, in parallel with additional extra-IGH locus variant analysis. In this follow-up study, we applied our method of IGH V-gene assessment to a cohort of CLL specimens and compared the results to our local clinical assay.

Methods: Ten randomly selected CLL specimens submitted for IGH V-gene somatic hypermutation testing were assayed using our novel method. By this method, a carefully curated set of probes for the functional IGH V-genes was employed for enrichment. NGS was performed on an Illumina MiSeq, followed by bioinformatic analysis using a custom pipeline. The results were then compared to those from independently performed IGH somatic hypermutation assessment using an Invivoscribe kit (v2.0; ABI Fluorescence Rearrangement Detection), followed by Sanger sequencing and analysis using the IMGT pipeline. Results: In all cases, the dominant clonotype identified by NGS demonstrated perfect concordance in named V-gene and junction sequence with that identified by Sanger sequencing; in the remaining six cases, somatic mutation varied from 4.9% to 14.6%. By NGS, the same four unmutated cases demonstrated perfect reference concordance when the V-gene pileup consensus sequence was considered; however, in the six mutated cases, the degree of mutation was decidedly lower, ranging from 0.3% to 9.7%. When we then performed a base-by-base coefficient of variation analysis of the V-gene pileup results (to account for coverage), the degree of correlation with Sanger results improved (Spearman r = 0.91, p = 0.0002), with encouraging performance metrics (AUC = 0.97; p = 0.0001).
Conclusions: Our data support previous assertions made by our group that IGH analysis, now including somatic hypermutation assessment, can be reliably performed using a hybrid-capture approach. Our data further support the assertion that direct application of consensus sequence analysis for computation of the degree of somatic hypermutation might yield erroneous results. Instead, a novel statistical approach is required for accurate interpretation.

H16. Comparison of Next-Generation Sequencing-Based TRG and TRB Assays for the Diagnostic Evaluation of T Cell Lymphoid Malignancies
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Introduction: High-throughput sequencing of the T-cell receptor beta (TRB) and gamma (TRG) genes is increasingly utilized for the diagnosis and monitoring of lymphoid malignancies due to its ability to specifically identify and track unique T-cell receptor (TCR) sequences harbored by malignant T-cells. However, selection of appropriate TCR gene targets for clinical testing requires a thorough cost-benefit analysis. A reflex testing algorithm has been favored by many labs to reduce cost and maintain clinical sensitivity while sacrificing turn-around-time (TAT). In this study, we compared results of two commercially available next-generation sequencing (NGS)-based TCR assays to provide evidences for effective testing algorithms. Methods: One hundred and eighty (75 peripheral blood, 18 bone marrow aspirate, and 87 formalin-fixed, paraffin-embedded (FFPE) tissue) samples from patients with suspicion of T cell lymphoid malignancies were tested using the commercially available LymphoTrack T-cell MRD assay (Thermo Fisher Scientific, Inc), and Ion AmpliSeq (InVivoScribe, Inc). Results from each target were classified as positive, negative, or oligoclonal based on the pre-determined interpretation criteria. Performance metrics, such as sequencing coverage, detection rate, library size, diversity, and evenness of immune repertoire, were compared. Results: With at least 50,000 sequencing reads as the cut-off, 96% of samples with tested for the TRG assay and 86% of samples tested with the TRB assay passed this QC requirement. The merged paired-end TRB reads after primer trimming were on average 50 bp greater than those of TRG. The sampled TRB repertoires were higher in diversity score and contained more unique clonotypes than the TRG repertoires. The TRB and TRG detection rates of clonal population were 90.38% and 57.69%, respectively, based on the current interpretation criteria. One-third of the discordant cases were oligoclonal by TRG but clonal by TRB, and one-fourth of the discordant cases had a predominant clone of >2.5% but failed to meet the defined fold-change criteria. Conclusions: Although the TRB assay showed a higher detection rate at least partially due to its greater structural complexity, it has a lower amplification rate for FFPE specimens likely attributed to its larger amplicon size. The TRG assay had higher successful amplification rate but lower detection rate; further optimization of the interpretation criteria may improve detection rate. Other important factors including TAT, subsequent residual disease testing, and differences in test performance characteristics for each TCR target also need to be considered. Whereas a sequential testing algorithm may lead to lower overall testing cost, parallel testing of TRG and TRB has advantages in reducing TAT and increasing test sensitivity.

H17. Characterization of the Immunoglobulin Heavy- and Light-Chain Repertoires in a Single Reaction
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Introduction: B cell repertoire analysis by next-generation sequencing (NGS) has shown particular utility in the field of hematological oncology research. Some advantages provided by NGS-based techniques include a lower limit-of-detection and simpler paths to standardization compared to flow-based methods, and the elimination of specifically designed primers often required for qPCR-based methods. Owing to primer-primer interactions and incompatibility of reaction conditions, current multiplex PCR assays require separate PCR reactions to survey each immunoglobulin chain (IGH, IGK, IGL), often leading to a longer time-to-answer for samples in which no marker is initially detected. We have developed an assay for receptor analysis based on Ion AmpliSeq technology to circumvent these issues, allowing the effective use of up to thousands of primers in a single reaction. The highly multiplexed, panclonality NGS assay provides for efficient detection of IGH, IGK, and IGL chain rearrangements in a single reaction. Methods: We developed a single primer panel targeting the framework 3 (FR3) portion of the variable gene and the joining gene region of heavy- and light-chain loci (IGH, IGK, IGL) for all alleles found within the IMGT database, enabling readout of the complementary-determining region 3 (CDR3) sequence of each immunoglobulin chain. To maximize sensitivity, we included primers to amplify IGK loci rearrangements involving Kappa deletion and C intron elements. To evaluate performance, we conducted clonality assessment and limit-of-detection testing using gDNA from a total of 45 research samples representing common B cell malignancies. We included samples derived from peripheral blood, bone marrow, and formalin-fixed, paraffin-embedded (FFPE)-preserved tissues at input levels ranging from 100 ng to 2 µg. Sequencing and clonality analysis was performed using the Ion GeneStudio SS System and Ion Reporter 5.16. Results: Clonality assessments performed using gDNA collected from both cell line and clinical research samples (CLL, B-ALL, multiple myeloma, Burkitt lymphoma, NHL, and DLBCL) show a >90% overall positive detection rate. Assessment of linearity-of-response and limit-of-detection was conducted using cell lines diluted in PBL to between 10-1 and 106 by mass. The multi-receptor assay performs as expected, with linear response to the cell line frequency across the range tested, including the ability to detect clones of interest at 10-6. Conclusions: These results demonstrate the robustness of our newly developed Ion AmpliSeq assay for B cell receptor heavy and light chains. We expect this assay to simplify the workflow for clonality assessment and rare clone detection in B cell malignancy research. For research use only.

H18. Assessment of a High-Throughput Sequencing Assay for Measurable Residual Disease (MRD) Monitoring in Patients with T-Cell Malignancies
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Introduction: High-throughput sequencing of the T-cell receptor (TCR) beta and gamma loci is becoming more widely utilized due to its high sensitivity, specificity, and versatility in the diagnosis of T-cell malignancies. Application of these technologies for tracking disease burden can be valuable in detecting recurrence, determining response to therapy, guiding future management of patients, and establishing endpoints for clinical trials. In this study, we assessed the performance of a commercially available high-throughput sequencing assay for determining residual disease burden in patients with various T-cell malignancies receiving care at our institution. Methods: Peripheral blood samples from 56 patients previously diagnosed with various T-cell malignancies were sequenced with the commercially available LymphoTrack T-cell MRD assay (InVivoScribe, Inc), which includes the TRB and TRG assay kits, internal and low positive controls, and the LymphoTrack MRD analysis software. Samples were sequenced and analyzed according to the manufacturer’s recommendations. Test performance characteristics including linearity, analytic sensitivity, specificity, and precision were assessed with both contrived and patient samples. The accuracy of the assay was further assessed by tracking 96 previously identified TRB clones and 130 previously identified TRG clones, and comparing results with concurrent MRD assessment by the clonoSEQ assay (Adaptive Biotechnologies). Results: The LymphoTrack T-cell MRD assay demonstrated excellent test performance characteristics for the DNA inputs tested. Assessment of intra-run and inter-run precision revealed coefficients of variation averaging less than 20% for samples containing 1,000 or more T-cell equivalents, whereas greater variances were seen for samples containing fewer than 100 T-cell equivalents. Both TRB and TRG assays reliably detected tracked clonotypes down to an order of 10 T-cell equivalents. Tracked clonotypes were also found to be highly specific to malignant cells when interrogated in pooled normal controls. Despite some notable differences with the clonoSEQ assay in the ability to detect certain rearrangements, MRD status was highly concordant with the LymphoTrack T-cell MRD assay at the sample level. Conclusions: High-
throughput sequencing of the TCR beta and gamma loci is a highly sensitive, specific, precise, and accurate method for determining disease burden for various T-cell malignancies and can be readily implemented in molecular diagnostic laboratories capable of performing high-throughput sequencing.

H19. Improved Clonality and Somatic Hypermutation Analysis of CLL with a Highly Multiplex IGHV Assay
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Introduction: IGHV somatic hypermutation (SHM) is an established
prognostic biomarker for chronic lymphocytic leukemia (CLL).
Traditional methods for quantifying IGHV SHM rely on amplification the rearranged
IGH chain via multiplex PCR employing consensus primers targeting the
variable gene leader and FR1 regions in combination with joining gene
primers, followed by Sanger or next-generation sequencing (NGS). The
success rate of this approach is limited by SHM, which may disrupt primer
binding and amplification. We have developed an assay for
analysis based on Ion AmpliSeq technology to circumvent these issues,
effectively enabling comprehensive SHM analysis of IGH within a
single reaction. The panel provides a highly robust multiplex assay for
IGHV somatic hypermutation analysis. We demonstrate the robustness of
the method by sequencing of synthetic rearrangements derived from
literature and a panel of CLL research samples. Methods: We developed a
multiplex panel to target the leader or Framework 1 (FR1) regions of
the IGH gene in combination with joining gene primers (Onconome SHM
IGHV Leader FR1 Assay). Primers were designed to amplify the
variable genes and alleles in the IMGT database. We created a companion
informatics pipeline in Ion Reporter to identify clonotypes and quantify
SHM. To evaluate accuracy, the assay was used to measure SHM from a
panel of synthetic rearrangements of known SHM status representing
unmutated and mutated CLL IGH chains from literature. Finally, 200 ng
gDNA was extracted from a set of 15 CLL research samples and used for
clonality testing and SHM analysis. Results: The assay was able to
correctly determine the SHM status of all synthetic rearrangements,
including two clones containing insertions/deletions in combination with
SHM-derived point mutations. The assay identified a dominant clone and
was able to assess SHM in 15/15 CLL research samples. Conclusions:
These results demonstrate the ability of our highly multiplex IGHV assay to
accurately quantify SHM while minimizing the need for secondary
analysis owing to SHM-derived interference with primer binding. We
anticipate this comprehensive IGHV assay will improve the detection
success rate and simplify the workflow for SHM research studies as
compared to traditional primer sets (e.g., BIOMED-2).

H20. The Development of an NGS Assay of Immunoglobulin Heavy Variable Gene Somatic Hypermutation in CLL
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Introduction: Chronic lymphocytic leukemia (CLL) is a highly
heterogeneous disease, which presents an indolent course in many cases but
transforms into more aggressive malignancies in others. Of particular
prognostic importance in this disease is the mutational status of the
immunoglobulin heavy variable (IGHV) genes, where a hypermutated
state predicts favorable outcome. Current Sanger sequencing
methodology requires gel separation of IGH ampiclons and the
identification of the dominant clone, which is hampered by the limited
resolution of gel electrophoresis. For instance, analysis could be
challenging when more than one clone co-exists. Here we attempted to
develop a new assay, taking advantage of the greater sequencing
capacity of next-generation sequencing (NGS) to increase the sensitivity
and accuracy of IGHV SHM determination. Methods: RNA samples were
extracted from normal and CLL blood specimens using a Promega RSC.
After reverse transcription (Bio-Rad iScript), the rearranged IGH loci were
amplified using leader forward primers and J leader primers. Pooled
libraries were sequenced on IonTorrent PGM 316 chips and analyzed
using Torrent Suite v5.10.1. The reads were mapped to a custom
reference downloaded from the International Immunogenetics (IMGT)
Information System database (imgt.org). The distribution of the reads to
IGHV genes was examined to assess clonality and identify the dominant
clone(s). The consensus IGHV gene sequence was uploaded to the V-
QUEST tool of IMGT to determine the mutation status. In addition,
representative reads were uploaded to the V-QUEST tool to determine the
productivity. Sanger sequencing results from a reference laboratory were
used for comparison. Results: In the initial development of this test, we
processed 8 randomly selected clinical samples, including 1 normal and 7
CLL blood specimens. Of note, 2 out of the 7 CLL specimens were
indeterminate by Sanger. Although the normal blood specimen displayed
characteristic polyclonal IGHV gene usage, the majority of the CLL reads
aligned to one or two IGHV genes. In 4 of 7 CLL cases, the dominant
IGHV gene represented more than 50% of the total base reads. In all 4
cases, the mutational percentage and IGHV gene identity by NGS were
highly consistent with Sanger results. When the base read representation
fell to 20% to 50%, the clonality became more complex and challenging to
resolve by Sanger (2 of 3 cases indeterminate). With NGS, we
successfully identified two mutated alleles in one case, a mutated allele
and an unmutated allele in another case, and one unmutated allele in a
third case. The complex clonality correlated with low white blood cell
counts. Conclusions: Extremely helpful in resolving complex cases, this
NGS assay has the potential to increase sensitivity and refine prognosis
stratification in CLL.

H21. Validation of MYD88 L265P ddPCR Assay and Application in Assessment of Primary CNS Lymphoproliferative Disorders
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Introduction: Myeloid differentiation factor 88 (MYD88)
L265P variant is a highly important and diagnostic marker that
constitutes 91% of known MYD88 mutations. It is present in 91% of
lymphoplasmacytic lymphomas and 30% to 50% of primary CNS
lymphomas (PCNSL). Given its prevalence, we validated our own
MYD88 L265P ddPCR assay using the Bio-Rad QX200 system, and applied it to
a variety of primary CNS lymphoproliferative disorders (CNS-LPD) including
diffuse large B cell lymphoma (DLBCL) in immunocompetent patients, and
Epstein Barr virus-associated post-transplant lymphoproliferative
diseases (PTLD) and HIV-associated lymphoproliferative disorders (HIV-
LPD) in immunocompromised patients to assess MYD88 L265P status.
Methods: Validation: Genomic DNA was isolated from 19 MYD88 L265P
positive samples detected by next-generation sequencing (NGS) across a
range of variant allele frequencies (VAF) and 14 known negative samples
that included bone marrow aspirates, cell suspensions, and formalin-fixed,
paraffin-embedded (FFPE) tissues. Studies to determine accuracy,
reproducibility, reportable range, sensitivity, and specificity in detection
of MYD88 L265P were performed using the Bio-Rad QX200 ddPCR system,
and included comparisons of NGS results and dilutional studies of cell
lines and known positive patient samples. Results were analyzed using
QuantaSoft software and reported as a percentage of fractional abundance (%FA), calculated as [(mutant copies/μL)/(mutant copies/μL + wild-type copies/μL)]x100. CNS-LPDs: Genomic DNA from 21 DLBCLs,
16 PTLDs, and 9 HIV-LPDs FFPE samples were assessed for MYD88
L265P using the Bio-Rad QX200 ddPCR system. Results: Validation:
Excellent correlation was seen with %FA by ddPCR and VAF by NGS (R-
square 0.93, slope 1.15) across a range of quantitative values, as well as
%FA with positive sample dilutional studies (R-square 0.99, slope 1.00).
There was 100% concordance for known positive and known negative
samples between ddPCR and NGS. specificity was 100% for
MYD88 L265P when tested against MYD88 wild-type cases. Reportable range for
clinical testing was set at 1% to 100% FA; however, maximum
reproducible sensitivity reached 0.1%FA. Intra-run/in-run reproducibility
showed a percentage of CV <25%. CNS-LPDs: A total of 12/21 (57%)
DLBCLs (median: 46%FA; range: 11% to 93.6% FA) and 2/9 (22%) HIV-
LPDs (range: 9% to 11.5%FA) were positive for
MYD88 L265P. The mutation was not detected in any CNS-LPDs. Conclusions:
Our MYD88 L265P ddPCR assay validation showed excellent accuracy,
reproducibility, specificity, and maximal reproducible sensitivity of
0.1%FA. This assay shows promising clinical applications, especially
in the setting of primary CNS lymphoproliferative disorders, and provides
further insights into the role of MYD88 L265P in CNS-LPDs arising in
immunocompetent and immunocompromised individuals.
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Introduction: Due to cryptic rearrangements and variations in MYC breakpoints, 15% of diffuse large B-cell lymphoma (DLBCL) with MYC rearrangements (R) remains undetectable by fluorescence in situ hybridization (FISH). Because of the clinical impact, we sought to develop an artificial intelligence (AI) system composed of recurrent cytogenetic aberrations (RCAs) and genetic progression score (GPS), derived from the number and type of RCAs, with high specificity to predict MYC status in DLBCL. In addition, identification of driver alterations, clinical evolution patterns, and clinical impact of RCAs on patient survival was performed.

Methods: Data from DLBCL with MYC from the published literature and from our institution were used to identify RCAs. Karyotypes were curated for RCAs and a Bonferonni adjusted p-value was used for correlations. Rheinmix package was used to calculate GPS, DriverNet algorithm was used to identify driver alterations, TRONCO Package was used to map the evolution of RCAs, and the NNET package was used to build an AI system. Both RCAs and GPS were applied to the AI system; 70% of the literature cases in the study were used to train the system, and 30% of the cases were used to test the system to predict MYC status. A receiver operating curve was performed to evaluate the AI system, and a Kaplan-Meier and log rank test was used to assess clinical impact of RCAs on patient survival. All p-values <0.05 were significant. Results: In the 583 karyotypes (524 from the literature and 59 institutional) evaluated, 25 RCAs were identified. One of the RCAs identified the MYC status, but chromosome losses were associated with MYC- status (p <0.001). In MYC+ cases, a MYC R was the sole driver alteration, but further evolution patterns revealed RCAs which associated with gene expression profiles, mainly FOXP1 protein expression and MYD88, CD79B, PIM1, CARD11 mutations. Derived GPS from 20 RCAs ranged in value from 0 to 5.81, the difference in GPS of MYC R (1.27) and MYC- (0.68) was significant (p <0.001). The RCAs, and a GPS greater than 2 to predict MYC status in 567 cases from the literature, obtained a diagnostic ability of 98.8%, with sensitivity of 100% and specificity of 90.4% at predicting the MYC status in 157 tumors from the literature. When predicting MYC status in 59 institutional cases, AI correctly classified 49 cases, an 89% positive predictive value (PPV). If clinically indicated, increasing AI specificity resulted in 100% PPV. In agreement with the literature, cases with MYC R showed a shorter survival.

Conclusions: Our findings support use of cytogenetic profiles combined with GPS and subsequent use of an AI system to predict MYC status in DLBCL.


Introduction: Classic Hodgkin lymphoma (CHL) is characterized by rare malignant Hodgkin and Reed-Sternberg (HRS) cells surrounded by abundant reactive or inflammatory cells in lymph nodes. Recently, bp24.1 alterations with amplification, rearrangement, and overexpression of the PDL1 and PDL2 genes has emerged as a biomarker in predicting response to checkpoint blockade. Such abnormalities can be detected by fluorescence in situ hybridization (FISH) tests but due to rarity and variable morphology of HRS cells, it is often difficult in formalin-fixed, paraffin-embedded (FFPE) FISH test to identify the HRS cells with DAPI staining only. To address this issue, we established a method of combined CD30 immunophenotyping and FISH using PDL1 and PDL2 probes, and applied it to 19 CHL cases with a comparison with a regular FISH test without CD30 staining. This combined FISH assay showed significant improvement in recognition of rare HRS cells, and improved detection of PDL1/2 amplification, gain or polysomy, which may enable accurate prediction of response to immunotherapy in CHL.

Methods: FFPE tissue sections of 19 CHL patients with various morphology were included. CD30 immunophenotyping employs tyramide-based signal amplification, followed by optimal FISH pretreatment procedures, which allows adequate retention of CD30 fluorescence and FISH signals for PDL1 and PDL2 probes. Simultaneous analysis of CD30 positive HRS cells and FISH signals was performed manually. In 17 cases, routine FISH analysis without CD30 immunophenotyping was included to compare accuracy and precision of analysis. Results: Simultaneous visualization of CD30 immunophenotyping and FISH signals of PDL1, PDL2, and CEP9 probes were successful in all 19 CHL cases, with adequate retention of CD30 staining and definite FISH signals on the HRS cells. In 17 cases with regular FISH without CD30 staining, a high concordance of results was observed in 8 cases with typical HRS morphology. In four of the remaining 9 cases, PDL1/2 amplification was detected only by combined CD30 staining and FISH assay, and the percentages of cells with amplification, gain or polysomy were substantially increased in five cases when compared to those without CD30 staining. In two other cases, FISH analysis was possible only with CD30 staining due to lack of morphological features of HRS.

Conclusions: Combining CD30 immunophenotyping and FISH procedures allows correct recognition of HRS cells and precise analysis of PDL1/2 copy number alterations in CHL, particularly those with a low number of HRS cells or with variant morphology, and may allow for accurate prediction of response to immunotherapy. This procedure is being adapted to automatic confocal scanning and analysis with a computer-based algorithm, and may be applied to other tumors with unique antigen expression with genomic abnormalities.

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Introduction: B-lymphoblastic leukemia (B-ALL) is the most common pediatric malignancy, and >90% of children are cured with frontline chemotherapy. However, 10% to 15% of patients relapse and require more intensive salvage therapies. We reviewed the outcomes of patients with the usually favorable hyperdiploid subtype of B-ALL at our institution to identify risk factors associated with relapse.

Methods: B-ALL patients diagnosed between 2001 and 2019 that had ≥50 chromosomes in their diagnostic specimens were included. Chart review was performed to collect demographics, additional genetic data, end of induction (EOI) minimal residual disease (MRD) and treatment outcome. Statistical significance was determined using Chi-square and Mann-Whitney U tests.

Results: Amongst 169 identified patients with hyperdiploid B-ALL, 19 experienced ≥1 relapse (11%). The average age at diagnosis was 6.8 years (2 to 19.5) for patients who relapsed and 5.5 (1 to 17) for patients who did not relapse (p = 0.15). Although there were similar numbers of males (88) and females (81), significantly more females relapsed (14/81 [17.3%] versus 5/59 [8.6%]; p = 0.017). Chromosome 1q gain was highly enriched in the relapsed group (7/19 [36.8%]) versus non-relapsed group (13/150 [8.7%]; p = 0.003). All three patients with constitutional trisomy 21 and somatic hyperdiploidy relapsed. The percentage of patients with positive EOI MRD (at a threshold of ≥0.1% by flow cytometry on day 29 bone marrow) was not statistically significant between those with (18.8%) and without (10.8%) subsequent relapse (p = 0.35). Seven patients died during therapy, one due to infection while in remission on frontline therapy, and six after relapse due to progressive disease during induction therapy (31.6%). Next-generation sequencing (NGS) results were available for 7/19 relapsed patients at time of relapse (paired diagnostic samples not available); all had ≥1 clinically significant variant identified. NGS data were available for 40/150 non-relapsed patients, 35 of whom had ≥1 clinically significant variant. RAS-pathway genes KRAS, NRAS, and PTEN/11 were most frequently mutated (24/35 [68.6%] non-relapse versus 5/7 [71.4%] relapse), followed by CREBBP and FLT3 in both groups. Though there was no notable difference regarding genes mutated and mutation rate between relapsed and non-relapsed groups, 9/33 RAS-pathway gene mutations in the non-relapsed group were subclonal.

Conclusions: Chromosome 1q gain and constitutional trisomy 21 were
associated with higher risk of relapse in our pediatric hyperdiploid B-ALL cohort, as well as female sex. Our data showed similar rates of E01 MRD positivity amongst patients who did or did not relapse. Subclonal mutations in RAS pathway genes were common in the non-relapse hyperdiploid ALL cohort.

H25. Novel Fusion of PVT1-RCOR1 in B-Cell Prolymphocytic Leukemia (BCPCLL) Producing False FISH Fusion of MYC-IGH with an Atypical Pattern

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Introduction: Plasmacytoma variant translocation t gene (PVT1), located at 8q24.21, is 55kb telomeric to MYC, and fine tunes MYC expression. PVT1 is overexpressed in tumors with increased MYC expression and confers poor prognosis. A fusion between PVT1 and IGH has been reported in Burkitt lymphoma. RCOR1, mapping to the centromeric side of IGH at 14q32, is a transcriptional co-repressor involved in the epigenetic regulation of blood cell development. RCOR1 fusions have not been reported in B-cell tumors. However, we present a unique case of B-cell prolymphocytic leukemia (BCPALL) with a t(8;14)(q24;q32) that showed an atypical fusion between MYC and IGH by fluorescence in situ hybridization (FISH); however, next-generation sequencing (NGS) analysis detected in-frame fusion between PVT1 and RCOR1 that has not been reported previously. Methods: Hematopathology evaluation of a 77-year-old patient showed leukocytosis with medium-sized to large, monotypic CD5+ B-cells that expressed MYC protein. Chromosome analysis of a peripheral blood specimen showed 46,XX;(8;14)(q24;q32) karyotype. Interphase FISH with probes for MYC and IGH showed an atypical pattern of translocation signal between MYC and IGH; metaphase FISH mapped the fusion signal on the der(8). FISH with MYC break-apart probe confirmed rearrangement in MYC in 95% of cells. From the results of hematopathology, cytogenetics, and FISH, a diagnosis of BCPALL with MYC rearrangement was rendered. Contrary to these findings, NGS analysis detected a fusion transcript that joined exon 2 of RCOR1 with exon 7 of PVT1 but no fusion of MYC-IGH. Results: Tumor-specific fusion genes created by reciprocal translocations can be detected by chromosome analysis, or by dual fusion FISH probes, or by fusion detection strategies in NGS; among them, using FISH probes is rapid and less expensive. This strategy sometimes can lead to false results emanating from three-way translocation, loss or gain of chromatin at the site of breaks, or from the probes of closely linked genes. Fusion of MYC-IGH is the most frequent alteration in large B-cell lymphoma and is associated with aggressive clinical course. Therefore, screening for this translocation performed by FISH in diagnostic specimens is essential. Although false negativity for the fusion has been reported, false positivity had not yet been reported. Conclusions: The case presented here is unique with discordance between chromosome and NGS findings. The (8;14) identified on the karyotype yielded an atypical fusion signal for MYC and IGH. NGS analysis identified that this atypical fusion was due to the fusion of PVT1 at 8q24 with RCOR1 at 14q32. Therefore, this case highlights the need to investigate the atypical FISH fusion signal pattern for tumor-specific translocations. Further studies are necessary to elucidate the molecular mechanism employed by this fusion to drive tumorigenesis.

H26. Identification of Clinical Molecular Targets for Childhood Burkitt Lymphoma

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Introduction: Burkitt lymphoma (BL) is a malignant tumor in children. Although BL is generally curable with rituximab, early relapse and refractoriness may occur. Whereas some molecular markers have been used for BL diagnosis, recent research and clinical practice have suggested that there is still large heterogeneity in childhood BL. Therefore, this study aimed at providing more accurate clinical molecular targets and methods that may help in precision diagnosis and improved treatment. Methods: Only children with BL were included in the study. Besides recently proved clinical molecular tests, targeted gene sequencing was conducted to identify tumor-specific mutations in these childhood BLs; the mRNA and protein level expression of potential target genes were measured by real-time PCR and immunohistochemistry. The relationship between BL-specific gene alterations and differential expression with clinical features was analyzed. Results: The results showed that MYC and IGH were the most common gene alterations (c-MYC, BCL2, and BCL6 gene translocation, mutation, and expression status would benefit childhood BL diagnosis, prognosis, and treatment determination; 2) TCF3 gene mutation may be a characteristic marker complementary to c-MYC translocation in childhood BL; 3) loss-of-function mutations in SOCS1 or CIITA gene might be used as malignant markers for diagnosis and prognosis; 4) although NF-kB pathway seems to have had no effect on BL tumorigenesis, specific mutations of genes related to the pathway, such as CD79A, MYD88, KL2L, CARD11, and NF-kB signaling pathway targets like benign clinical outcomes; and 5) the high expression of MYC, TCF3, CD3D, and loss-of-function ID3 genes in tumors may be potential therapeutic targets for childhood BL and could be used for treatment monitoring. Conclusions: In summary, specific gene mutations and differential expressions have been identified as potential clinical molecular markers for childhood BL and might be used together with existing markers to improve diagnosis, prognosis, and treatment.

H27. Characterization of TP53 Mutations in Myeloid Neoplasms for Targeted Therapy

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Introduction: Mutant p53 has been considered therapeutically non-targetable for various reasons; however, there are 20-30 growing number of small molecules that promote reactivation of missense-altered p53 due to altered protein structure/folding or decreased thermostability. TP53 gene alterations are found in ~50% of all neoplasms, including all types of hematologic neoplasms, and its presence is typically associated with worse survival outcomes. Here we characterize TP53 variants in a myeloid neoplasm cohort, sequenced at our institution, to ascertain details from whose case subsets that may potentially be suitable for targeted therapy. Methods: We evaluated all Illumina TruSight Myeloid Sequencing Panel data from 752 cases performed on blood and bone marrow biopsies from 692 patients, between 2017 and 2020. Diagnoses included confirmed myeloid neoplasms of all types, CHIP, few non-malignant malignancies, and marrow with no evidence of disease. Sequencing was performed on the MiSeq platform. Base-calling and sequence alignment were performed using MiSeq Reporter Software and analyzed on the PierianDx CGW platform. TP53 gene alterations were evaluated in relation to type, numbers/frequency, neoplasm subtype, and co-mutational profile. Results: Of 367 cases with at least one gene variant, 62 cases had an altered TP53 (n = 75 total TP53 variants). These included missense (n = 58, 77%, all within p53 DNA-binding domain), nonsense (n = 2, 3%), frameshift/truncating (n = 8, 11%), and splice-site (n = 7, 9%) variants. Twenty-two cases had a paired deletion or structural alteration involving chr-17p13 detected by karyotype/fluorescence in situ hybridization (FISH) (19/50 cases with one TP53 alteration, 3/12 cases with ≥2 TP53 variants). TP53 was the sole variant in 28 cases. A second TP53 variant was the most frequent co-alteration (n = 12), but co-mutation in 16 other genes was seen (ASXL1, DNMT3A, SF3B1, STAG2, TET2 most frequent, each n = 5). The TP53 altered cases included de novo AML (n = 4), secondary AML (n = 18), therapy-related myeloid neoplasms (n = 6), MDS (n = 19), MDS/MPNs (n = 5), CHIP (n = 3), and few non-malignant neoplasms (n = 8). Of the cases with detectable variants, 58% with mut-TP53 were deceased at the time of data collection, versus 20% of those with wt-TP53. Conclusions: TP53 mutated myeloid neoplasms are known to have poor survival outcomes and new treatment strategies are needed. Novel therapies aimed at reactivating mutant p53 are currently in clinical trials (such as APR-246), and these presumably work via stabilization and/or refolding of the p53 core domain. We found that 82% of cases with mut-TP53 may be suitable for targeted therapies with these novel agents, emphasizing the importance of incorporating sequencing analysis into standard of care for patients with myeloid neoplasms.
H28. Chromosome Arm Gain or Loss by Next Generation Sequencing
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Introduction: Vxxeos (liposome packed daunorubicin and cytarabine) has been recently approved for the treatment of acute myeloid leukemia with myelodysplasia-related changes (AML-MRC), therapy-related AML, and secondary AML. Most of these cases carry myelodysplastic cytogenetic abnormalities defined AML-MRC by WHO Hematopoietic Neoplasms Classification 2016. The goal of the current study was to preliminarily evaluate whether the most frequent cytogenetic abnormalities in these patients including -5/del(5q), -7/del(7q), -5q, -11q, -12p, and -13q could be detected by modifying the Archer VariantPlex Myeloid panel. Methods: We added 149 primer sets into the current Archer VariantPlex Myeloid panel to have an average of 0.03 Kb coverage over the above chromosomes to enable the detection of chromosome arm gain or loss. A total of 25 AML patients (11 with normal karyotype and 14 AML-MRC cases) were studied by this customized panel. Next-generation sequencing (NGS) was performed using Illumina MiSeq, and the data analysis was done by Archer Analysis 6.2. Results: The NGS results of 11 patients with normal karyotype (7 males, 4 females) were used to build the normal data set for analysis. Among the 14 AML-MRC cases, cytogenetic studies showed the following abnormalities in ranging from 35% to 100% of 20 cells analyzed: -5/del(5q) in 6 cases, -7/del(7q) in 5, gain of 11q in 4, -12p in 3, and -13q in 3. The NGS study results demonstrated almost complete concordance (15/16 or 94%) for all chromosome arm abnormalities occurring in more than 70% of cells analyzed by conventional cytogenetics. The only abnormality missed by NGS is -7 in a single case. Of note, NGS showed a small segment loss of chromosome 7 not shown by karyotyping likely due to the resolution limitation of karyotyping. However, NGS was not able to reliably detect any chromosomal abnormalities (0/4) occurring in less than or equal to 70% of cells. Conclusions: This customized NGS panel has the potential to accurately detect the chromosome arm level abnormalities commonly occurring in AML-MRC cases. Since NGS is now routinely used for AML cases to detect mutations of AML-associated genes for treatment and prognosis, this added capacity further increases the clinical utility of NGS. This is particularly useful when karyotyping studies fail due to no dividing/visible cells. The current approach is to use fluorescence in situ hybridization (FISH) studies to detect the AML-MRC-associated abnormalities. This adds significant cost and delays in turn-around time. The sensitivity of the NGS approach can be further increased by enriched blasts using CD34 magnetic beads, modifying panel design, and/or improving the bioinformatics analysis. Additionally, the clinical significance of small segmental gain or loss detected by NGS needs further evaluation.

H29. Clinical Significance of CEBPA Double Mutants: Challenges in Variant Classification and Subtyping of Acute Myeloid Leukemia
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Introduction: Acute myeloid leukemia (AML) with biallelic CEBPA mutations comprises a clinically distinct subgroup of patients, associated with better prognosis and earlier ages at diagnosis. The classic pattern for biallelic CEBPA mutations includes two distinct variants located in the N- and C-terminal portions of the gene. These mutations result in loss of the p42 isoform and loss of the DNA binding ability of the p30 isoform, respectively. However, two CEBPA variants often cannot be phased using conventional sequencing platforms, and variant classification can be challenging. Methods: CEBPA was sequenced on the Illumina MiSeq using long-range PCR and tagmentation. Among the cases with two or more CEBPA variants, cases were designated as “classic” double mutant when the combination included both an N-terminal frameshift/nonsense variant (within the first 120 amino acids in NM_004364.4) and a C-terminal in-frame in/del/missense variant within the DNA-binding domain (amino acids 278 to 358). Results: Of 862 AML patients who underwent CEBPA sequencing, 85 CEBPA variants were identified in 62 patients. A total of 2162 patients were found to have two or more CEBPA variants, with 14 having the classic N- and C-terminal variant combination. All 14 patients with the classic CEBPA double mutant pattern were most consistent with AML with biallelic CEBPA mutations, based on their clinical history and other genetic findings. In contrast, 6/7 of non-classic double mutant patients were more consistent with other AML subtypes (1 with myelodysplasia-related changes, 2 therapy-related, and 3 with NPM1 mutations). A total of 31/41 of monallelic cases could be subtype (1 inv[16], 12 MDS-related changes, 4 therapy-related, 9 NPM1-mutated, and 5 RUNX1-mutated). The variants seen in the classic double mutant cases showed a large overlap in terms of the location and types of CEBPA variants with other CEBPA-mutated cases. The patients with the classic CEBPA double mutant pattern had significantly better overall survival (log-rank test p = 0.01995), whereas the survival curve for the non-classic double mutant cases largely overlapped with that for monallelic cases. The patients with classic CEBPA double mutant patterns were also younger at diagnosis. Conclusions: Although allelic mutism is uncertain in most double mutant cases, CEBPA variants are better interpreted in the context of clinical history and other genetic findings. The clinical features seen in our cohort of CEBPA classic double mutant cases support the variants are likely biallelic. The clinical significance of non-classic double mutant patterns remains unclear and warrants careful clinico-patho-molecular correlations for subtyping.

H30. Cytogenetic and Molecular Landscape in Hispanic Acute Myeloid Leukemia Patients from Puerto Rico
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Introduction: Acute myeloid leukemia (AML) is a clonal hematopoietic malignancy with complex and heterogeneous cytogenetic and molecular features. Current WHO classification (2017) recognizes eight disease-defining balanced translocation categories and three specific gene mutation categories of AML. Although adequately studied in different populations, the incidence and prevalence of different genetic mutations and chromosomal translocations in Hispanic AML patients are underreported. Here, we have endeavored to bridge that knowledge gap by retrospectively studying the cytogenetic and molecular profile of 1,165 AML patients from Puerto Rico (PR) of Hispanic descent. Methods: Demographic data, clinically relevant cytogenetic results, and next-generation sequencing (NGS) data of a 40-gene panel of 1,165 Hispanic AML patients from PR were obtained and analyzed. Results: The age at diagnosis for our patient population ranged from 1 to 92 years (median age = 62 years). The male-to-female ratio was 1:1.08. Of the 1,026 patients whose karyotypes were available, 37% (380/1,026) of the patients showed normal karyotype, and only 7.12% (73/1,026) of patients showed complex karyotypes (≥3 chromosome abnormalities). Of 405 patients tested, FLT3-ITD was detected in 139 patients (34.3%). Out of all AML with balanced chromosomal translocations, acute promyelocytic leukemia (12.78%), AML with inv[16] (5.46%), and AML with t(8;21) (2.85%) were found to be most prevalent. A 40-gene panel NGS was performed in 204 AML patients. DNM3A mutation was found to be most prevalent (22.5%), followed by NPM1 mutation (14.7%), RUNX1 mutation (13.7%), and CEBPA mutation (4.4%). Conclusions: Previous reports suggested that in the majority of the Caucasian patient population with AML, the incidence of complex karyotype was 10% to 12%, and the incidence of normal karyotype was 40% to 45% (Mrozek et al., 2019). Our data indicate that Hispanic AML patients from PR have a higher rate of cytogenetic abnormalities with a relatively lower incidence of complex karyotype. The frequency of NPM1 mutation is also relatively low in the population (14.7% in our population, as opposed to 27% to 35% of the population described in WHO 2017 classification); however, the co-incidence of NPM1 mutation and the normal karyotype is maintained. The incidence of biallelic CEBPA mutation (4.4%) is similar to that reported in WHO 2017 classification (4% to 9%), but we observed this mutation exclusively in adult patients (median age 49 years) as opposed to its higher incidence reported in children and young adults. The incidence of RUNX1 mutation in our population (13.7%) falls towards the higher end of the range provided by WHO 2017 classification (4% to 16%). These findings raise the possibility of a different and unique genetic landscape in Hispanic AML patients.
H31. Evaluation and Follow-up of JAK2 V617F Positive Patients with Low Allele Burden: A Single-Center Experience
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Introduction: Patients with features suspicious for BCR-ABL1 negative myeloproliferative neoplasms (MPN) typically undergo an algorithmic approach, with testing for JAK2 V617F mutation being the first step. If positive, often no further testing is performed. If negative, testing for JAK2 exons 12-15 is performed for polycythemia vera and, if other MPNs are under consideration, testing for CALR and MPL mutations is also added. This algorithmic testing approach is often followed whenever JAK2 V617F results are positive, whether the allele burden (AB) is low or high. Whereas some studies have indicated that V617F allele burden at diagnosis provides important information, current evidence exists regarding the clinical significance of detecting low AB mutation. The most widely used method for JAK2 V617F detection is based on allele-specific PCR. Methods: A retrospective review was done on 43 patients with low AB (<1%) JAK2 positivity detected by Competitive Allele-Specific TaqMan Polymerase Chain Reaction (castPCR) over a period of 1 year with follow-up for 1-2 years. At the discretion of the hematologist or pathologist, if clinical or laboratory values were worrisome for MPN, reflex testing by next-generation sequencing (NGS) was performed, which comprised a gene panel including JAK2, CALR, MPL, and other myeloid-related targets. Results: Hematologists ordered the JAK2 V617F castPCR assay for various reasons: elevated hemoglobin (14/43), thrombocytosis (13/43), leukocytosis (6/43), venous thrombosis (2/43), cytopenias (1/43), both thrombocytosis and erythrocytosis (1/43), and others (7/43). Twenty-three of 43 patients (53%) had repeat JAK2 V617F testing, of which 2/43 (5%) showed increased (JAK2 AB), whereas 4/43 (9%) showed persistent low AB, and in 2/43 (5%), the AB was below the level of assay sensitivity. Four patients (9%) went on to test positive for CALR, MPL, or other MPN-related mutations by NGS. All the remaining patients (~91%) were found to be negative for any additional mutations. Seven patients with positive, low AB JAK2 V617F had subsequent bone marrow studies and of those, 3 received a diagnosis of MPN. Conclusions: Our study, as outlined by similar reports, shows that low AB JAK2 positivity can be present often in the absence of MPN. Such cases may be true low-level clonal hematopoiesis and may uncommonly expand to result in disease such as MPN. Thus, such cases should prompt follow-up, whether in the form of repeat testing or bone marrow biopsy, depending on other clinical factors. Low AB JAK2 V617F positivity should be reported and may not reflect assay variability, which can be seen in such analysis at low levels. Additionally, low AB JAK2 V617F positivity should not preclude testing for other MPN-related driver genes, particularly CALR and MPL.

H32. Number of Variants and Pathogenic Variants in ASXL1, STAG2, and RUNX1 Correlate with High Ogata Score by Flow Cytometry in Myelodysplastic Syndromes: A National Reference Laboratory Experience
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Introduction: Although the diagnosis of myelodysplastic syndromes (MDS) largely relies on morphologic, cytogenetic, and molecular evaluation, flow cytometry is an evolving technique and can also be used to reinforce MDS diagnosis. Here we assess flow cytometric findings using Ogata score in patients with unexplained cytopenia, and their correlate with concurrent molecular aberrations by next-generation sequencing (NGS). We aim to describe the NGS findings associated with low and high Ogata score with the ultimate aim of improving our understanding of flow cytometry as a diagnostic tool in MDS. Methods: We retrospectively evaluated 127 bone marrow aspirates submitted to ARC Laboratories for both flow cytometry and a myeloid malignancies NGS panel over 9 months from 2019 to 2020. The NGS panel results were used to classify the cases as confirmed myelodysplastic syndrome (n = 60), reactive cytopenia (n = 50), or clonal cytopenia of undetermined significance (CCUS) (n = 17) cases. Ogata scores, where points are assigned for increased CD34+ myeloblasts, decreased B-progenitor-related cluster size, and alterations in CD45 expression on myeloblasts and in the side scatter of granulocytes, were calculated based on the original flow cytometry plots. A Mann-Whitney U test analysis was used to examine associations between Ogata score and genes with tier 1 variants detected in more than 5 cases by the NGS panel. Results: Ogata scores were significantly higher in molecularly confirmed MDS cases than in reactive (p = 0.04) cytopenia or CCUS (p = 0.03) cases. Based on this study cohort, Ogata scores of 2 and above showed a more than 50% sensitivity and a more than 85% specificity in diagnosing MDS, confirmed by NGS studies, and the positive predictive value was 81%. Higher Ogata scores are in association with more pathogenic variants in MDS cases. Eight genes had pathogenic variants detected in more than 5 cases examined: ASXL1, STAG2, RUNX1, DNMT3A, U2AF1, SF3B1, TET2, and TET3. Of these, three showed a significant correlation with higher Ogata scores: ASXL1, STAG2, and RUNX1 with p-values of 0.0037, 0.0053, and 0.0293, respectively. Conclusions: Ogata scores by flow cytometry were significantly correlated with the positive variant scores compared to reactive cytopenia or CCUS cases, and high Ogata scores have a high specificity to reinforce MDS diagnosis. Variability in the Ogata score within confirmed MDS cases correlated with the presence of pathogenic variants in particular genes, including higher Ogata scores associating with ASXL1, STAG2, and RUNX1 variants, which are among the most frequently mutated genes in MDS. This study suggests that Ogata score by flow cytometry is a reliable tool to assist the diagnosis of MDS, in addition to morphologic, cytogenetic, and molecular analyses.

H33. Clinical Implementation of a Custom Myeloid NGS Assay and Overview of NPM1 and IDH1/IDH2 Mutation Status in a Clinical Cohort
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Introduction: Next-generation sequencing (NGS) is important in the clinical-pathologic work-up of myeloid hematopoietic disorders. In AML, NPM1 is an important prognostic factor, and IDH1/IDH2 are therapeutic targets. We implemented a custom Myeloid NGS assay for clinical use and have assessed the features of myeloid cases with NPM1 and/or IDH1/IDH2 mutations in our clinical patient cohort. Methods: A custom NGS 45-gene panel using RainDance library preparation with Illumina MiSeq sequencing was validated for clinical testing. Analytical sensitivity is 1% to 2% VAF for SNVs/indels. Results: A total of 838 diagnostic and/or post-therapy samples were sequenced: AML (274 samples, 33%), high grade MDS in transformation to AML (45 samples, 5%), MDS (121 samples, 14%), CMML (18 samples, 2%), cytopenia (100 samples, 12%), leukocytosis (37 samples, 4%), polycythemia (29 samples, 4%), P. Vera (61 samples, 7%), among others. NPM1 was mutated in 30 samples (24 unique patients) and was almost entirely restricted to patients with AML (except for 1 CMML and 2 cases with history of AML where which were negative for concurrent AML by flow cytometry and morphology). Type A (TCTG) NPM1 mutations represented 83% of all NPM1 variants and VAF range was 1% to 45% across all samples. Among NPM1+ samples, FLT3 mutations were enriched in the DNMT3A+ subset (Fisher’s Exact Test: 0.047). Conversely, IDH1+ or IDH2+ appeared enriched in samples lacking DNMT3A mutations (trend not statistically significant, Fisher’s Exact Test: 0.08) and in samples lacking FLT3 mutations (Fisher’s Exact Test: 0.013). IDH1/IDH2 were mutated in 61 samples (44 unique patients, predominantly with concurrent AML (23/44 patients), MDS/NPM1 (5/44 patients), cytopenia (4/44 patients), P. Vera (4/44 patients), MDS (4/44 patients), and few patients with history of AML which were negative for concurrent AML by flow cytometry and morphology (4/44 patients). IDH1 mutations occurred at Arg132 and IDH2 mutations occurred at Arg140, and VAF range was 1% to 50% across all samples. IDH1 mutations were found to be largely exclusive of IDH2 mutations (Fisher’s Exact Test: <0.00001). NPM1 mutations were enriched among IDH1+ cases compared to IDH2+ cases (Fisher’s Exact Test: 0.0179). Lastly, follow-up samples from IDH+ patients undergoing therapy revealed gain of variants in histone methylation pathway (SETBP1, SUZ12) and evolution of NRRAS/PTPN11 pathway mutation patterns. Conclusions: We have implemented a custom Myeloid NGS panel for clinical use which has been successfully applied to a broad range of patients with myeloid disorders. NGS has revealed that NPM1 variants are largely restricted to AML. IDH+ samples were predominantly AML, but were found in a broader range of
myeloid disorders than NPM1. Evolution of co-mutations in histone methylation- and RAS-pathway genes was observed in IDH+ patients during therapy.

H34. Development of FIP1L1-PDGFRα Real-time RT-PCR Assay
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Introduction: Myeloid and lymphoid neoplasms with eosinophilia are rare disorders, with a significant subset characterized by PDGFRα gene rearrangements and associated with chronic eosinophilic leukemia (CEL). The FIP1L1-PDGFRα gene fusion is the most commonly encountered finding generated by an approximately 800 Kbp cryptic chromosome deletion, with loss of the intervening CHIC2 locus; this abnormality is typically identified by fluorescence in situ hybridization (FISH) method. However, due to limitations of FISH probe design, small deletions may be missed with potential false negative results. RT-PCR provides a very sensitive method for fusion detection, but is challenging because of breakpoint heterogeneity in FIP1L1. We developed a multiplex real-time RT-PCR strategy to reliably detect FIP1L1-PDGFRα fusions.

Methods: Bone marrow (BM) and blood (PB) samples were collected and total RNA was extracted (Promega Maxwell). Reverse transcription (RT) was performed with the High Capacity RT kit (Thermo Fisher). Primers and probes were sourced from IDT. Real-time RT-PCR was performed using the delta delta CT method, with gblock oligo correction. All potentially involved exons (exon 8 to exon 18) of FIP1L1 and exon 12 of PDGFRα were included for forward and reverse primer design. The reporter hydrolysis probe was situated in exon 12 of PDGFRα. The ABL1 gene was used as reference transcript. The fusion positive cell line EOL-1 (10 ng/µl) was used as a calibrator in each run. This calibrator was corrected by 1:1 ratio of target and reference gblock oligo. Results: Analytic sensitivity showed that cell line and gblock oligo log dilution achieved at least 4 log limit of detection (LOD = 0.01%) with excellent linearity (R² value at 0.998). Assay specificity assessed in 20 normal donors showed 100% specificity (absence of detection at LOD). Ten “artificial patients” were created by mixing EOL-1 and HL-60 cell lines, and showed the expected detection values; in addition, multiple breakpoint forms assessed by gblock oligo mixes also demonstrated very high assay sensitivity and detection specificity. Following repetitive training of the assay over multiple runs, a stable correction factor was established for cell line calibration relative to the synthetic gblock. Conclusions: We developed a highly sensitive and specific real-time RT-PCR assay for FIP1L1-PDGFRα. The assay design covers nearly all exons of the FIP1L1 gene and is predicted to detect more FIP1L1-PDGFRα fusions compared to standard FISH tests. Detection of the FIP1L1-PDGFRα in CEL is critical to identify patients who are highly responsive to imatinib therapy. This assay can achieve clinically sensitive detection at diagnosis, as well as provide monitoring capability following treatment.

H35. FLT3-ITD Mutant Allelic Ratio: Impact of Using Non-standardized Published Calculations and Potential Correction Based on Marrow Blast Percentage
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Introduction: FLT3-ITD (ITD) mutant allelic ratio (MAR) is used for risk stratification of AML patients by the (2017) European Leukemia Network, with patients having a MAR ≥0.51 (ITD high) stratified to an adverse risk category. Increasingly, the MAR is being reported by US laboratories. There are at least two published methods for calculating the MAR, and no calculation corrects for percent blasts in the marrow. We studied 33 ITD positive patients with AML from 2017 to present, and determined the impact on the ITD MAR using area under the curve (AUC) versus peak height (PH) ITD mutant and wild-type (WT) values in samples at diagnosis, and using two published MAR equations. We also examined the potential of correcting for marrow blast percentage to more closely approximate the ITD leukemic burden.

Methods: Using fluorescence-based PCR DNA fragment analysis, AUC and PH for ITD and WT values were used in two published equations for determining MAR: 1) ITD/Total: ITD/total FLT3 (ITD + WT), and 2) ITD/WT: ITD/FLT3 WT. To correct for the blast versus non-blast (presumably, non-leukemic) marrow, we determined the blast percentage by flow cytometry or morphologic assessment (differential of aspirate smears, touch imprints, or CD34/CD117 immunostains), and used the highest value. The MAR was corrected by dividing the MAR by the blast percentage. Results: Using AUC analysis, 6 (18%) versus 14 (42%) of 33 patients were classified as ITD high (MAR ≥0.51) using equations 1 and 2 above, respectively; using PH analysis, 5 (15%) versus 12 (36%) of 33, respectively, were classified as ITD high. Thirty cases had marrow blast percentages ranging from 7% to 92% by flow cytometry (median 61%), and 22% to 100% by morphology (median 65.5%). Correcting the MAR for blast percentage using AUC, 12 (40%) versus 18 (60%) of 30 were classified as ITD high using equations 1 and 2 above, respectively; using PH analysis, 8 (27%) versus 16 (53%) of 30, respectively, were classified as ITD high. Conclusions: The wide degree of variability (15% to 42%) for classifying patients as ITD high using AUC and PH in different published MAR equations demonstrates the need for standardizing calculations to one universally accepted method, and the importance of detailing on lab reports the method by which the MAR is derived. Using the blast percentage in the calculation to determine MAR corrects the FLT3-ITD burden to the leukemic content of the marrow. There are no published papers on standardizing or correcting the MAR. This study is small; institutions with larger data sets should consider standardizing to AUC values using the ITD/WT equation and correcting with the FLT3-ITD MAR blast percentage calculation to standardize and re-validate original datasets. Doing so will allow for better cross-institutional comparisons.

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Introduction: The presence of CBFB rearrangement or CBFB-MYH11 fusion is a confirmatory diagnostic marker for inv(16)(t(16;16))(CBFB-MYH11). Both CBFB break-apart FISH (BAP FISH) and CBFB-MYH11 fusion fluorescence in situ hybridization (FISH) (FUSION FISH) tests are widely applied in the clinical laboratory. However, atypical signal patterns encountered in certain cases can pose diagnostic challenges with potential significant clinical implications.

Methods: Karyotyping and BAP FISH using Vysis Dual Color Break Apart Probe were routinely performed in all new AML cases potentially with inv(16)(t(16;16)) in our Clinical Cytogenetics Laboratory. CytoTest CBFB-MYH11 Fusion Probe was additionally performed on a challenging cases of an atypical fusion. Acute Leukemia Translocation Panel was performed for screening several recurrent fusion genes including CBFB-MYH11 in new acute leukemia cases. Real-time reverse transcriptase PCR (RT-PCR) was subsequently performed to quantitatively monitor the CBFB-MYH11 transcript levels in positive cases.

Results: From January 1, 2000, to June 30, 2020, 2,425 BAP FISH tests were performed in 1,370 AML cases. Among them, 248 (18.1%) cases (336 tests) were positive, 58 (4.2%) cases (60 tests) were reported as abnormal results. e.g., gains of 1-5 intact CBFB signals (12 tests/cases), loss of one CBFB signal (35 tests/cases), 3' CBFB deletion (12 tests/10 cases), and 5' CBFB deletion (1 test/case). Correlating with karyotype and RT-PCR results, 246 (99.2%) BAP FISH positive cases presented a classic inv(16)(p13q22) (n = 227) or t(16;16)(p13;q22) (n = 19) and were RT-PCR positive as well. The remaining 2 positive cases exhibited a t(16)(q22;p22) and a t(16)(q22;p22), and were not tested with RT-PCR due to lack of RNA. A CBFB read-out was further confirmed by metaphase FISH with the 5' CBFB signal on the abnormal 16q in both cases and the 3' CBFB signal on the abnormal 1q and 2q, respectively. Identifying CBFB rearrangement with partner genes other than MYH11 in these 2 cases. Seven (70%) 3' CBFB deletion cases and 1 CBFB FISH negative case were RT-PCR positive for CBFB-MYH11 fusion, whereas the remaining cases (n = 51) with abnormal BAP FISH results were RT-PCR negative. As demonstrated by FUSION FISH, 2 cases with normal karyotype had their CBFB-MYH11 fusion through an insertion of part of the MYH11 into the CBFB gene. Conclusions: Although the CBFB FISH tests are widely applied in laboratory diagnostics, atypical signal patterns, including those with 3' CBFB deletion, may not clearly indicate a
CBFβ rearrangement in all cases. Our data suggest that a few cases with a negative FISH result may nonetheless harbor a CBFβ-MYH11 fusion. Therefore, further investigations are necessary to establish an inv(16)(p13;16)(q22) AML diagnosis in these cases.

H37. Curation of FLT3 Variants in Acute Myeloid Leukemia by Clinical Genome Resource Somatic Hematologic Cancer Taskforce (ClinGen HCT)

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Introduction: The ClinGen Somatic Cancer Clinical Domain Working Group (CDWG) is a multi-disciplinary team of experts engaged in developing standard processes to curate and interpret somatic variants across cancer types using the AMP/ASCO/CAP guidelines. The primary goals of the CDWG are to facilitate accurate clinical interpretation of somatic variants associated with hematological malignancies. HCT has curated 25 evidence items from clinical and preclinical studies on FLT3-ITD in AML, but the prognostic and therapeutic significance of the less frequent tyrosine kinase domain (TKD) mutations remains uncertain.

Results: FLT3 has identified 14 common FLT3-TKD variants. To date, the HCT has curated 25 evidence items from clinical and preclinical studies on FLT3-TKD variants in CIVIC. In addition, three AMP tier I, level A variant assertions of FLT3-ITD, D835, and I836, which predict response to gilteritinib, an FDA-approved drug for relapsed or refractory AML, have been curated. After the initial pilot curation phase, the HCT will seek to become an official ClinGen FLT3 Variant Curation Expert Panel.

Conclusions: Curation of FLT3 TKD variants in AML has been undertaken by the HCT to achieve standardization of their clinical interpretation and reporting.

H38. Persistent IDH Mutations in AML Patients in Remission on IDH Inhibitors

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Introduction: Isocitrate dehydrogenase (IDH) is an enzyme that catalyzes the conversion of isocitrate to α-ketoglutarate in the tricarboxylic acid (TCA) cycle. About 20% of acute myeloid leukemia (AML) patients harbor IDH mutations, which change the functions of the enzyme, and lead to accumulation of the oncometabolite 2-hydroxyglutarate and cause DNA and histone hypermethylation. Ivosidenib and enasidenib, the IDH1 and IDH2 inhibitors, have been approved by the FDA for the treatment of adult relapsed or refractory (R/R) AML with IDH1 and IDH2 mutations, respectively. It has been shown that IDH mutations can remain detectable in patients who have otherwise achieved remission, especially in patients on IDH inhibitors. The aim of this study is to better characterize IDH mutation status in AML patients with IDH mutations at the time of remission.

Methods: We searched the database of our institution from January 2018 to June 2020 and identified 45 AML patients with IDH mutations. The clinical and pathological information were obtained from the EMR. IDH mutational analysis was done using the Sequenom MassARRAY Analyzer 4 mass spec system using iPLEX reagents targeting codon 132 of IDH1 and codons 140 and 172 of IDH2.

Results: Out of a total of 43 patients with IDH-mutated AML, 14 (33%) had IDH2 R1402 mutations, 8 (19%) had IDH1 R132C mutations, 8 (19%) had IDH2 R172K mutations, and 7 (16%) had IDH1 R132H mutations. Less commonly found mutations include IDH1 R132G (4.6%), IDH1 R32S (4.6%), IDH2 R140L (2.3%), and IDH2 R140W (2.3%). Twenty-four patients achieved remission, with 17 on IDH inhibitor therapies. Only patients on IDH inhibitors had detectable IDH mutations at the time of remission (13/17, 76%). IDH mutations became undetectable in subsequent samples within a one- to six-month time frame in 8 patients. In contrast, none of the 7 patients who did not receive IDH inhibitor treatment (0/7) had detectable IDH mutations at remission (p = 0.003). There was no statistically significant difference between the groups in terms of overall survival.

Conclusions: More than 70% of AML patients on IDH inhibitor therapies had detectable IDH mutations at the time of remission. Indicating that IDH inhibitors induce blast differentiation/maturation rather than cell death. For AML patients with IDH2 mutations on enasidinib, all the patients with R140Q mutations (6/6), but none of the patients with R172K mutations (0/3), had detectable mutations at the time of remission (p = 0.003). The patients with IDH2 mutations on enasidinib are more likely to have persistent mutation at the time of remission than those with R172K mutations.

H39. Diagnostic Value of Molecular Markers in the Work-up of Myelodysplastic Syndromes

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Introduction: Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders characterized by cytopenia, morphologic dysplasia, ineffective hematopoiesis, and variable risk of progression to acute myeloid leukemia. Diagnosis is based on morphologic evaluation and clinical and cytogenetic findings. As karyotype is normal in 50% to 60% of MDS, and myelodysplasia can be morphologically subtle, we explored the diagnostic value of molecular markers in the work-up of MDS.

Methods: We retrospectively screened...
cases that had a targeted 35-gene next-generation sequencing (NGS) panel performed. The 2016 WHO revision-based bone marrow (BM) pathologic diagnoses were verified by at least 2 hematopathologists. Pertinent laboratory and clinical findings were collected by chart review. DNA was extracted from BM aspirate/peripheral blood using Qiagen EZ1 (Qiagen, Germantown, MD). Sheared DNA (200 ng) was target-enriched with a custom hybridization-capture reagent (SureSelectXT, Agilent, Santa Clara, CA) and sequenced on the MiSeq or HiSeq platforms (Illumina, San Diego, CA). NGS data were processed through a bioinformatic pipeline using CLC Bio Genomics Server v6.0 (Qiagen, Redwood City, CA).

Genetic variants were curated following the American College of Medical Genetics and Genomics (ACMG) five-tier system. Results: There were 206 MDS cases meeting the diagnostic criteria of MDS and 124 cytopenia cases not fulfilling the diagnostic criteria of myeloid neoplasm (moMN). In MDS cases, 164 (80%) showed at least one pathogenic/likely pathogenic (P/LP) mutation (median and range, 2 and 0-8) with a median variant allele fraction (VAF) of 35% (5% to 100%). Among the MDS cases, 25% (51), 21% (44), and 34% (69) showed 1, 2 or 3 mutations. Pathways involved included epigenetic modifiers (EM, 56%, 115), splicing factors (SF, 48%, 99), transcription factors (TF, 19%, 39), tumor suppressors (TS, 14%, 26), kinase pathway (KP, 10%, 21), NPM1 (1%, 2) and SETBP1 (2%, 5). In the 124 moMN cases, 76% (94) had no mutations and 24% (30) showed ≥1 P/LP mutations (0, to 4), with 16% (20), 4% (5), and 4% (5) harboring 1, 2 or 3 mutations, respectively. The median VAF was 33% (6% to 87%). Pathway involved included EM (15%, 19), SF (13%, 16), and KP, TS, and SETBP1 (0.8%, 1%). Of the molecular markers, highest sensitivity (80%) for MDS diagnosis was seen in ≥1 P/LP mutations, with a corresponding specificity of 76%. At least 95% specificity was observed with presence of ≥3 P/LP mutations, presence of non-DTA (DNMT3A, TET2, ASXL1) EM mutations, or KP, TF, TS mutations. Conclusions: In working up cytopenia patients for MDS, molecular markers may supplement morphologic and cytogenetic evaluation, and aids in the diagnosis of MDS, particularly in cases with subtle morphology and normal karyotype.

H40. Clinical Validation of Mutant IDH1 and IDH2 Detection by Multiplex Digital Droplet PCR
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Introduction: In AML, IDH1/IDH2 are targets of FDA-approved therapy. Although our in-house, custom Myeloid NGS assay can detect IDH1/IDH2 mutations, there is a clinical need for more rapid detection of the various IDH1/IDH2 mutations, without compromise of analytical sensitivity.
Methods: Three different multiplex (Mpx) digital droplet PCR (ddPCR) assays were designed (bio-rad.com/digital-assays) to screen for various IDH1 and IDH2 mutations in patient samples using genomic DNA (100 ng) extracted from peripheral blood and bone marrow aspirates. The results are reported as a percentage of fractional abundance. Results: Concordance: ddPCR results were compared to known Myeloid NGS findings. A total of 42 patient samples (29 positive and 13 negative) (IDH1 p.R132 positive: 10; IDH2 p.R140 positive: 10; IDH2 p.R172 positive: 3; Dual IDH1 p.R132 positive/IDH2 p.R140 positive: 3) were tested and showed 100% concordance between ddPCR and next-generation sequencing (NGS). Three additional samples with a history of prior IDH mutation showed additional IDH variants found at very low VAF (0.2% to 0.4%) by ddPCR. Accuracy: Correlation of VAFs between NGS and ddPCR was excellent: IDH1 R132: R2 0.97; IDH2 R140: R2 0.99. Analytical sensitivity: Patient samples were serially diluted to evaluate the analytical sensitivity of each assay. All three ddPCR multiplex assays showed a lower limit of detection of 0.1% VAF. Specificity: Each patient sample was tested using all three ddPCR multiplex assays. Twenty-six samples with known IDH1 or IDH2 mutations showed the same IDH variant by ddPCR and did not show cross-reactivity with the other ddPCR reactions. As noted above, 3 samples showed additional low level IDH variants (0.2% to 0.4% VAF) by ddPCR, which are beyond the usual reportable range of the NGS assay. Inter- and Intra-Run Reproducibility: For both inter- and intra-run comparison, IDH1 p.R132 (7 positive and 3 negative samples), IDH2 p.R140 (4 positive and 3 negative samples), IDH2 p.R172 (2 positive and 3 negative samples) were tested. All samples showed 100% reproducibility across different runs. The anticipated turnaround time (TAT) for the multiplex ddPCR assay is 2-4 days, which is shorter than TAT for routine NGS assay, which can take 10 to 14 days.
Conclusions: We have validated a multiplex ddPCR assay for the rapid and sensitive detection of the various IDH1/IDH2 mutations in patient samples. We have found this method is sensitive and specific, and showed excellent concordance with NGS VAFs for a broad range of mutation types in IDH1 and IDH2. With shorter TAT compared to NGS, this platform offers a robust molecular approach for rapid screening of AML patients to identify candidates for therapeutic targeting of IDH1/IDH2.

H41. Comparison of Targeted Myeloproliferative Subpanel versus Comprehensive Myeloid Panel in the Evaluation of Suspected BCR-ABL1−Negative Myeloproliferative Neoplasms
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Introduction: The BCR-ABL1−negative myeloproliferative neoplasms (MPNs) are characterized by the overproduction of functional granulocytes, red blood cells, and platelets. The following entities have been recognized by the World Health Organization (WHO): chronic neutrophilic leukemia (CNL), polycythemia vera (PV), primary myelofibrosis, essential thrombocythemia, chronic eosinophilic leukemia, and myeloproliferative neoplasm unclassifiable. Mutational analysis is an essential part of diagnosing these entities. Next-generation sequencing (NGS) has allowed for the simultaneous testing of multiple genes implicated in the diagnosis of MPNs (i.e., JAK2, CALR, MPL). Targeted panels including these genes have been developed and are often ordered on peripheral blood or bone marrow aspirate by clinicians who suspect MPNs. The lack of mutations in these limited panels is often seen as evidence to exclude a neoplastic process. Our aim is to look at patient samples that were clinically studied with a limited MPN gene subpanel (CALR, CBL, CSF3R, ETNK1, JAK2, MPL, SETBP1) and reanalyze the data with a 50-gene comprehensive myeloid panel to assess if doing so adds clinically significant findings.
Methods: Ninety-three cases of suspected myeloproliferative neoplasms (63 peripheral blood, 24 marrow aspirates) that had been initially profiled by NGS based on a 7-gene MPN subpanel (CALR, CBL, CSF3R, ETNK1, JAK2, MPL, SETBP1) using an external bioinformatics pipeline were reanalyzed with a 50-gene comprehensive myeloid panel using our in-house developed pipeline.
Results: The MPN subpanel alone detected diagnostic variants in 29/93 (31%) of cases (JAK2 59%, CALR 24%, MPL 14%, CSF3R 3%). Fifteen of these 29 cases (52%) had additional pathogenic variants detected by the comprehensive myeloid subpanel in the following genes: ASXL1 47%, DNMT3A 40%, TET2 33%, CUX1 13%, SRSF2 20%, NRSF 13%, TP53 7%, BCR 7%, and U2AF1 7%). More importantly, pathogenic variants were detected by the comprehensive myeloid panel in 13/84 (20%) cases with no diagnostic variants detected by the MPN subpanel (DNMT3A 43%, TET2 36%, ASXL1 21%, SF3B1 14%, ZRSR2 7%). Conclusions: Our findings suggest there is value in evaluating patients with suspected MPNs with a more comprehensive myeloid panel. Additional variants with diagnostic and prognostic implications may be uncovered in cases with diagnostic MPN subpanel mutations, and most importantly, in cases that are negative for mutations after screening with a limited MPN panel.

H42. Haplootype Phase of CEBPA Mutations in Acute Myeloid Leukemia
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Introduction: The CCAAT enhancer binding protein alpha (CEBPA) gene is a 1.077-base-pair-long, intronless gene that maps to chromosome 19q13.1 and encodes a transcription factor. Mutations in the CEBPA gene contribute to leukemic transformation of myeloid progenitors and occur in approximately 10% to 15% of cases of cytogenetically normal acute myeloid leukemia (CN-AML). The presence of two CEBPA mutations is a favorable prognostic indicator in AML in CN-AML and constitutes a separate diagnostic AML entity in WHO 2017 classification. Clinically significant mutations are predicted to cause loss of protein function and tend to cluster in the N-terminal and C-terminal regions of the gene. Regardless of the location, CEBPA mutations show three typical patterns in AML: single mutated, single mutated with loss of heterozygosity, and
double mutated. Double CEBPA mutations are assumed to be biallelic because the haplotypic phase of double CEBPA mutations has not been thoroughly investigated. This is primarily due to the limitations of standard short-read sequencing technologies, namely, Sanger sequencing and next-generation sequencing (NGS). Recently, long-read sequencing technologies, such as Pacific Bioscience's Single Molecule Real-Time (PacBio's SMRT) sequencing platform, have been developed to overcome some of the shortcomings of Sanger and NGS. Although the maximum read length obtained by NGS is approximately 500 bp, PacBio SMRT sequencing yields average read lengths of 10 to 14 kb, enabling direct determination of the haplotypic phase of variants. Methods: In this pilot study, we developed and validated a PacBio's SMRT sequencing platform for detection and phasing of CEBPA mutations. The "training set" of samples included a combination of negative samples, a single CEBPA mutation positive sample, and samples with single mutations and common CEBPA polymorphisms. Results: We established that PacBio's SMRT is an effective method for phasing of CEBPA variants. We are in the process of sequencing additional ~80 samples with single and double CEBPA pathogenic variants. The direct haplotype phasing will reveal whether double CEBPA mutations occur in cis and/or trans configuration. We will subsequently explore any associations between haplotype phase of double CEBPA mutations with event-free survival (EFS) and overall survival (OS) in patients treated with induction chemotherapy. Conclusions: We established that PacBio's SMRT is a viable option to phase CEBPA double variants in CN-AML cases. If cis and trans configurations of double CEBPA mutations do occur and have different clinical outcomes, this may lead to major changes in WHO AML classification. To our knowledge, this is the first time a SMRT sequencing was applied to clinical CEBPA mutation testing, and the first time that haplotype phase of CEBPA double mutations was elucidated.

H43. Identifying Non-canonical Mutations in Myeloproliferative Neoplasms: Our Experience with JAK2 Sequencing

Introduction: The JAK2 exon 12 hotspot V617F mutation is the most common driver mutation of non-chronic myeloid leukemia myeloproliferative neoplasms (MPNs), is present in approximately 95% of polycythemia vera (PV) and 50% of primary myelofibrosis (PMF) and essential thrombocythemia (ET) cases. JAK2 exon 12 mutations, as well as CALR and MPL mutations (in ET and PMF), are present in a smaller percent of MPN cases. JAK2 exon 12 testing is often reflexed in the setting of a negative targeted V617F result. Additionally, rare alternative mutations in exon 12 have been reported recently in suspected MPN. The WHO does not mention such mutations, and the clinical significance of these mutations is still evolving. We performed a retrospective review of cases submitted for next-generation sequencing (NGS)-based myeloid panel testing to identify non-canonical JAK2 mutations. Methods: Institutional records from 2016 to present were reviewed to identify cases run on a targeted myeloid NGS panel, including coverage for exons 12 and 14 of JAK2, in which non-canonical JAK2 mutations were detected. Patient clinical histories, additional molecular testing, and other mutations detected by NGS were also analyzed through a chart review. Results: We identified four cases tested by the myeloid NGS assay with non-canonical JAK2 mutations in exons 12 and 14. Two patients had alternative V617F mutations in exon 14: c.1849_1851delinsTTT; p.V617F and c.1849_1853delinsTT; p.V617_C618delinsF. Neither of the alternative exon 14 mutations was detected by a V617F targeted allele-specific PCR assay. Mutations in exon 12 included one patient with two in cis missense mutations (c.1609T>C; p.F537V and c.1616A>T; p.K539I) and one with an in-frame insertion (c.1638_1639ins33; p.I546F_S547insN11). Additional mutations in DMNT3A, TET2, IDH2, ASXL1, and U2AF1 were also identified. Review of clinical notes revealed 3 cases of PV and one ET with progression to AML. Conclusions: Our data demonstrated a high concordance (up to 98%) between conventional cytogenetics and WGS in detecting large-scale CNVs and SVs. In addition to the advantage in solving complex karyotype, WGS offers an unbiased approach to the discovery of a wide range of other clinically significant genetic variants that are below the resolution of conventional cytogenetics, including SNVs/Indels, focal CNVs (<5 Mb), and cryptic gene fusions.

H45. Workforce Comparison between Two NCCN Guideline Recommended Myeloproliferative Neoplasms Screening Workup: A Single Institution’s Experience

Introduction: The Journal of Molecular Diagnostics because the haplotypic phase of double CEBPA mutations has not been thoroughly investigated. This is primarily due to the limitations of standard short-read sequencing technologies, namely, Sanger sequencing and next-generation sequencing (NGS). Recently, long-read sequencing technologies, such as Pacific Bioscience’s Single Molecule Real-Time (PacBio’s SMRT) sequencing platform, have been developed to overcome some of the shortcomings of Sanger and NGS. Although the maximum read length obtained by NGS is approximately 500 bp, PacBio SMRT sequencing yields average read lengths of 10 to 14 kb, enabling direct determination of the haplotypic phase of variants. Methods: In this pilot study, we developed and validated a PacBio’s SMRT sequencing platform for detection and phasing of CEBPA mutations. The “training set” of samples included a combination of negative samples, a single CEBPA mutation positive sample, and samples with single mutations and common CEBPA polymorphisms. Results: We established that PacBio’s SMRT as an effective method for sequencing and phasing of CEBPA variants. We are in the process of sequencing additional ~80 samples with single and double CEBPA pathogenic variants. The direct haplotype phasing will reveal whether double CEBPA mutations occur in cis and/or trans configuration. We will subsequently explore any associations between haplotype phase of double CEBPA mutations with event-free survival (EFS) and overall survival (OS) in patients treated with induction chemotherapy. Conclusions: We established that PacBio’s SMRT is a viable option to phase CEBPA double variants in CN-AML cases. If cis and trans configurations of double CEBPA mutations do occur and have different clinical outcomes, this may lead to major changes in WHO AML classification. To our knowledge, this is the first time a SMRT sequencing was applied to clinical CEBPA mutation testing, and the first time that haplotype phase of CEBPA double mutations was elucidated.

Methods:

Institutional records from 2016 to present were reviewed to identify cases run on a targeted myeloid NGS panel, including coverage for exons 12 and 14 of JAK2, in which non-canonical JAK2 mutations were detected. Patient clinical histories, additional molecular testing, and other mutations detected by NGS were also analyzed through a chart review.

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Conclusions:

Our data demonstrated a high concordance (up to 98%) between conventional cytogenetics and WGS in detecting large-scale CNVs and SVs. In addition to the advantage in solving complex karyotype, WGS offers an unbiased approach to the discovery of a wide range of other clinically significant genetic variants that are below the resolution of conventional cytogenetics, including SNVs/Indels, focal CNVs (<5 Mb), and cryptic gene fusions.
genetic processes and include chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), and others. Besides characteristic morphological changes, most CMLs feature BCR-ABL1 fusion, whereas other MPNs are BCR-ABL1 negative. In patients with suspicion of MPNs and negative for BCR-ABL1, NCCN guidelines currently recommend two molecular workshop pathways in blood: 1) a multi-step reflex mutation testing algorithm including JAK2, CALR, MPL, JAK2 exon 12, or 2) a multigene next-generation sequencing (NGS) panel that includes at least JAK2, CALR, and MPL genes. Aiming at improving test efficiency and patient care, we compared the workflows of these two screening algorithms in our health system and evaluated their clinical impact including test turn-around time (TAT), test cost, and hematology/oncology service feedback.

Methods: A total of 420 cases between 2019 and 2020 were included in this study which were clinically suspicious for MPNs with negative BCR-ABL1. Ninety-five peripheral blood samples were tested with a multi-step sequential testing algorithm including qualitative detection of mutation in JAK2 V617F, CALR exon 9, MPL exon 10, JAK2 exon 12, and CSF3R. This testing proceeds by reflex through each gene in the above order until CSF3R. Also, a cohort of 325 samples were tested by a 7-gene NGS panel which includes at least JAK2, CALR, MPL, and CSF3R genes. The cost of the test, wet-bench time, and TAT were compared between the two different workflows. Results: The average TAT of the multi-step sequential testing algorithm is 22.8 days, whereas that of the multi-gene NGS panel is 8 days. There is also at least 50% cost and wet-bench time savings by using the multigene NGS panel compared to the multi-step sequential testing algorithm. Conclusions: We have evaluated two NCCN guideline-recommended MPN workshop algorithms. The multi-gene NGS panel is significantly cost and time saving compared to the multi-step sequential testing algorithm. The shortened form ordering to reporting time could further contribute to a better patient care experience and a more efficient clinical management course.

Infectious Diseases

ID01. Multisite Evaluation of the ARIES MRSA ASSAY for the Detection of Methicillin-Resistant Staphylococcus aureus (MRSA) from Nasal Swabs


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Introduction: Methicillin-resistant Staphylococcus aureus (MRSA), one of the most common nosocomial infections, can cause a variety of illnesses including skin and soft tissue, bloodstream infections, and pneumonia. Routine screening for MRSA can guide antibiotic prophylaxis, and can be used to support infection control measures through identification of colonized patients. Culture methods remain the “gold standard” for identification of MRSA in nasal swab specimens; however, the requirement of 18 to 48 h for results limits real-time interventions. We evaluated the Luminex ARIES MRSA ASSAY for detection of MRSA directly from nasal swabs. Methods: A total of 1,762 residual de-identified nasal ESwabs were enrolled across 4 U.S clinical centers. Specimens were tested using the sample-to-result, qualitative, real-time PCR ARIES MRSA ASSAY (ARIES). ARIES identifies MRSA based on the presence of mecA or mecC in combination with only, or in combination with, the SCCmec junction region. An aliquot of each specimen was sent to a central reference laboratory within 48 h of collection for direct and enriched culture. Culture included both non-selective (blood agar) and selective chromogenic MRSA medium. Isolates characteristic of S. aureus were confirmed with biochemical tests and MALDI-TOF MS. Cefoxitin disc diffusion was used to determine susceptibility in accordance with CLSI M-100. Two clinical sites used the BD MAX MRSA XT or StaphSR, which enabled direct comparison to other molecular assays. Results: Direct culture identified 93 specimens containing MRSA; 11 additional specimens contained MRSA following broth-enrichment only. The sensitivity and specificity of ARIES was 93.3% (97/104) and 93.5% (1,550/1,658), respectively. ARIES reported a positive MRSA result in 108 culture-negative specimens, 63 of which were found to contain methicillin-susceptible Staphylococcus aureus (MSSA) by culture. Culture isolates of all 63 specimens were tested using ARIES, and 62 reported as MRSA-negative, suggesting the presence of other mecA carriers, or low concentration or non-viable MRSA. A total of 794 specimens were tested by alternative molecular assays for routine care. ARIES demonstrated positive percent agreement of 95.9% (707/73) and negative percent agreement of 94.9% (684/721).

Conclusions: The ARIES MRSA ASSAY demonstrated sensitivity and specificity comparable to reference culture and alternative FDA-cleared molecular assays for the detection of MRSA in nasal specimens. ARIES requires ≤2 h to perform, with minimal hands-on time, providing a viable method to identify patients colonized with MRSA.

ID02. Comparison of a Cartridge-Based Host Gene Expression Test to a Manual Method for Use in the Diagnosis of Sepsis

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Introduction: A novel approach to sepsis diagnosis is to characterize the host immune response to infection by measuring differential expression of immune response genes. Based on this approach, SeptiCyte LAB was the first-in-class sepsis diagnostic to gain FDA clearance with results reported on a scale of 1 to 10 based on increasing probability of sepsis. However, due to the high complexity and lengthy turnaround time (TAT) the assay was not commercialized. A just-developed second-generation assay, SeptiCyte RAPID (on Biocarts Idylla) is a fully integrated sample-to-result test that includes in-cartridge sample preparation and quantitative polymerase chain reaction (qPCR) with a TAT of <1 h. The purpose of this study was to compare performance and output of a prototype SeptiCyte RAPID test to its predicate. Methods: We conducted retrospective testing of prospectively collected clinical samples to compare performance and output of SeptiCyte RAPID with SeptiCyte LAB (N = 28), and to compare interoperator performance using SeptiCyte RAPID cartridges (N = 20). Whole blood was collected into PAXgene blood RNA tubes prior (“VENUS,” NCT02127502, Miller et al., 2018). Retrospective samples originated from patients suspected of sepsis and admitted to an intensive care unit at Intermountain Medical Center. Residual, cryopreserved samples were selected to represent the full score range of SeptiCyte LAB. Testing was conducted at two clinical laboratories: Intermountain Healthcare Central Lab and Oregon Regional Laboratory; and Biocarts. SeptiCyte scores were calculated using a formula and qPCR Cq values. Hands-on-time (HoT) and assay TAT were recorded. Results: High correlations (r2 = 0.97 and r2 = 0.98) were found for 28 clinical samples run using the manual SeptiCyte LAB test and SeptiCyte RAPID at the two clinical laboratories. Average HoT was 2 mins, and average TaT was 65 mins. Interoperator correlation for 20 clinical samples tested on Idylla systems located at three laboratories above was 0.984 for SeptiCyte RAPID, with a coefficient of variation of 1.25%. Conclusions: SeptiCyte RAPID prototype assay results, run on the Biocarts Idylla instrument, correlate strongly with SeptiCyte LAB, and are reproducible. This is the first demonstration of a fully integrated, rapid, reproducible sepsis test that has the potential for near patient testing.

ID03. Comparison of Two Multiplex Real-Time PCR Assays for Detection of Tick-Borne Pathogens

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Introduction: Ticks are ecologically important vectors of pathogens that cause both human and animal diseases. The prevalence of these diseases is increasing due to factors such as environmental spread of the tick’s home range and further encroachment of humans into these areas. With overlapping symptomology among these diseases, broad panels inclusive of the pathogens endemic to an area are favored by clinicians for investigation of potential infections following tick exposure. In this study,
we compared the performance of our lab-developed standard of care (SOC) real-time PCR assay to the Diasorin Tick-Borne analyte specific reagents (Tick-ASR). **Methods:** A total of 350 prospective and 25 retrospective (previously positive) blood samples (195 µl) from patients with SOC testing ordered were extracted using the BioMerieux EasyMag instrument. Extracted DNA (5 µl) was tested using the two methods for shared targets of *Anaplasma phagocytophilum, Ehrlichia*, and *Babesia* species. The Diasorin Tick-ASR includes these targets as well as detection of *Borrelia* species. Where possible, data were obtained for each patient, including age, sex, environment/tick exposure, clinical manifestations, Lyme serology, immune status, and antibiotic therapy. Limit of detection (LoD) was compared for both methods using dilutions of Exact Diagnostics control material for each target. **Results:** Among these 325 samples, we identified 24 *A. phagocytophilum*, 3 *Babesia*, and 4 *Ehrlichia* infections, with 100% correlation between the two methods. No sample contained more than one pathogen by PCR, however; four patients were diagnosed with *Anaplasma/Lyme* co-infections based on positive PCR results for *Anaplasma* and positive serology (IgM and Western blot) for Lyme. The Diasorin Tick-ASR also detected 1 *Borrelia* infection that would have been missed using our SOC panel. The LoDs of the two methods were excellent ranging from < 125 genomic copies /µL to 747 genomic copies /µL. Full analysis of chart review data will be provided in the poster. **Conclusions:** The Diasorin Tick-Borne ASRs demonstrated excellent correlation with our LDT SOC assays and detected additional *Borrelia* infection that would otherwise have been missed with our SOC panel. The LoDs determined by each method also showed good correlation and sensitivity for all targets. Inclusion of the *Borrelia* target in the Diasorin ASR panel will provide enhanced clinical sensitivity for tick transmitted pathogens beyond our more limited lab-developed PCR panel in conjugation with Lyme serology testing.

**ID04. Development and Performance of a Multiplex Polymerase Chain Reaction (PCR)-Based Assay for Detection of Bacteria in Sterile Body Fluids**

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**Introduction:** Infection of normally sterile body sites often results in severe morbidity and mortality; therefore, rapid and accurate microbiological assessment of these samples is important for successful patient management. Culture from bodily fluids such as pleural, synovial, and bone/joint aspirates are frequently negative in the pediatric setting due to several factors including low specimen volume and organism burden. Common causes of pleural space and bone and joint infections in children include *S. aureus*, *S. pyogenes* and *S. pneumoniae*. *Kingella kingae* is a fastidious organism that is increasingly recognized as a cause of osteoarticular infections in children. *N. meningitidis* causes severe invasive infections like meningitis, septicaemia, and occasionally septic arthritis and joint infections. The aim of this study was to assess the performance characteristics of a lab-developed, real time PCR assay for detection of these bacterial pathogens in sterile body fluids. **Methods:** A total of 272 prospectively collected body fluid specimens (75 CSF, 94 pleural fluid, and 103 bone/joint specimens) from 241 patients had DNA extracted using the DNA Tissue Kit and Bacterial Card on the QIAGEN EZ1 Advanced XL instrument, and amplification and detection were performed on the ABI 7500 instrument. PCR primer/probes were designed and multiplexed to detect organism-specific gene targets. PCR results were compared to contemporaneous cultures and reference lab molecular testing, if ordered. **Results:** Based on comparator culture and ancillary testing, the calculated sensitivities and specificities, respectively, for the 6 targets on the panel were: *N. meningitidis* 2/2 (100%) and 270/270 (100%), *S. pneumoniae* 6/6 (100%) and 224/2246 (94.2%), *S. pyogenes* 5/5 (100%) and 253/263 (96.2%), methicillin-resistant *S. aureus* (MRSA) 14/14 (100%) and 257/258 (99.6%), methicillin-susceptible *S. aureus* (MSSA) 12/12 (85.7%) and 257/258 (99.6%), and *K. kingae* 3/3 (100%) and 263/269 (97.8%). There were 166 true negative samples by all methods. Notably, PCR on direct specimens detected 42 additional *S. pneumoniae*, 10 *S. pyogenes*, and 6 *K. kingae*. The overall sensitivity of culture compared to PCR was 25.5%. **Conclusions:** The sterile body fluid PCR assay provided a more rapid turnaround time (~3 h) than traditional culture (~3 days). It was also much more sensitive than culture and was able to detect a larger number of fastidious organisms known to be challenging to recover in culture (e.g., *K. kingae*). Implementation of this sterile body fluid PCR allowed for more timely and accurate detection of bacterial pathogens that can cause bone and joint, central nervous system, and pleural space disease.

**ID05. Automated Multiplex Real-Time PCR Detection of *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* Using the Panther Fusion Open Access System**

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**Introduction:** The incidence of tick-borne infections in the United States has risen significantly in the past decade. Most notably, Eastern New York State has seen a rise in anaplasmosis. As a result, test volumes have increased dramatically, and automation is needed. The Panther Fusion (Fusion) has an Open Access functionality to perform lab developed tests on a fully automated system for nucleic acid extraction and multiplex real-time PCR amplification and detection. Our laboratory adapted an *Anaplasma phagocytophilum* (AP) and *Ehrlichia chaffeensis* (EC) multiplex real-time PCR on the Fusion (APEC). This assay targets the AP *msp2* gene and the EC dsb gene. **Methods:** DNA from two plasminids, each containing one of the target regions, obtained from Kimberlee Musser, Wadsworth Center, New York State Department of Health, were used to create a series dilution panel (6 concentrations at 1:10 dilutions in whole blood) and tested six to eight times for limit of detection (LOD) and precision analysis. A panel of 39 organisms that can be present in the blood were spiked into whole blood for analytical specificity studies. To evaluate the performance of APEC on Fusion with clinical specimens, 80 whole blood samples, which were previously tested with our test of record (TOR, DNA extraction on the MagNA Pure 24 and PCR amplification on SmartCyclers) were analyzed. This sample set included 50 specimens previously shown to be positive for AP. Since there were no clinical specimens positive for EC available, 30 specimens (including 20 AP positive ones) were spiked with EC plasmid DNA at concentrations of 100 or 10,000 copies/reaction. **Results:** From these studies, the LODs for AP and EC were 11 and 10 copies/reaction, respectively. The coefficient of variance (CV) for the intra-assay and inter-assay Ct values, across three concentrations of DNA, ranged from 0.5% to 2.1% for AP and 0.3% to 2.6% for EC. The panel of 39 blood associated pathogens were all negative with the APEC assay, demonstrating lack of cross-reactivity. Among the 80 clinical whole blood specimens, AP was detected on the Fusion in 49 out of 50 positive specimens. The 1 false negative sample was retested by both methods and was now positive by both, albeit with high Ct values (35.6 on Fusion and 34.8 by TOR). EP was detected on Fusion in all 30 spiked specimens. **Conclusions:** APEC PCR was successfully developed and optimized on the Panther Fusion with performance characteristics comparable to our TOR. Furthermore, automation of this assay will help the lab meet the demand for increased testing. This assay complements a separate Open Access PCR for *Babesia microti* and expands our automated testing capabilities on the Fusion for tick-borne diseases.

**ID06. Automated Real-Time PCR Detection of *Babesia microti* Using the Panther Fusion Open Access System**

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**Introduction:** Babesiosis is caused by the parasite *Babesia microti* (BM) which infect red blood cells and are spread by the tick *Ixodes scapularis*. In the United States, transmission mostly occurs in the Northeast and upper Midwest, and usually peaks during the warm months. Babesiosis is an emerging zoonosis with important public health implications, as the incidence of the disease has risen dramatically over the past decade. The Panther Fusion (Fusion) has an Open Access functionality to perform lab developed tests on a fully automated system for nucleic acid extraction and multiplex real-time PCR amplification and detection. PCR for the detection of BM provides for more accurate diagnosis. Our laboratory adapted a BM real-time PCR, which targets the 18S rRNA gene of the
Evaluation of an Automated rRNA Quantitation System for Rapid AST in Clinical Lab Diagnostics


Introduction: The GeneFluidics multiplex biosensor based-rRNA quantitation system represents a sensitive, low-cost strategy for molecular diagnostics. This electrochemical sensor is a robust platform and does not require nucleic acid amplification. This technology has been incorporated into several automation systems to streamline the rapid identification and antimicrobial susceptibility testing (AST) of pathogenic microorganisms. In this study, we have evaluated the performance of ProMax and assessed the application of phenotypic transcriptional responses to antibiotic exposure for AST. This study indicates that the integration of rRNA quantitation and electrochemical sensor technology in an automated system could provide a powerful and versatile diagnostic tool.

Methods: AST is measured by electrochemical-based sandwich hybridization. After culturing a diluted inoculum in a strip well for 2h at preconfigured antibiotic breakpoints of ciprofloxacin (CIP), gentamicin (GEN), and meropenem (MEM), lysate from each well was transferred to sensor chips for 16S rRNA sequence-specific hybridization-based measurement. A built-in multi-channel potentiostat reads the electrical current from the steady-state enzymatic cycling amplification. As the reduction current from the HRP enzymatic REDOX amplification is proportional to the bound 16S rRNA content from lysate, the relative signal strength from wells containing the tested organism is susceptible, intermediate, or resistant to each antibiotic based on a built-in classification algorithm.

Results: The categorical classification of 35 reference bacterial strains (CDC, ATCC) and 36 clinical isolates (10 species) obtained on ProMax was compared to the disk diffusion result to assess the percentage of categorical agreement (CA), major error, and very major error. The CA of the reference strains was 92.3% (CIP), 95.5% (GEN), and 94.1% (MEM). The CA of all antibiotics was 100% for 35 of 36 isolates, and 1 isolate of M. morganii for CIP AST was between R and I by disk diffusion. The reproducibility was assessed by testing 35 reference strains with various operators using different batches of consumables over 30 days. The reproducibility of the results was 95.8% for CIP, 96.1% for GEN, and 96.7% for MEM.

Conclusions: We have demonstrated the accuracy and reproducibility of testing results on ProMax in this study. Measurement of the phenotypic response of bacterial rRNA transcription to antibiotics using the biosensor provides a complementary alternative to the genotypic detection of antibiotic resistance genes, as antibiotic resistance mechanisms of most pathogens are diverse and continue to evolve.
TI values) and NanoString counts were input into two neural network classifiers (BNV-2 and SEV-2) which output scores for the presence of bacterial infection, presence of viral infection, and condition severity, respectively. Results: The score correlations between LAMP and NanoString were 0.98 for BNV-2 bacterial score, 0.93 for BNV-2 viral score, and 0.88 for the SEV-2 severity score. A Bland-Altman analysis gives a 95% LOA of -0.17 to 0.14, -0.26 to 0.30, and -0.15 to 0.12, respectively. Furthermore, the bead-based sample prep took just 12 minutes, which, combined with a 15-minute LAMP process, yields a rapid turnaround time. Conclusions: Our rapid LAMP assay demonstrated accuracy for quantifying mRNAs. The rapid and isothermal features of our assay approach advance the InSep test’s development into a <30-minute point-of-care diagnostic test, towards the ultimate goal of antimicrobial stewardship.

ID10. In silico Performance of a Rapid Sepsis Test in Patients with Candidemia
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Introduction: SeptiCyte RAPID is the first-in-class sepsis diagnostic to gain FDA-clearance for differentiating infection negative systemic inflammation (INSI) from sepsis. The assay in Biocartis Idylla cartridge format is called SeptiCyte RAPID. We have previously demonstrated that SeptiCyte RAPID biomarkers can differentiate both bacterial and viral sepsis from INSI, and here we demonstrate the assay’s ability to differentiate candidemia from three control cohorts. Methods: The two genes of SeptiCyte RAPID were used in an in silico analysis of microarray data. For microarray data, patients with positive blood cultures for Candida (n = 6) were compared to healthy (n = 42) patients initially suspected of sepsis and treated with antibiotics but subsequently demonstrated not to have an infection (“no infection,” n = 26) and INSI (43) patients. Patients and samples were selected from the MARS database and biobank based on discharge diagnosis and/or clinical microbiology results. For blood culture positive samples, only those with a single organism isolated were used. Diagnostic performance was measured using Receiver Operating Characteristic Area Under Curve (ROC-AUC). Results: Using in silico data from microarrays, the biomarkers of SeptiCyte RAPID had AUCs of 1.00, 0.942, and 0.942 for differentiating candidemia from healthy subjects and patients with discharge diagnoses of INSI or no infection. Conclusions: In silico analysis of microarray data has demonstrated that the two genes of SeptiCyte RAPID differentiated patients with candidemia from blood culture negative patients, those with INSI, and healthy subjects. In combination with prior work, we have now demonstrated that SeptiCyte RAPID differentiates patients with bacterial, viral, or Candida sepsis from clinical controls.

ID11. Development of ViroKey SARS-CoV-2 RT-PCR Test v2.0 for the Sensitive and Accurate Automated Detection of the SARS-CoV-2 Virus
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Introduction: As of early July 2020, a novel coronavirus SARS-CoV-2, responsible for COVID-19, has infected more than 10.2 million people across more than 103 countries. At point of writing, the global death toll has topped 503,000. These numbers are expected to increase, as SARS-CoV-2 has demonstrated the capability to rapidly spread, leading to significant impact on health care systems and social systems. Thus, there is an urgent need for a fast, high-throughput, and accurate diagnostic method to provide timely treatment for those infected and to prevent community infections. Methods: The ViroKey SARS-CoV-2 RT-PCR Test v2.0 is a real-time RT-PCR-based in vitro diagnostic test intended for qualitative detection of SARS-CoV-2 RNA in clinical specimens (nasopharyngeal, oropharyngeal swabs, and saliva). The assay can be run on an automated workflow using the Sentosa SX101 liquid handler instrument with the ViroKey SX Virus Total Nucleic Acid Kit extraction kit, followed by the detection of SARS-CoV-2 RNA on the ABI 7500 Fast Dx or Sentosa SA201 Real-Time PCR instrument, and automated data analysis and reporting on the SA201 Reporter software. The assay utilizes two targets (ORF1a and N genes) in a single multiplex reaction that is highly specific to the SARS-CoV-2 RNA. The assay performance was evaluated using SARS-CoV-2 genomic RNA as well as heat-inactivated SARS-CoV-2 virus. Lastly, clinical performance was evaluated with 30 positive and 30 negative nasopharyngeal clinical samples. Results: The ViroKey SARS-CoV-2 RT-PCR Test v2.0 was determined to be specific to SARS-CoV-2, and showed no cross-reactivity to closely related coronaviruses and other respiratory viruses causing similar febrile illnesses based on wet-testing and in silico analysis. The analytical limit of detection (LoD) of the assay was determined as 200 GE/mL of sample input or 9.2 GE/reaction in nasopharyngeal samples. In saliva, LOD was 300 GE/mL of sample input or 13.8 GE/reaction. Assay performance was not affected by potentially interfering agents such as common cold medications. Assay reagents are stable for up to 5 freeze-thaw cycles, storage at 2°C to 8°C for 7 days and 30°C for 3 h. Clinical performance when tested with clinical nasopharyngeal samples showed sensitivity of 96.7% (29/30), specificity of 100% (30/30), PPV of 100% (29/29), and NPV of 96.8% (30/31). Turn-around time including automated analysis and reporting for 48 tests (46 samples, 1 positive and 1 negative control) is 4 h to 4.5 h with a 0.5 h to 1 h hands-on time. Conclusions: We have developed a highly specific and sensitive automated SARS-CoV-2 nucleic acid test assay that can process 46 samples and 2 controls simultaneously. This may be beneficial for rapid mass screening, thus contributing significantly to the fight against COVID-19.

ID12. Comparison of Four Commercial Molecular Diagnostic Kits for Detection of SARS-CoV-2: A Pilot Study
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Introduction: Coronavirus disease 2019 (COVID-19) pandemic has resulted in the need for the rapid implementation of diagnostic testing centered on detection of SARS-CoV-2 RNA using real-time RT PCR. Testing for COVID-19 enables infected individuals to be identified and isolated to reduce spread, allows contact tracing for exposed individuals, and provides knowledge of regional and national rates of infection for interventions. Though many COVID-19 RT-PCR kits are currently commercially available, an independent assessment of these products by a diagnostic lab is a mandate prior to patient testing. This study aims to test multiple commercially available CE-IVD real-time PCR options for detection of SARS-CoV-2. Methods: A total of 27 clinical specimens (nasopharyngeal swabs in VTM) were analyzed using nCoV Real-Time Detection kit (RdRp, E gene) from SD Biosensor, Alplex 2019-nCoV Assay (RdRp, N gene, E gene) from Seegene Inc., RealStar SARS-CoV-2 RT-PCR Kit (S gene, E gene) from Altona Diagnostics, and Xpert Xpress SARS-CoV-2 cartridge based assay (N2 and E gene) from GeneXpert. RNA was extracted using 200 µl specimen on QIAasympn (Qiagen), an automated RNA extraction system using DSP Virus/Pathogen kit. SD Biosensor, Altona, and Seegene assays were run on RotorGene Q Real Time cycler. For Xpert Xpress SARS-CoV-2 assay, 300 µl specimen was directly loaded on the cartridge and was run on the GeneXpert platform. Results: Out of 27 specimens analyzed 16 (59.25%) were positive and 11 (40.75%) were negative for the presence of SARS-CoV-2 by Altona, Seegene, and SD biosensor kits as well as by cartridge based assay. SD biosensor kit was found to be the best amongst real time PCR kits, as the Ct values obtained were much lower in comparison to the other assays. GeneXpert showed best turnaround time of 50 minutes for detection in comparison to the other kits (3.5 to 5 h) due to an additional RNA extraction step. Conclusions: GeneXpert Xpert Xpress SARS-CoV-2 assay offers good sensitivity and TAT for rapid detection of COVID-19. Other CE-IVD Real Time PCR kits discussed in the study provide cost-effective detection of SARS-CoV-2 in comparison to GeneXpert and can be used for routine clinical testing.
Abstracts

**ID13. Evaluation of Ion AmpliSeq SARS-CoV-2 NGS Research Panel**

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**Introduction:** The presence of SARS-CoV-2 viral nucleic acid in clinical samples such as nasal swabs can be detected using PCR or next-generation sequencing (NGS) methods. NGS analysis of clinical isolates can provide insight into virus evolution and help elucidate strain origin and transmission routes for contact tracing. It can also help identify mutation (e.g., D614G) causing increased infectivity. Here we evaluated Ion AmpliSeq SARS-CoV-2 NGS Research Panel, a targeted NGS panel consisting of two primer pools with 237 amplicons covering >99% of the 30 kb SARS-CoV-2 genome, including all published variants. **Methods:** Two sets of clinical samples VR-1986D (USA-WA1) and VR-1991D (Hong Kong) were purchased from ATCC. The virus copies (cps) were provided by ATCC as measured by ddPCR. Each of the two strains (40, 400, or 4,000 cps) and human lung RNA (Thermo Fisher) as negative control were analysed using 10 ng RNA. Samples with mixed ratio 1:4, 1:9, 1:39, and 1:99 of VR-1986D versus VR-1991D were also analysed. Library preparation was performed manually based on manufacturer’s instructions. PCR cycles varied depending on the different viral loads with 18 cycles for 4,000 cps input and 24 cycles for 400 and 40 cps input and negative control. Libraries (n = 12) were enriched on IonChef and sequenced on a 530 chip using S5. Data were analysed using Torrent Suite software and COVID-19 plugins, including Variant Caller for germline and somatic mutations, COVID19AnnotateSnvEff for variant annotation, and AssemblerTrinity and IRMAreport for assembly. **Results:** Both strains were detected from 4,000 to 40 cps with mean read depth >9,000, on target depth >95.3% and uniformity >98%. Negative control showed low reads of human RNA reference genes included in the panel design. VR-1986D showed 100% sequence match with published sequence with 3 variants compared with Wuhan-Hu-1: 8782C >T, 18060C >T, and 28144T >C. Positions 8782 and 28144 are previously identified hotspots of hypervariability. In addition to these two hotspot variants, VR-1991D showed 5 more variants compared with Wuhan-Hu-1: 1663C >T, 22661G >T, 24034C >T, 26729T >C, and 28077G >C. All these variants were concordant with published sequence. Five more variants were detected for VR-1991D but were not reported by the GISAID database. These are true variants between the original primary isolate and passage 6 (from ATCC). Variants with <10% allele frequency were detected across samples with 4,000 to 40 cps. Additional work is needed to determine the exact nature of these variants. Samples with 40 cps showed numerous false variants with AF <10% that are likely to be RT-PCR error. **Conclusions:** In summary, our data demonstrate that the panel has the potential to provide a sensitive, automated NGS workflow to detect and monitor SARS-CoV-2 infection.

**ID15. SARS-CoV-2 Cycle Number as a Metric for Population Trends in New Hampshire**

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**Introduction:** SARS-CoV-2 is a single-stranded RNA virus that causes the severe acute respiratory syndrome known as COVID-19. Using reverse transcriptase, real time PCR (RT-PCR), it is possible to semi-quantify viral load in patients who have tested positive for the virus. SARS-CoV-2 viral burden can be associated with severity of disease and worsening outcomes. Here, we describe general trends for positive SARS-CoV-2 tests performed over a three-month period in the clinical laboratory of a rural New Hampshire medical center. Methods: Our institution implemented SARS-CoV-2 testing using the Abbott Real Time SARS-CoV-2 assay on the m2000. Positive SARS-CoV-2 results from March 24 to June 27, 2020, were compiled from testing performed at the Dartmouth-Hitchcock Medical Center (n = 652). Testing was performed on nasopharyngeal swabs collected from both symptomatic and asymptomatic patients from network hospitals and other client locations in New Hampshire and Vermont. Cycle number (CN) values from the m2000 assay were used to monitor trends in viral burden. Patient results were compared and sorted into very low, low, medium, high, and very high viral concentration cohorts based on CN values. Average monthly values were determined as a metric for spread of the virus. **Results:** Positive SARS-CoV-2 results from 652 patients were analyzed. Of the 652 cases, 0.77% had a very high viral load (CN <3, n = 5), 29.14% had a high viral load (CN between 3 and <10, n = 190), 35.73% had a medium viral load (CN between 10 and <20, n = 233), 32.26% had a low viral load (CN between 20 and <30, n = 211), and 1.99% had a very low viral load (CN >30, n = 31). The range of CN values was 28.6 (lowest CN = 2.84, highest CN = 31.47). March, April, May, and June all had medium viral load averages. March had an average CN of 13.78 (n = 33), April had an average CN = 14.20 (n = 138), May had an average CN = 14.98 (n = 358), and June had an average CN of 19.41 (n = 141). March had the highest viral load, and June had the lowest viral load. **Conclusions:** Viral loads as indicated by real time PCR cycle numbers (CN) showed a downward trend of the amount of virus (increasing CN). This trend followed the incidence of positive cases during the outbreak in our region. The CN given by the Abbott assay can be a metric for monitoring trends in the population. There are also possible implications for viral concentration and correlation with individual patients’ clinic presentation (time between onset of symptoms and testing, type and severity of symptoms) and testing algorithms (symptomatic versus asymptomatic, repeat testing of positive patients, etc.).
Use Authorization (EUA) status from the FDA. Here we describe our verification of the Centers for Disease Control and Prevention (CDC) 2019-Novel Coronavirus (2019-nCoV) Real-Time Reverse Transcriptase (RT)-PCR Diagnostic Panel, using the Applied Biosystems 7500 Fast Dx Real-Time PCR instrument. Methods: Previously tested nasopharyngeal (NP) swabs (n = 57) collected from patients at the Dartmouth-Hitchcock Medical Center and extracted RNA samples (n = 4 positives, n = 4 negatives) received from the New Hampshire Public Health Laboratory were used for the verification of the CDC EUA assay. This test includes primer/probe sets for the N1 and N2 viral sequence targets and an RNaseP gene target that are detected in three separate reactions. RNA was extracted on an EZ1 Advanced XL instrument from 120 µl NP swab UTM using the EZ1 Virus Mini 2.0 kit (QIAGEN). The accuracy, precision, specificity, and limit of detection were verified. Results: Accuracy was determined by testing 41 samples consisting of 6 known positive NP samples and 35 known or presumed negative NP samples. All of the negative samples tested negative, and 83.3% of the positive samples tested positive with 1 sample being indeterminate. This sample repeated as positive. Precision was determined using 6 aliquots of the same 4 specimens extracted in two runs of the EZ1 and by 2 separate laboratory technologists. The 4 specimens were human specimen controls pooled from negative NP swabs and spiked with a high positive patient specimen from the State Laboratory. The same specimens spiked and extracted at different times differed by <0.1 Ct. Of 39 NP swabs from known or presumed negative patients for SARS-CoV-2, 11 were positive for other respiratory pathogens, but tested negative for COVID-19. The lack of cross-reactivity demonstrates the assay’s specificity. Limit of detection (LOD) per the CDC protocol was established at 3.16 copies per µl. Conclusions: The CDC EUA assay met the performance characteristics as indicated for specificity, precision, accuracy, and LOD. Up to 29 specimens can be run per 96-well PCR plate, using three primer/probe sets with approximately 1.5 h of hands-on time and 2 h for the real-time RT-PCR.

ID17. Analytical Validation of a SARS-CoV-2 Whole Genome Sequencing Method by Amplicon-Based NGS

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Introduction: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the ongoing pandemic of coronavirus disease 2019 (COVID-19), a public health emergency of international concern. Whole-genome sequencing (WGS) can aid in characterization of therapeutic targets, and the tracking viral transmission and changes in the viral genome. A number of studies utilizing SARS-CoV-2 WGS have been reported, but the clinical performance of these assays remains uncertain. We developed an amplicon-based SARS-CoV-2 WGS method using next-generation sequencing (NGS) and evaluated its analytical performance using clinical specimens. Methods: Viral RNA was extracted from de-identified sequential clinical specimens collected in March 2020 for SARS-CoV-2 testing by real-time reverse transcription PCR. A total of 141 unique SARS-CoV-2 positives (Ct range between 31 and 9, approximately 40 copies to 163 million copies), 24 pooled positives, and 24 negatives were used for validation. Remnant extracted RNA of confirmed positive or negative specimens was reverse transcribed to cDNA and PCR amplified in two pools using a total of 98 overlapping ARTIC network amplicons with optimized primer concentrations. NGS libraries were sequenced on an Illumina MiSeq sequencer. We utilized an in-house bioinformatics pipeline to generate consensus genomes and identify sequence variants relative to the MN908947.3 reference genome. Results: From inter- and intra-assay precision studies, 96% of specimens (inter-assay, 66/69; intra-assay 66/69) with a Ct ≤30 generated 100% full genome consensus sequence coverage. For 22 inter-and 22 intra-assay replicates, amino acid variants present in ≥10% of the reads were 100% concordant in all the replicates. More than 90% (127/141) of unique patient specimens yielded high-quality sequence data for clade classification and further analyses. Clade G, defined by the S-gene mutation D614G, was identified in 60% of the patient specimens, followed by clade D (25%), clade O (13%), and clade V (2%). In addition, 117 of the 127 specimens generated ≥99% full genome consensus coverage. For these 117 specimens, variant analysis identified a median of 7 [IQR: 6 to 8] amino acid substitutions per genome. The median count of variants per genome differed significantly by clade and was higher in clade S, a median of 8.5 [IQR: 7 to 11] (p < 0.0001). Viral coding regions of S3a and orf8 harbored a significantly higher proportion of variants relative to other genomic coding regions (p < 0.001). Conclusions: SARS-CoV-2 WGS NGS can be used to generate near-complete genome coverage from clinical specimens. The method may be widely used for classification of SARS-CoV-2 subtypes and identification of genetic relationships.


Thermo Fisher Scientific, South San Francisco, CA.
Introduction: The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiologic agent of the 2019 coronavirus disease (COVID-19). In addition, human influenza type A and B viruses (Flu A and Flu B) cause epidemics of disease in many countries each year. Thus, a respiratory panel that captures these critical human infectious agents such as SARS-CoV-2, Flu A, and Flu B is urgently needed for research in the upcoming 2020 flu season during the COVID-19 pandemic. Methods: To reduce costs and meet the need of high-throughput testing of research samples, we developed a 4-plex real-time PCR assay in 1-tube, for nucleic acid detection of SARS-CoV-2, Flu A, Flu B viruses, along with a process control. We utilized some of the SARS-CoV-2 assays from our existing TaqPath COVID-19 Combo Kit. SARS-CoV-2 assay targets both spike (S) protein and nucleocapsid (N) protein regions having higher specificity and exhibiting lower risk for mutation. We further leveraged our proprietary bioinformatics pipeline and designed specific assays for Flu A and Flu B with great coverage. Strain coverage is 99.9% for SARS-CoV-2 based on data download from GISAID on July 6, 2020, with 35,833 high-quality complete sequences. Strain coverage for Flu A and Flu B is 6,730,854 or 98.2%, and S15053,127 or 99.3%, respectively, as of April 13, 2020. Assays were tested with qPCR instruments such as Q5S and 7500 Fast Dx using a modified RT-PCR protocol based on TaqPath COVID-19 Combo Kit. Results: These 4-plex real-time PCR assays can simultaneously detect and differentiate SARS-CoV-2 and influenza type A and B viral nucleic acids down to 10 genomic copy equivalent (GCE) per reaction (Rxn). The assays have been tested on multiple qPCR instruments and demonstrated great sensitivity for all three viral targets. The 4-plex assay showed robustness with a linear dynamic range of detection 10^7 to 10 GCE/Rxn with both synthetic DNA controls and genomic RNA controls of these viruses, as well as excellent PCR efficiency and clean NTC. We also performed inclusivity testing and exclusivity testing for the panel, and data showed high strain coverage and high specificity. Conclusions: We developed this 1-tube multiplex real-time PCR assay panel detecting 3 critical and challenging viral nucleic acids with high sensitivity, high specificity, and maximum strain coverage. The delivery of this 4-plex assay has a significant impact on public health research, especially on simultaneous molecular detection and differentiation of SARS-CoV-2, Flu A, and Flu B viruses. This new 4-plex panel will play an important part in the global battle against SARS-CoV-2 and COVID-19, and provide meaningful differentiation among the three most important viruses in respiratory infection. For research use only. Not for use in diagnostic procedures.
ID19. Comparison of Test Performance of Two Rapid SARS-CoV-2 Viral Assays  
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Introduction: The performance of the ID NOW COVID-19 rapid test to detect viral SARS-CoV-2 has been reported in the literature using residual specimens in viral transport medium (VTM). The revised indications by the Food and Drug Administration’s Emergency Use Authorization for the ID NOW test specify that the assay should be performed using direct swabs (dry swabs) since studies showed that dilution of the sample in viral transport media results in reduced detection sensitivity. Here we report a validation study comparing nasopharyngeal swabs obtained in parallel from the same patient to compare the performance of the ID NOW COVID-19 (Abbott), which targets the RdRp gene and the RT-PCR Xpert Xpress SARS-CoV-2 (Cepheid) which targets the E and N genes of SARS-CoV-2.

Methods: Samples from 303 patients were collected in parallel with 1 nasopharyngeal swab submitted in VTM for the Xpert Xpress SARS-CoV-2 RT-PCR to be run in the Cepheid Infinity 48 platform and 1 nasopharyngeal dry swab to be tested directly in the Abbott ID NOW platform, for validation of the ID NOW COVID-19 test for detection of SARS-CoV-2. Samples were tested for Abbott ID-NOW within 2 h of being received in the laboratory. Ct values for the Xpert RT-PCR were recorded. Three samples that were positive by the Xpert RT-PCR but were negative by ID NOW were subjected to reverse transcriptase loop-mediated amplification. The Xpert RT-PCR Ct values of the 6 cases that were false negative by ID NOW and 1 case that was false negative by Cepheid. The Xpert RT-PCR Ct values of the 6 cases that were ID NOW false negative for the N target were Ct = 38.5 to 42.1, with only 1 case amplifying the E2 target Ct = 37.7. Three samples that were positive by the Xpert RT-PCR but negative by ID NOW were subjected to viral culture and were negative. Taking the Xpert RT-PCR as the gold standard, Abbott ID NOW sensitivity for cases with Xpert RT-PCR amplification of E2 target Ct <37.7 and N2 target Ct <38.5 was 100% (CI = 93% to 100%), whereas the sensitivity for amplification at Ct ≥37.7 was 66.7% (CI = 41% to 84%).

Conclusions: The Abbott ID NOW rapid COVID-19 viral test showed lower sensitivity as compared to the Xpert RT-PCR. However, for samples tested with Xpert RT-PCR Ct <37.7, ID NOW detected cases with 100% sensitivity. All ID NOW false negative cases had Ct values by Xpert RT-PCR ≥37.7. The ID NOW false negatives may represent, in some cases, viral RNA remnants rather than live virus since cultures failed to grow SARS-CoV-2 virus.

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Introduction: The SARS-CoV-2 pandemic necessitated expedited diagnostic test platforms with varying turnaround times. Abbott IDNOW received scrutiny for its high false-negative rate, attributed to a dilutional effect caused by the viral transport media (VTM). A multi-institutional analysis of performance of this test was conducted to further clarify performance characteristics of the assay.

Methods: Ten facilities, including large quaternary care centers, community hospitals, and emergency departments, participated in the study. Two swabs, nasopharyngeal (NP) or nasal, were obtained on all participants in the study; 1 swab was analyzed on the IDNOW, with Roche Cobas 6800 RT-PCR platform serving as the gold standard comparator. Transport media for the IDNOW included VTM until the instructions for use (IFU) update in April 29, 2020, at which time only dry swabs (transported with no liquid media) were accepted for testing.

Results: A total of 534 specimens were tested; 5 were eliminated due to an initial invalid IDNOW result, 3 were eliminated due to improper transport media, and 7 were eliminated due to positive IDNOW tests not originally being reflexed to confirmatory RT-PCR. Overall, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the Abbott IDNOW were as follows, respectively: 89.66%, 99.57%, 96.30%, and 98.71%.

Conclusions: Ten institutions tested a total of 534 specimens, providing a representation of performance across several hospitals and populations. A total of 519 specimens were analyzed with the overall sensitivity and specificity (89.66% and 99.57%) generally matching those reported in literature. Performance variation did occur among institutions, though this is likely multifactorial with no one specific identifiable root cause. For example, the best performance was seen at the largest hospital in the network where testing was restricted to the lab and delivery department (sensitivity and specificity 100% each). Another unexpected finding is the increased sensitivity of swabs transported in VTM versus the dry swabs on IDNOW.

ID21. Detecting Signatures of SARS-CoV-2 Using Clusters Regularly Interspaced Short Palindromic Repeats (CRISPR)  
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Introduction: Novel applications exploiting the genome editing ability of the CRISPR-Cas system have triggered significant advances in CRISPR diagnostics. This has prompted an interest in developing new biosensing applications for nucleic acid detection. Recently, such applications have been engineered for detection of SARS-CoV-2. Increased demand for testing and consumables of RT-qPCR assays has led to the use of alternative testing options in some cases. Here we evaluate the accuracy and performance of the Sherlock CRISPR SARS-CoV-2 kit using the CRISPR-Cas13a system.

Methods: The Specific High-Sensitivity Enzymatic Reporter UnLOCKing (Sherlock) technology forms the basis for this approach. Extraction of nucleic acids from nasopharyngeal swabs was performed using the Qiagen EZ1 Advanced system. Following the Sherlock CRISPR SARS-CoV-2 kit IU, the extracted material was subjected to reverse transcriptase loop-mediated amplification. The amplified products were incubated with Cas enzyme complexed with CRISPR guide RNAs specific to SARS-CoV-2 targets. Fluorescent readouts of the cleaved reporter were taken at 2.5 min intervals completing in 10 minutes on a microplate reader (BioTek). Data output of relative fluorescence ratios were normalized to a no-template control. The relative fluorescence ratios on negative samples ranged from 16.45 to 49.17 and 33.82 to 48.15 for N and ORF1ab gene targets, respectively. Conversely, fluorescence ratios on positive samples ranged from 0.54 to 1.28 and 0.84 to 4.93 for N and ORF1ab targets, respectively.

Results: The experimental strategy included selection of COVID-19 patient samples from previously validated RT-qPCR assays (CDC, Abbott m2000). Positive samples were selected based on a broad range of cycle thresholds (Ct). These comprised an average of low (µ = 7.11), mid (µ = 17.2), and high (µ = 27.9) Ct values. A total of 20 COVID-19 patient samples (10 positives and 10 negatives) were correctly diagnosed with up to 100% accuracy. All controls, including RNAse P, showed expected findings with 5.500 copies/µL detected for diluted positive control (BEI). Specifically, for COVID-19 positive samples, normalized ratios ranged from 16.45 to 49.17 and 33.82 to 48.15 for N and ORF1ab gene targets, respectively. Conversely, fluorescence ratios on negative samples ranged from 0.54 to 1.28 and 0.84 to 4.93 for N and ORF1ab targets, respectively.

Conclusions: We have evaluated the accuracy of detecting conserved targets of SARS-CoV-2 across a range of viral loads using the Sherlock CRISPR collateral detection reaction. These findings demonstrate encouraging results, especially at a time when COVID-19 clinical diagnosis is in high demand, often with limited resources. This approach highlights new thinking in infectious disease identification and can be expanded to measure nucleic acids in other clinical isolates.
ID22. A Practical Comparison of Seven Molecular SARS-CoV-2 Methods

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Introduction: The SARS-CoV-2 pandemic demanded a rapid and unprecedented response from clinical laboratories across the country. Access to accurate and timely SARS-CoV-2 testing remains critically important for both patients and healthcare workers. With major operational challenges including supply constraints and growing clinical demand, our multi-institute health system deployed numerous methodologies for SARS-CoV-2 viral RNA detection. This strategy afforded scalability, redundancy, and the flexibility to accommodate both rapid and routine testing. Leveraging our experience with seven methodologies, a practical comparison of key operational and analytical metrics is presented.

Methods: Comparator samples were generated by combining quantified positive clinical samples (n = 8, nasopharyngeal swabs [NPS] in viral transport media [VTM]) at different dilution levels with pooled negative samples (n = 17, NPS in VTM). Positive samples were quantitated with a standard curve generated using synthetic RNA (N gene) on a CDC-based methodology. Dilutions ranged from 50,000 to 50 copies (cp/mL). Comparator samples were tested on five commercial SARS-CoV-2 assays (Abbott m2000, Cepheid, GenMark, Roche, and Thermo Fisher) and two laboratory developed tests (based on CDC and BDXmax methodology). Dilutions <1,000 cp/mL were evaluated on selected methods based on the stated limit of detection (LOD). Other critical variables (e.g., cost, turnaround time [TAT], batch size, training requirements, etc.) were compared among methods to identify the use case for each methodology.

Results: All methods detected viral RNA at 50,000 cp/mL in 0.8 reactions; however, only five methods detected viral RNA at 1,000 cp/mL in all reactions. Compared to the stated LODs, four methods were equivalent, two methods were twice as sensitive (Abbott and CDC based), and 1 method was 10-fold less sensitive (Roche). Methods utilized for rapid testing (≤2 h TAT; Cepheid, GenMark, BDXmax) often have higher cost, reduced sensitivity, and decreased throughput. High-throughput methods (up to 96 tests/run; Abbott-m2000, Roche, Thermo Fisher) had higher sensitivity, a higher throughput, and can provide redundancy, and the flexibility to accommodate both rapid and routine testing. Leveraging our experience with seven methodologies, a practical comparison of key operational and analytical metrics is presented.

Conclusions: This study showed that many of the methods evaluated have similar levels of analytical sensitivity (≤1,000 cp/mL). Factors such as ease of use and cost play an important role in determining which assays to develop for clinical use and which should be carefully evaluated to determine if the benefits outweigh their limitations. Having multiple testing platforms for SARS-CoV-2 testing allows the lab to adapt to changes in testing volumes and reagent supply as well as to prioritize rapid and routine testing to the appropriate methodology.


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Introduction: Coronavirus disease 2019 (COVID-19) is a respiratory tract infection caused by a newly emergent coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was recognized in Wuhan, Hubei Province, China, in December of 2019. The standard diagnostic method of SARS-CoV-2 is a real-time PCR testing with nasopharyngeal specimens. Although many companies have produced PCR assays for SARS-CoV-2 RNA extraction reagents, our laboratory was performed on a limited number of automatic instruments and extraction reagents. This study was performed to compare clinical performance of the SARS-CoV-2 assay after a simple boiling step instead of a conventional RNA extraction process using an automated platform.

Methods: We developed the multiplex real-time PCR assay (Aliplex SARS-CoV-2 assay, Seegene) to detect 4 target genes (E, N, RdRp, and S gene) of SARS-CoV-2 in a single tube. A total of 134 nasopharyngeal specimens including 33 negative and 101 positive samples were used to compare the RNA extraction using Magnapure96 instrument (Roche) and extraction-free, simple boiling condition. Various virus transport media (VTM) including ESwab, UTM, and MSwab were tested to optimize the dilution fold using nucleic-acid-free water. The sensitivity and specificity were analyzed between extraction and extraction-free process. Results: Fifteen microliters of specimen from each VTM were aliquoted and tested in different dilution conditions between 1:1 and 1:3 with nucleic-acid-free water. Extraction-free conditions including incubation time between 1 and 5 min and also boiling temperature between 95°C and 100°C were tested. Sensitivity and specificity in optimized extraction-free conditions with clinical specimens were compared to those in automated extraction condition. Overall agreement of 90% was achieved. Especially in specimens with less than Ct 35, agreement rate was dramatically increased to about 100%. Conclusions: Applying an extraction-free PCR protocol would avoid limitations on COVID-19 screening capacity in low-to-middle-income countries (LMIC) caused by global PCR and extraction reagent supply constraints. We provided a workflow-based, extraction-free method with the appropriate SARS-CoV-2 assay, be explored further by other medical laboratories to improve the testing capacity of virology laboratories during the pandemic.

ID24. The Combination Assay for SARS-CoV-2 and Other Respiratory Viruses in Symptomatic Patients and the Statistical Outcome

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Introduction: SARS-CoV-2 is rapidly spreading globally and has become a major public health threat. Its reported symptoms are wide ranging, from mild cold-like illness to complicated pneumonia, severe inflammatory response, and death. Recently, case and prevalence studies on SARS-CoV-2 co-infected with the most common respiratory viruses and other coronaviruses have been reported in different communities and nations. To meet the global interests and needs for understanding complications related to the most common respiratory pathogens including SARS-CoV-2, Seegene Inc., initiated extensive clinical studies using a combination assay called OVERCOMM (Overseas Validation Experiments for Respiratory virus and Coronavirus co-testing in Multi-centers, Multination). Additionally, OVERCOMM provides a web-based statistics tool for global trends would be essential tools to prepare for a potential second wave of COVID-19 and co-infection with other respiratory viruses shortly.

Methods: Eight hundred thirteen nucleic acids of respiratory symptomatic patients were tested with Aliplex SARS-CoV-2 Assay and Allplex RV-EA detecting seven respiratory viruses (Flu A, Flu B, RSV, MPV, HRV, AdV, and PIV), and results were sequentially uploaded using Seegene Viewer plus software. After login, results were analyzed with various functions (i.e., summary of infection, trend of infection, and co-infection). Results: A total of 319 (38.4%) specimens were positive; 276 (33.3%) and 52 (6.3%) samples were detected with SARS-CoV-2 and other respiratory viruses, respectively. HRV was the most prevalent pathogen, followed by RSV, MPV, AdV, Flu A, and PIV, and Flu B, each of which appeared in 2 to 17 infections. Out of 319 positive, 9 (2.8%) samples were co-infected with SARS-CoV-2 and other respiratory viruses. Conclusions: The combination assay for accurate diagnosis and the statistical tool for global trends would be essential tools to prepare for a potential second wave of COVID-19 and co-infection with other respiratory viruses shortly.

ID25. Temporal Spatial Heterogeneity of Immune Response to SARS-CoV-2 Lung Infection

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Methods: We developed the multiplex real-time PCR assay (Aliplex SARS-CoV-2 assay, Seegene) to detect 4 target genes (E, N, RdRp, and S gene) of SARS-CoV-2 in a single tube. A total of 134 nasopharyngeal specimens including 33 negative and 101 positive samples were used to compare the RNA extraction using Magnapure96 instrument (Roche) and extraction-free, simple boiling condition. Various virus transport media (VTM) including ESwab, UTM, and MSwab were tested to optimize the dilution fold using nucleic-acid-free water. The sensitivity and specificity were analyzed between extraction and extraction-free process. Results: Fifteen microliters of specimen from each VTM were aliquoted and tested in different dilution conditions between 1:1 and 1:3 with nucleic-acid-free water. Extraction-free conditions including incubation time between 1 and 5 min and also boiling temperature between 95°C and 100°C were tested. Sensitivity and specificity in optimized extraction-free conditions with clinical specimens were compared to those in automated extraction condition. Overall agreement of 90% was achieved. Especially in specimens with less than Ct 35, agreement rate was dramatically increased to about 100%. Conclusions: Applying an extraction-free PCR protocol would avoid limitations on COVID-19 screening capacity in low-to-middle-income countries (LMIC) caused by global PCR and extraction reagent supply constraints. We provided a workflow-based, extraction-free method with the appropriate SARS-CoV-2 assay, be explored further by other medical laboratories to improve the testing capacity of virology laboratories during the pandemic.
analyzed lung specimens from 24 autopsies from patients who succumbed to SARS-CoV-2 infection. We visualized viral RNA by in situ hybridization (RNA-ISH) and assessed the immune response using GeoMx Digital Spatial Profiler (DSP), immunohistochemistry (IHC), and total RNA sequencing (RNA-seq). **Methods:** We evaluated 89 pulmonary and extrapulmonary samples from 24 COVID-19 autopsies. All patients were confirmed for SARS-CoV-2 infection through qRT-PCR assays performed on nasopharyngeal swab specimens. We performed RNA-ISH for SARS-CoV-2 and IHC for CD3, CD8, CD20, CD163, CD123, IDO1, PD-1, napsin, keratin, and SARS-CoV N. qRT-PCR, RNA-seq and the Nanostring GeoMx DSP were performed on selected cases. **Results:** Quantitative assessment of RNA-ISH on cases were classified as high versus low viral RNA, with a notable shorter time of disease to death in high compared to low viral cases. To validate the RNA-ISH data, we performed molecular confirmation through qRT-PCR and RNA-seq on 15 COVID-19 cases, and 5 non-COVID-19 autopsy lung specimens as controls. A correlation was noted across the orthogonal technique of viral detection. Total RNA-seq analysis demonstrated a separation of high from low viral RNA samples. On gene set enrichment analysis for genes that were different between the clusters, the virus high cluster had high expression of interferon stimulated genes, antiviral genes, and genes related to wound healing. Low virus samples had notable elevated surfactant genes, mucins, and keratins suggesting higher presence of airway and alveolar lining cells. Noting variable morphological appearance across the lobes of the lung in individual patients accompanied by variable distribution of the virus, an intrapulmonary RNA and protein analysis of viral positive and negative areas was performed with the GeoMx DSP. The analysis highlighted the spatial heterogeneity within different regions of lung specimens, which showed a consistent spatial distribution of virus and interferon driven immune response. **Conclusions:** We demonstrate heterogeneous levels of SARS-CoV-2 RNA across patients with high viral cases having a shorter duration of disease to time of autopsy that correlated with a robust interferon immune response. Moreover, we find spatial heterogeneity of interferon immune response within individual lung specimens from patients. Altogether, the temporal and spatial changes of host immune response to SARS-CoV-2 present a complex picture for the timing of therapeutic interventions for COVID-19.


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**Introduction:** Part of the strategy to combat spread of SARS-CoV-2 is widespread testing of symptomatic persons under investigation (PUIs). Testing has also been deployed to screen asymptomatic patients being admitted to the hospital or scheduled for potential aerosol-generating procedures, so-called surveillance (SURV) testing. The goal of this study is to evaluate the real-world performance of six nucleic acid detection test systems for detection of SARS-CoV-2 in pediatric and maternal PUI and SURV patients. The following test systems were utilized: Aptima SARS-CoV-2 on Hologic Panther; Altona RealStar SARS-CoV-2 RT-PCR kit 1.0 with nucleic acid extracted on the Roche MagNA Pure 96 (AlitMP) or bioMérieux EMAG (AlitEM); CDC SARS-CoV-2 RT-PCR assay with nucleic acid extracted on the Roche MagNA Pure 96 (CDCMP) or bioMérieux EMAG (CDCDEM); and the Diasorin Molecular Simplexa COVID-19 Direct system. **Methods:** A total of 153 unique patient NP swab specimens collected in 3.0 mL of MARD viral transport media were enrolled. Sample selection was enriched to obtain an approximate 1:1 ratio of positive and negative specimens for PUI and SURV patients. These included 56 pediatric and 42 maternal SURV patients and 60 pediatric and 35 PUI patients. All testing was performed on the same specimen aliquot after identical storage conditions. The Aptima and Diasorin assays were performed according to the manufacturer’s FDA Emergency Use Authorization (EUA) instructions for use. Amplification and detection using the Altona and CDC assays were performed on the ABI 7500 instrument. A specimen was considered a true positive if the results of two or more of the test systems were positive. **Results:** For both PUI and SURV, the overall clinical sensitivities and specificities were, respectively, as follows: Aptima, 96.3% (78/81) and 100% (112/112); AlitMP, 96.3% (78/81) and 100% (112/112); CDCMP, 100% (81/81) and 98.2% (110/112); AlitEM, 90.1% (73/81) and 100% (112/112); CDCDEM, 98.6% (80/81) and 100% (112/112); and Diasorin, 91.4% (74/81) and 99.1% (111/112). The false negative rates for each test system in PUI versus SURV patients were, respectively, as follows: Aptima, 2.3% versus 5.4%; AlitMP, 0% versus 8.1%; CDCMP, 0% versus 0%; AlitEM, 9.1% versus 10.8%; CDCDEM, 0% versus 2.7%; and Diasorin, 9.1% versus 8.1%. There was no significant difference in false negative rates among pediatric PUI versus SURV patients or maternal PUI versus SURV patients. **Conclusions:** The CDCMP assay was the most accurate test system for viral RNA detection in both PUI and SURV patients, whether pediatric or maternal. For most other test systems, lower false negative rates were observed in PUIs compared to SURV patients.

**ID27. Evaluating the Clinical Utility of Next-Generation Sequencing of Nasopharyngeal Specimens for SARS-CoV-2 in the COVID-19 Pandemic**

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**Introduction:** RT-PCR tests detect the presence of SARS-CoV-2 through primers and probes that target specific sequences in the viral genome. These sequences are susceptible to change as strains mutate and evolve, which can lead to false negative test results. Next-generation sequencing (NGS) can provide the full sequence of the virus and, in cases of co-infection, the genomes of other pathogens present in the sample. We hypothesized that NGS would allow for more sensitive detection of SARS-CoV-2 or infection of another etiology, especially in repeat negative RT-PCR but high suspicion for COVID-19 disease patients (NHSCPs). We also hypothesized that phylogenetic analysis of sequences could help in determining nosocomial transmission among hospital patients. **Methods:** We used two different platforms, the Ion Ampliseq SARS-CoV-2 test on the Ion Torrent S5 sequencer for detection and whole genome characterization of SARS-CoV-2, and the RNA workflow of the Respiratory Virus Oligos Panel on the NextSeq500 sequencer for detection and whole genome characterization of up to 40 respiratory viruses, including SARS-CoV-2. NGS was performed on 565 symptomatic clinical nasopharyngeal swab specimens that were previously tested on institutional SARS-CoV-2 RT-PCR assays and included the following sample types: positive RT-PCR across a range of Ct values, negative RT-PCR, NHSCPs, and transplant patients with suspected nosocomial transmission. **Results:** Both platforms detected SARS-CoV-2 in clinical samples across a wide range of Ct values. There were no cases where NGS revealed presence of high or moderate levels of SARS-CoV-2 when RT-PCR testing was negative. Infections with Influenza A and human coronaviruses HKU1 and NL63 were found in two NHSCPs, and co-infection with Influenza A and SARS-CoV-2 was found in 1 patient. Analysis of background reads revealed the presence of commensal respiratory tract bacteria in several patients. More than 90% of patients had the D614G variant present, consistent with the dominant strain in the New York City area. Phylogenetic analysis alone could rule out nosocomial transmission, but not rule in nosocomial transmission. **Conclusions:** Early in the pandemic, it was unclear whether SARS-CoV-2 strains were evolving rapidly and could develop mutations at primer binding sites in RT-PCR assays. So far, this has not been the case with SARS-CoV-2, and our data support this conclusion. Although NGS can help with ruling out nosocomial transmission, ruling in transmission requires close coordination with hospital epidemiology and infection and control teams. As more outbreaks occur across the United States, new predominant variants may emerge that can be detected with sequence level data provided by NGS. With collaborators, we are exploring if certain variants are associated with more severe disease.
Validation of an Emergency Use Authorization RT-PCR Test for SARS-CoV-2 in PVS from clinical NP swab samples was used for reproducibility studies of 4 replicates of a negative, a low positive and a high positive sample in 5 runs over a total of 3 days. Limit of detection was determined using the 100,000 copies/mL of the AccuFlex SARS-CoV-2 Verification panel, serially diluted between 10,000 and 100 copies/mL in pooled negative saliva. Results: Intra-run and inter-run reproducibility for the target cycle number (CN) had a percentage of CV of less than 2.51 for the low positive sample (~3,500 copies/mL) and 2.0 for the high positive sample (~105,000 copies/mL) and perfect reproducibility for the negative result. The limit of detection was determined to be approximately 1,000 copies/mL. A total of 5/57 saliva specimens in the clinical validation and amplification (8.8%), of the remaining 52 saliva specimens, the positive predictive agreement was 80% and the negative predictive agreement was 100%. Stability of the straight saliva was up to 7 days in the fridge. In our cohort of patients there is no trend towards saliva generally having a higher or a lower viral load compared with the matching NP swab. Of all positive NP swabs for which CN data were available, 9 saliva samples had a higher CN or were negative, and 8 saliva samples had a lower CN compared with the NP swab. Conclusions: Even though saliva is an attractive specimen type it is easy to collect for the patient and does not require special collection and transport devices or personal protective equipment, several challenges affect widespread implementation. The limit of detection on the m2000 instrument is notably higher than the one observed with NP swabs, and there is a failure rate of about 9%, which is lower than NP swabs, and there is a failure rate of about 9%, which is higher than the one observed with NP swabs. It is helpful to be able to offer this alternative specimen type, but there has to be the awareness that the performance characteristics are different from NP swabs.

Validation of an Emergency Use Authorization RT-PCR Test for Detecting SARS-CoV-2 in Upper and Lower Respiratory Tract Specimens

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Introduction: Quick and accurate diagnosis of the novel coronavirus SARS-CoV-2 is critical for COVID-19 management. However the shortage of FDA-approved tests in the beginning of the pandemic limited access to adequate clinical diagnostics. On February 29 the FDA issued a new Emergency Use Authorization (EUA) that allowed testing for SARS-CoV-2 in laboratories proficient at high-complexity molecular testing to ensure analytical and clinical validity of the test. This study described the validation and performance characteristics of a research use only (RUO) SARS-CoV-2 EUA test in upper and lower respiratory tract (LRT) specimens. Methods: Accuracy, specificity, and limit of detection (LoD) studies were performed on nasopharyngeal (NP) swabs and sputum specimens (n = 124), using the dual-target (S and E genes) RT-PCR RealStar SARS-CoV-2 Reagent (Altona Diagnostics). Following automated extraction (QIAasymp) of total nucleic acid from respiratory samples, viral RNA targets were amplified by a single step RT-PCR using the Rotor-Gene Q (Qiagen). A validation panel consisting of 124 patient and contrived specimens, including NP (64) and sputum (60) specimens, was used for the validation. Contrived samples were spiked at a concentration of 1x to 2x LoD, with the remainder of samples spanning the assay testing range. To verify the LoD of the assay, a panel of 10-fold dilutions (1x10⁵ to 1x10⁷) was prepared by spiking inactivated SARS-CoV-2 RNA control (~6x10⁶ genomic copies/mL, strain USA, WA1/2020, BEI Resources) into pooled SARS-CoV-2 negative patients' specimens. Samples with cycle threshold (CT) values >40 were considered negative. Results: Accuracy testing demonstrated excellent agreement between the expected and observed SARS-CoV-2 RT-PCR results. The LoD was 2.7 and 23.0 gene copies/reaction for the NP and sputum specimens, respectively. A total of 30 each negative NP and sputum specimens were not detected by the assay (100% specificity). No cross-reactivity was observed with other human coronaviruses (NL63, 229E, OC43, or HKU1)-positive specimens. Using this assay we have tested 1,372 NP and 327 LRT specimens, the majority of which were tracheal aspirates (TAs), with an overall positivity rate of ~40% and 11%, respectively. More than 70% of the positive samples could be classified as having medium to high viral burden (CT <30). Conclusions: The study demonstrates that the RT-PCR RealStar SARS-CoV-2 test is accurate and sensitive, allowing the identification of SARS-CoV-2 in multiple specimen types in the clinical setting. The difference in detection rates between NP and LRT specimens could be attributed, at least in part, to TA testing in a manner that is less invasive and unpleasant for the patient.

Development of a Multiplexed External Control for Monitoring Performance of a Qualitative Laboratory Nucleic Acid Testing Panel Used for Identification of Respiratory Infections, Including SARS-CoV-2


Maine Molecular Quality Controls, Saco, ME.

Introduction: Acute respiratory infections are a significant public health issue that require rapid and accurate diagnosis. The emergence and rapid spread of SARS-CoV-2 worldwide necessitates preparedness and response in public health laboratories, as well as healthcare facilities and other areas of society in general. Best practice and regulations require routine use of quality controls to monitor test systems for accuracy, shifts, trends, and random errors. Clinical laboratories require controls that are ready to use, non-infectious, and provide a streamlined QC scheme to reliably confirm valid results, ensuring that consumables and test systems are performing as expected. A synthetic, multiplexed control panel, consisting of 2 positive controls and 1 negative control, has been developed for use with QIAstat-Dx Respiratory SARS-CoV-2 to monitor the performance of the integrated test system capable of differentiating SARS-CoV-2 from 20 other respiratory infections in a single sample from patients with upper respiratory symptoms. Methods: Synthetic, multiplex control constructs, containing genome segments of all respiratory pathogens detected by the QIAstat-Dx RP SARS-CoV-2 test, including SARS-CoV-2, were designed in silico, dispersed among several pieces of synthetic DNA. The in silico DNA strands were synthesized, ligated, and transformed to create stable frozen clone stocks. In vitro RNA transcrpts were generated, quantified, and formulated in a proprietary matrix to stabilize and carry the RNA through the entire test process, including the extraction. Two positive controls covering all assay targets were further optimized at a concentration close enough to the limit of detection to accurately monitor the test system. Results: Verification studies across multiple control lots and cartridge lots resulted in 100% correct calls (54/54 valid runs) for all targets with a percentage of CV <10%. The negative control demonstrated 100% accurate results (27/27 valid runs across multiple cartridge lots. Accelerated and ongoing real-time stability studies confirm 18 months at -20°C. Conclusions: A multiplex external control covering all targets within the QIAstat-Dx Respiratory SARS-CoV-2 Panel offers a simple, ready-to-use, non-infectious, robust solution to monitor a comprehensive, integrated molecular assay that can be used in any laboratory.


Maine Molecular Quality Controls, Saco, ME.

**Introduction:** According to Johns Hopkins CSSE, as of July 2020, there were over 11.6 million confirmed cases of coronavirus disease 2019 (COVID-19), with a range of asymptomatic to severe symptoms that have led to hundreds of thousands of deaths worldwide. In response to this global pandemic, it is imperative to test individuals who are suspected carriers to decrease community spread and to diagnose symptomatic patients for proper treatment. The FDA has approved several Emergency Use Authorization (EUA) molecular diagnostic tests that can detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19. The Xpert Xpress SARS-CoV-2 Assay is a qualitative, rapid assay for use on GenExpert systems (Cepheid) that can detect the N2 and E gene of SARS-CoV-2 in upper respiratory specimens of infected individuals. A synthetic, extracellular control has been developed for use with Xpert Xpress SARS-CoV-2 to assist in assuring test accuracy by identifying shifts, trends, and errors caused by variations in the test system. **Methods:** Genome segments of SARS-CoV-2 were designed in silico to create synthetic DNA segments, and cloned to create a stable frozen clone stock. In vitro RNA transcripts were generated, quantified by 260/280 UV spectrophotometry and formulated in proprietary matrix that carries the RNA through the extraction process and provides additional stability. Traceability was established by performing bi-directional, quality-scored Sanger sequencing of all the target sequences. Validation studies for precision were conducted on multiple lots of Xpert Xpress SARS-CoV-2 cartridges and by an internal SARS-CoV-2 qPCR on the LC480 (following protocol for CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel) to demonstrate robustness and reliability as an external control. **Results:** Three lots of positive and negative controls were tested at two testing sites over multiple days, across multiple Xpert Xpress SARS-CoV-2 cartridge lots, and operators. A total of 156 samples were run with 100% correct results for all positive and negative controls. The synthetic external control demonstrated 100% correct results for all positive and negative samples tested across multiple reagent lots. Stability studies and historical data for similarly formulated MMQCI control material demonstrate >18 months stability when stored at -20°C. **Conclusions:** The synthetic SARS-CoV-2 external control demonstrated reproducible and robust performance when tested on Xpert Xpress SARS-CoV-2. Reliable performance on the GenExpert systems establishes the usefulness of the control for routine monitoring of the test system, training, verification, and troubleshooting.

**ID32. Development of a Multiplex Respiratory Panel and a Singleplex SARS-CoV-2 External Control for Use in a Rapid Nucleic Acid Amplification Detection System**


Maine Molecular Quality Controls, Saco, ME.

**Introduction:** SARS-CoV-2 caused a global pandemic resulting in hundreds of thousands of deaths worldwide. During this time, numerous assays were quickly developed and FDA expedited their release using Emergency Use Authorization (EUA). Accurate and reliable testing, particularly for a virus that spreads asymptptomatically, is critical to prevent community spread of COVID-19 from patients who test positive, as well as to distinguish SARS-CoV-2 from other respiratory viruses, especially during flu season. External controls have never been more important for clinical use to confirm that laboratories report valid results, to ensure consumables are performing as expected, and to monitor assay consistency over time. Here, we describe development of a singleplex SARS-CoV-2 control for use with the ePlex SARS-CoV-2 Test, as well as an extractable multiplexed control for use with GenMark’s ePlex RP2 Panel. **Methods:** Synthetic constructs containing genome segments of all 17 respiratory pathogens and SARS-CoV-2 were designed in silico. The in silico DNA inserts were synthesized and transformed to create stable frozen clone stocks. In vitro RNA transcripts (IVTs) were generated, quantified, and formulated in a proprietary matrix to stabilize and carry the RNA through the entire ePlex process, including extraction, amplification, and detection. Formulations studies were conducted to determine the optimum concentration close enough to the limit of detection to accurately monitor the test system. **Results:** Three lots of the ePlex SARS-CoV-2 Control Panel M435 were tested across 6 ePlex SARS-CoV-2 cartridge lots and by an internal SARS-CoV-2 qPCR on the LC480 (following protocol for CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel). All positive controls (20/20) and negative controls (18/18) gave correct results. Early development of a multiplex respiratory, two-positive control panel tested across 3 cartridge lots of ePlex RP2 resulted in 100% correct calls for all targets, including SARS-CoV-2 and 17 additional respiratory pathogens. Stability studies and historical data for identically formulated external control material demonstrate >18 months’ stability when stored at -20°C. **Conclusions:** ePlex SARS-CoV-2 Control Panel M435 shows stable behavior across multiple control lots and cartridge lots. A multiplex ePlex RP2 control panel, offered in a 2-positive control format, performed robustly across multiple cartridge lots, demonstrating an effective synthetic control to detect and differentiate SARS-CoV-2 and other respiratory pathogens.

**ID33. Comparison of Two High-Throughput qPCR Assays for SARS-CoV-2**

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Virginia Commonwealth University, Richmond, VA.

**Introduction:** One tenet of controlling infectious disease outbreaks is access to adequate and reliable diagnostic testing. To meet the demand of rapidly expanding testing requirements during the COVID-19 pandemic, many laboratories have added Emergency Use Authorization (EUA) authorized real-time PCR (qPCR) testing for SARS-CoV-2 detection to their testing menus. This rapid increase in testing has led to global constraints in the supply of reagents, thus requiring laboratories to diversify test offerings by developing multiple qPCR assays simultaneously. In addition, in many academic laboratories’ testing demand has required utilization of all available resources, often resulting in testing being performed in multiple certified laboratories across an institution. In this study, we evaluated the concordance of two high-throughput SARS-CoV-2 qPCR assays performed between two pathology laboratories at Virginia Commonwealth University Health Systems (VCUHS). **Methods:** Testing was performed on 119 patient specimens using the cobas SARS-CoV-2 test (Roche) and the TaqMan 2019-nCoV Assay Kit v1 (Thermo Fisher). The Roche assay was performed on the cobas 6800 System following the EUA authorized manufacturer guidelines. The Thermo Fisher assay was performed using a Microlab STAR automated liquid handler (Hamilton) for nucleic acid extraction and qPCR plate set-up, and an Applied Biosystems 7500 Real-Time PCR instrument (Thermo Fisher) for qPCR analysis. The Thermo-Hamilton assay was performed in the Clinical Microbiology laboratory and the Roche cobas 6800 assay in the Molecular Diagnostics laboratory at VCUHS. **Results:** Of the 119 total specimens tested, 58 were considered positive and 61 were negative for SARS-CoV-2. The overall agreement between the assays was 96.8% (115/118). Of the 58 positive results included in this analysis, 55 agreed (94.9%) and 60 of 61 negative specimens agreed (98.4%). The 4 discrepant results were found to have low target amplification (Ct >35) for at least 1 qPCR target, suggesting either differences in sensitivity or, more likely, issues with sample stability post-freeze-thaw cycle. **Conclusions:** With academic laboratories offering SARS-CoV-2 qPCR testing using multiple assay systems across very diverse laboratory types, it is imperative to determine test comparability. The multiple high-throughput qPCR assays offered by VCUHS pathology laboratories are highly comparable, allowing for better monitoring of positivity rates across the institution regardless of the testing platform utilized.
ID34. Development of a Multiplexed Synthetic Control for Rapid Detection of SARS-CoV-2 and Other Respiratory Pathogens Using a Nucleic Acid Syndromic Testing Panel
Maine Molecular Quality Controls Inc., Saco, ME.

Introduction: A novel coronavirus identified in late December 2019, now known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread rapidly throughout the world, causing a global pandemic. Individuals infected with SARS-CoV-2 can develop a variety of symptoms that range from asymptomatic to severe disease.

Asymptomatic and pre-symptomatic transmission has made containment of SARS-CoV-2 infections challenging. Accurate and reliable testing is critical to prevent community spread of SARS-CoV-2 by quickly identifying infected individuals and isolating them, as well as to distinguish SARS-CoV-2 from other respiratory viruses. External controls are essential for this process, as clinical laboratories require appropriate external controls to confirm valid patient results. An extractable multiplexed control has been developed for the BioFire Respiratory 2.1 (RP2.1) and RP2.1plus Panel which includes SARS-CoV-2 and 21 other common respiratory pathogens. Methods: Synthetic constructs containing genome segments of 21 respiratory pathogens and SARS-CoV-2 were designed in silico. The in silico DNA inserts were synthesized and transformed to create stable frozen clone stocks. In vitro RNA transcripts (IVTs) were generated, quantified, and formulated in a proprietary matrix to stabilize and carry the RNA through the entire process, including extraction, amplification, and detection.

Formulations studies were conducted to determine the optimum concentration to be delivered to the limit of detection to accurately monitor the test system. Results: Three lots of BioFire RP2.1/RP2.1plus Control Panel M441 were manufactured and tested across three RP2.1plus pouch lots and between two testing sites (172 runs). Of the 172 runs, there were 171 valid runs used for analysis. BioFire RP2.1/RP2.1plus controls showed 100% accuracy, with all positive control runs (86/86) and negative control runs (85/85) with correct calls. Conclusions: The BioFire RP2.1/RP2.1plus Control Panel M441 covering all assay targets plus control lots and between two testing sites (172 runs). Of the 172 runs, there were 171 valid runs used for analysis. BioFire RP2.1/RP2.1plus controls showed 100% accuracy, with all positive control runs (86/86) and negative control runs (85/85) with correct calls.

ID35. Comparison of Nasopharyngeal Swabs and Saliva Samples for the Detection of SARS-CoV-2 RNA
Memorial Sloan Kettering Cancer Center, New York, NY.

Introduction: The ongoing outbreak of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has continued to place a burden on the availability of nasopharyngeal swabs (NPS), especially since surveillance testing is necessary to identify both symptomatic and asymptomatic patients. The goal of this study is to determine the reliability of self-collected, posterior oropharyngeal saliva as an alternative sample type for the detection of SARS-CoV-2 RNA in healthcare workers (HCW) at our center.

Methods: Samples were collected from symptomatic HCW at Memorial Sloan Kettering Cancer Center (MSKCC) between April and May 2020 during the peak of our regional outbreak. For the purpose of this study, paired samples of both NPS and saliva were collected. Saliva specimens were collected by first asking HCW to rinse and gargle with mouth closed, swish for 15 seconds without gargling, and then bring up saliva from the back of the throat and spit at least 3.0 mL of saliva into an empty sterile container. NPS were collected by dedicated nurses at test sites who were trained in the swab technique, whereas saliva samples were self-collected.

Results: Eighty-seven paired NPS and saliva samples were collected for this study. The detection rate for SARS-CoV-2 RNA was 19.5% (n = 17/87). Overall agreement between the two sample types occurred in 88% (n = 85/97) of samples including 16 positive pairs and 69 negative pairs. For 2 paired samples, SARS-CoV-2 RNA was detected only in the nasopharyngeal swab for 1 pair and only in saliva for the other pair. Using detection rate in nasopharyngeal swabs as the gold standard, the sensitivity and specificity of SARS-CoV-2 RNA was 94.1% (95% CI: 70.2% to 99.7%) and 98.6% (95% CI: 92.4% to 99.9%).

Conclusions: In this study, we provide data to support the use of saliva for the detection of SARS-CoV-2 RNA based on evaluation of samples from HCW at risk for COVID-19. Our results collectively suggest that saliva is an acceptable alternative sample for SARS-CoV-2 RNA detection by RT-PCR with the distinct advantage of offering a viable solution to the pervasive test collection supply shortages, and for implementing broader test approaches for SARS-CoV-2.
spit the water into a sterile container. NPS samples were collected using flocked swabs (Copan Diagnostics, Murrieta, CA) and placed in viral transport media (VTM). Samples were stored at room temperature until transport to that lab within 2 h of collection. Results: For the oral rinses and NPS samples, 98 paired samples were collected. The detection rate for SARS-CoV-2 RNA was 33.7% (n = 33/98) in NPS and 23.4% (n = 23/98). Overall agreement between the two sample types occurred in 85.7% (n = 84/98) of samples including 21 positive pairs and 63 negative pairs. SARS-CoV-2 RNA was detected in 12 additional NPS only and in 2 oral rinses only. Using NPS as the reference standard, the sensitivity and specificity of SARS-CoV-2 RNA detection in oral rinses was 63.6% (95% CI: 46.6% to 77.8%) and 96.9% (95% CI: 89.5% to 99.5%). Given that the N1 and N2 Ct values were mostly similar (i.e., within ± 1 to 2 Ct, data not shown), we focused comparison on N2 Ct values. The mean and median Ct values for N2 in nasopharyngeal swabs were 22.6 (95% CI: 19.9 to 25.3) and 22.6 (IRQ 18.5 to 24.3), respectively, whereas the mean and median Ct values in the corresponding oral rinses were 29.9 (95% CI: 29.7 to 34.1) and 32.0 (IRQ 25.4 to 33.5). Conclusions: We report the most extensive data set on SARS-CoV-2 RNA detection in oral rinses to date. With an overall sensitivity of 63.6%, we do not find it to be a suitable alternative when compared to nasopharyngeal swabs.

ID38. Lung Injury Due to COVID-19 Relative to Influenza and Non-viral ARDS and Normal Controls
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Introduction: Lung injury and morbidity due to COVID-19 disease is severe, temporally heterogeneous, and involves both epithelial and vascular compartments. As the disease is novel, the molecular and cellular underpinnings of the progressive tissue injury are poorly understood, as is the role of direct versus indirect viral-induced injury. This study compared gene expression of lungs from COVID-19 patients who suffered from acute respiratory distress syndrome (ARDS) to that of other types of ARDS as well as normal donors. Methods: RNA from fresh, TRizol extracted, and fixed lung tissue from 29 COVID-19 autopsies, 4 non-COVID-19 lung injury, and 3 controls was evaluated by the nanoString nCounter PanCancer IO 360 Panel Plus probes for 8 SARS-CoV-2 viral RNAs. Immunochemistry (IHC) for spike protein and ACD RNA in situ hybridization (ISH) targeting spike RNA were also performed. Selected cases of COVID-19 lung injury (4), non-viral ARDS lung (3), post-flu lung (2), and normal lung (n = 3) were evaluated using GeoMx Cancer Transcriptome Atlas (CTA) plus COVID-19 spike-in module. This spatial transcriptomics analysis included 24 regions of interest per tissue guided by immunofluorescent labeling with PanCK, CD45, CD68 and STYO 13. Alveolar airway, and vascular zones were analyzed. These CTA results were then compared to a set of 85 RNAseq profiles from COVID19 and COVID patient samples from nasopharyngeal swabs, as well as 156 RNAseq profiles from whole blood and autopsy tissues. Results: Bulk profiling via clustering and dimensional reduction revealed that virus-related expression showed 3 distinct groups of high, medium and low expression, which corroborates with complementary studies of IHC and ISH and control groups. CTA data showed that normal and flu samples clustered well, but COVID-19 and non-viral ARDS samples were more heterogeneous. Given the heterogeneity of the COVID-19 lung, the number of differentially expressed genes that were identified was more limited. However, pathway analysis revealed several upregulated pathways including the immune signaling in immune system, hemostasis, interleukin signaling, apoptosis, and cellular senescence. Cell type deconvolution was performed, and notable for enrichment in macrophages and fibroblasts was observed in COVID-19 disease, and interferon signaling and olfactory receptor genes were significantly disrupted, with viral presence and variants annotated across deceased patients’ lungs, kidneys, and livers. Conclusions: COVID-19 lung injury has at least 3 distinct groups by level of virus in tissue. Initial comparison with flu lung injury shows a distinct gene expression profile, and expansion of the CTA cohort is needed to capture the heterogeneity of potentially three distinct COVID-19 injury subgroups.

ID39. Evaluation of Sample Pooling for the Detection of SARS-CoV-2 RNA Using the Cobas SARS-CoV-2 Test
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Introduction: It has been well established that there remains a consistent strain on the supply chain with regards to severe acute respiratory-coronavirus-2 (SARS-CoV-2) testing. Due to the shortage of supplies and low prevalence, evaluation of alternative methods has become necessary to maintain our testing capabilities. The goal of this study was to evaluate sample pooling for SARS-CoV-2 testing at an oncology center using the Cobas 6800 platform. Methods: Positive and negative remnant samples previously tested using the Cobas 6800 SARS-CoV-2 assay were used. Both negative and positive pools were composed of 8 samples, using 100 µL of each sample for a total volume of 800 µL. Thirty negative pools were made using previously tested negative samples. Thirty positive pools were made using 7 negative samples and 1 positive sample. Negative samples were chosen at random, whereas positive samples were based on the cycle threshold (Ct) value of Target 2. Twenty positive samples with Ct values of 33 or above and 10 samples with a Ct value of 34 or below were selected. Testing was performed according to manufacturer’s instructions. Results: For samples with a Ct value of 34 or lower 10/10 (100%) were detected in their respective pool. Samples with a Ct value of 35 or higher had a detection rate of 6/20 (30%) for an overall sensitivity of only 53% (add 95% CI: 34.33 to 71.66). Specificity was 100% (95% CI: 88.06 to 100.0). Conclusions: Using a pool of 8 samples, the sensitivity of detection near the LOD was inadequate to support pooling. Sensitivity may be increased by reducing the number of samples per pool, and with the prevalence in our center being low, this remains a viable option to explore. Studies to evaluate the optimum number of samples in a pool are ongoing.

ID40. RT-PCR Detection of SARS-CoV-2 Infection in Formalin-Fixed, Paraffin-Embedded Tissue Sections in Autopsy Cases
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1New York Presbyterian Hospital, New York, NY; 2Well Cornell Medical College, New York, NY. Introduction: Identification of COVID-19 in patients at autopsy can be integral to determining the cause of death. Autopsy was performed on several patients prior to the development of reliable detection methods of the novel coronavirus, SARS-CoV-2. Additionally, autopsies have been performed on patients when there was a high suspicion for COVID-19, but the patient expired outside of a healthcare setting or before testing was done. This study describes the validation and performance characteristics of an RT-PCR SARS-CoV-2 test used on formalin-fixed, paraffin embedded (FFPE) tissue from autopsy specimens of documented SARS-CoV-2 autopsies by nasopharyngeal swab PCR. The goal is to develop a sensitive test detecting SARS-CoV-2 in patients with an unknown COVID-19 status to determine if the disease contributed to their cause of death or current disease. Methods: Representative FFPE tissue blocks (n = 14) of trachea, bronchus, and/or lung tissue from 12 autopsy cases were collected for analysis. Six cases, confirmed positive by nasopharyngeal (NP) swab RT-PCR testing, were used for positive controls and 6 cases and 6 cases that predated the existence of the novel coronavirus were used for negative controls. Three positive controls were confirmed by immunohistochemistry for SARS-CoV-2 viral spike protein (Genetex clone 1A9 at 1:75 dilution with 20-min antigen retrieval at ph 9.0 on Leica Bond III automated instrument) and confirmed using RNAscope technology (Advanced Cell Diagnostic). Viral RNA was extracted with Thermo Fisher reagents and quantified with Qubit HS RNA assay (Agilent). A minimum of 10 ng total RNA was used for the reaction. SARS-CoV-2 testing was performed using the dual-target RT-PCR RealStar SARS-CoV-2 reagents (Altona Diagnostics) and the Rotor-Gene Q (Qiagen) thermal cycler via a single step RT-PCR. Samples in which both the E gene (all lineage B-3CoV) and the S gene target (SARS-CoV-2 specific), or the S gene target only, were detected within the first 40 cycles of amplification were considered “Detected.” Results: The average RNA extracted was 15.1 ng/µL (range 1.4 to 40.1 ng/µL). In patients confirmed positive for COVID-19 by NP swab in pre-mortem testing, 7 of 8 blocks obtained from 4 patients, were positive for the S gene and 6 of 8 blocks were positive for
the E gene. The average cycle threshold values were 26.7 and 23.9, respectively. All 6 negative controls collected prior to COVID-19 tested negative for both lineages B-beta coronavirus and SARS-CoV-2 specific RNA, with appropriate internal controls. **Conclusions:** The study demonstrates that RT-PCR methodology can be used to identify COVID in FFPE tissue samples obtained during autopsy to confirm clinical suspicion of COVID-19 as a contributing factor in the cause of death of the patient.

**ID41. The Evaluation of Oropharyngeal Swabs and Saliva Samples for the Detection of SARS-CoV-2 RNA**

Memorial Sloan Kettering Cancer Center, New York, NY.

**Introduction:** The ongoing outbreak of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has continued to place a burden on the availability of swabs, including oropharyngeal swabs (OPS), especially since surveillance testing is necessary to identify both symptomatic and asymptomatic patients. The goal of this study is to determine the reliability of self-collected, posterior oropharyngeal saliva as an alternative sample type for the detection of SARS-CoV-2 RNA in healthcare workers (HCW) compared to OPS. **Methods:** During the peak regional epidemic between April and May 2020, paired samples were collected from symptomatic HCW at Memorial Sloan Kettering Cancer Center (MSKCC) in New York City. Self-collection of saliva samples was performed by the HCW who were asked to first swallow, then bring up saliva from the back of their throat. A total volume of at least 3 mL was collected in a sterile container. OPS swabs were collected by designated nurses that had been trained in swabbing techniques. **Results:** A total of 100 paired saliva and OPS samples were collected for the study. The SARS-CoV-2 RNA detection rate for the paired OPS and saliva samples was 30% (n = 30/100) in OPS and 35% (n = 35/100) in saliva samples. Overall agreement between the two sample types occurred in 93% (n = 93/100) of samples including 29 positive pairs and 64 negative pairs (Table 2). For 7 paired samples, SARS-CoV-2 RNA was detected only in OPS for 1 pair and only in saliva for 6 pairs. Using detection rate in OPS as the gold standard, the sensitivity and specificity of SARS-CoV RNA was 96.7% (95%CI: 83.3 to 99.8%) and 91.4% (95% CI: 82.5 to 96.0%). **Conclusions:** Our study concluded that saliva was superior to oropharyngeal swabs for detection of SARS-CoV-2 RNA in HCW. These results indicate that self-collection of saliva is an advantageous sample source regarding both sensitivity and availability of test collection supplies when compared to oropharyngeal swabs.

**ID42. Evaluation of a Sample-to-Answer Cartridge-Based SARS-CoV-2 Assay**

J. Lefferts, D. Green, G. Tsongalis
Dartmouth-Hitchcock Medical Center, Lebanon, NH.

**Introduction:** For routine testing of symptomatic and asymptomatic individuals for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), tests with turnaround times of 12 to 24 h are acceptable. However, certain clinical situations arise that require hospitals to have rapid testing options. We describe our evaluation of the Biocartis Idylla COVID-19 assay, which provides a 1.5-h sample-to-answer testing option with minimal hands-on time. **Methods:** Idylla COVID-19 assay cartridges were prepared by adding 200 µl of viral transport media directly into the sample compartment. Cartridges were then decontaminated and loaded into the instrument. Each cartridge acts as a closed system for nucleic acid isolation, real-time reverse transcriptase polymerase chain reaction, and fluorescent detection. Lower limit of detection was assessed using the AccuPlex SARS-CoV-2 Verification Panel (SeraCare), which has samples at concentrations of 100,000 copies/mL, 10,000 copies/mL, 1,000 copies/mL, and negative. The 1,000 copies/mL sample was diluted to 500 copies/mL to assess the LLOD. The AccuPlex SARS-CoV-2 Positive Reference Material (5,000 copies/mL) was run diluted to 1,000 copies/mL and 500 copies/mL in duplicate. A pool of residual positive patient samples was tested after diluting to 500 copies/mL. Cross-reactivity was assessed with coronavirus 229E (ZeptoMetrix). Four residual positive clinical samples with a variety of viral copies and three residual negative clinical samples, as determined by the Abbot m2000 SARS-CoV-2 assay, were also tested. The residual sample with the lowest copy number was run in duplicate (two different instruments on two different days). **Results:** All residual patient samples had 100% concordance with previous results. The coronavirus-229E sample was negative, demonstrating specificity of the assay for SARS-CoV-2. All reference material at 1,000 copies/mL and above were detected. The pooled positive sample diluted to 500 copies/mL was negative; however, its internal control was depressed, likely due to the presence of saline in the pool. The 500 copies/mL dilution from AccuPlex SARS-CoV-2 Verification Panel was negative due to an insufficient number of amplified targets. However, both replicates of AccuPlex SARS-CoV-2 Positive Reference Material diluted to 500 copies/mL were positive. **Conclusions:** The Idylla COVID-19 assay provides a rapid method for SARS-CoV-2 detection. However, the limit of detection of this assay may be slightly higher for swabs collected in saline instead of viral transport media, as recommended.

**ID43. Viral Sequencing Suggesting Transmission of SARS-CoV-2 from a Patient with False-Negative Molecular Results to Health Care Providers**

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Dartmouth-Hitchcock Medical Center, Lebanon, NH.

**Introduction:** In addition to accurately diagnosing COVID-19 for the purpose of treating patients, molecular testing for SARS-CoV-2 has played a critical role in reducing transmission of the virus in the healthcare and community setting. Testing has been used in the current pandemic to determine eligibility for elective procedures, as a prerequisite before discharging patients to other facilities, and to determine appropriate infection control precautions. One suspected COVID-19 in-patient treated in our institution who repeatedly tested negative for SARS-CoV-2 by nasopharyngeal (NP) swab collections was later found to be SARS-CoV-2 positive after transferring to another institution when a lower respiratory specimen (BAL) was tested. Because of the initial false-negative test results, providers treating this patient used reduced precautions. Contact tracing identified multiple providers who were exposed, and three tested positive for SARS-CoV-2. Due to additional potential sources of exposure for these providers, viral sequencing of lower respiratory specimens from the patient and NP collections from the infected providers was pursued in an attempt to determine if these infections were caused by exposure to this patient. **Methods:** NP swab samples were tested at different times during the patient’s hospitalization using three different Emergency Use Authorization (EUA) assays validated in our laboratory (Abbott m2000 RealTime, Alita iAMP, and Diasorin Simplexa). The exposed providers were tested by NP swabs using the Abbott m2000 assay. These NP samples were sequenced along with lung biopsy tissue and BAL cell blocks using the Ion AmpliSeq SARS-CoV-2 Research Panel and the Ion AmpliSeq Kit for the Chef DL8 (Thermo Fisher). Sequence analysis was performed with standard methods suggested by the manufacturer, and variants were called relative to the reference sequence NC_045512.2 (isolate Wuhan-Hu-1). **Results:** Residual specimens were available for two of the three positive providers. The viral sequence from the patient and both providers share eight variants with high variant allele fractions (VAF: >0.65). One provider’s sample also contained three additional high VAF variants (4860A >C; 11575C >T; 13115C >T). Of the eight shared variants, six are common in the SARS-CoV-2 samples sequenced in our patient population, 1 variant (9479G >T) has only been detected in our facility in these three patients, and the final variant (16887C >T) has only been seen in one other case. Both providers also contained several low VAF variants, possibly due to the sequenced sample being collected later in disease progression. **Conclusions:** Molecular diagnostic testing of SARS-CoV-2 is extremely important in treating and preventing infections. Viral sequencing may be useful in confirming suspected routes of nosocomial infection.
ID44. **Digital Droplet PCR to Detect Low-Titer SARS-CoV-2 in Nasopharyngeal, Nasal, and Salivary Specimens**


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

**Introduction:** Multiple testing platforms have been implemented to diagnose SARS-CoV-2 in the expanding pandemic. Some point-of-care devices are rapid but are limited by high false-negative rates. Real Time PCR (RT-PCR) platforms can detect low levels of virus in a high-throughput manner, but weak positives can be difficult to distinguish from technical artifacts after many PCR cycles. Digital droplet PCR (ddPCR) is an orthogonal method that is well suited to detect low levels of nucleic acid. The BioRad SARS-CoV-2 Droplet Digital PCR (ddPCR) test was the first ddPCR assay that received Emergency Use Authorization (EUA) approval, but has not been evaluated clinically. Therefore, we evaluate the performance characteristic of ddPCR and its ability to confirm weak positive SARS-CoV-2 results.

**Methods:** We clinically validated a triplex probe ddPCR assay (SARS-CoV-2 Digital Droplet PCR kit, Bio-Rad) which used probes based on the Centers for Disease Control and Prevention design (N1, N2, and Rnase P). The Maxwell RSC 48 instrument (Promega Corp.) extracted RNA from the following specimen types: nasopharyngeal swab, nasal swab, and saliva. The limit of detection was determined using serial dilutions of SARS-CoV-2 RNA in an artificial viral envelope (Accuplex SARS-CoV-2, SeraCare). The ddPCR assay was performed according to the manufacturer’s specifications on specimens confirmed to be positive (n = 30) or negative (n = 30) by an RT-PCR assay (m2000 Real Time System, Abbott). Samples were considered positive when: >5,000 droplets were measured with at least 2 droplets positive for N1 and at least 2 droplets positive for N2, and at least 4 droplets were Rnase P positive. Results: The limit of detection was confirmed as 50 copies/mL (19/20 positive). Thirty RT-PCR positive samples spanning a range of quantification cycles (2.5 to 25.9 Cq) were positive by this assay (30/30, 100% PPA) and 30 negative samples were confirmed as 50 copies/mL (19/20 positive). The Biorad SARS-CoV-2 Droplet Digital PCR (ddPCR) test was the first ddPCR assay that received Emergency Use Authorization (EUA) approval, but has not been evaluated clinically. Therefore, we evaluate the performance characteristic of ddPCR and its ability to confirm weak positive SARS-CoV-2 results.

**Results:**

- Of the first 45 samples sequenced, 31 had sufficient coverage. Of these, 20 contained the same six variations g.3037C>T, g.241C>T, g.23403A>G, g.14408C>T, g.25563G>T, and g.1059C>T, which corresponds to Nextstrain clade 20C.
- Additionally, nine more samples shared five of these variants but lacked g.1059C>T, which corresponds to Nextstrain clade 20A. Both of these clades are associated with a European origin. Most samples contained unique secondary mutations not seen in the global SARS-CoV-2 populations. The most frequent types of variants seen were missense followed by synonymous. Most variants were detected at ~100% variant allele fraction, but lower VAFs in some samples suggest the possibility of inter-host viral evolution. Despite the high degree of viral genome similarity in most samples, two contained none of the previously mentioned variants. These two viral genomes belong to a subset of clade 19A, which also localizes to Europe.

**Conclusions:** Resequencing residual SARS-CoV-2 samples has provided insight into the genomic diversity of infections in New Hampshire. This understanding may prove useful in tracking the origin of new nosocomial or community-acquired infections. Sequencing additional samples will inform on the clustering of viral strains in our cohort and enhance our understanding of the SARS-CoV-2 landscape in our patient population.

ID45. **Leveraging Clinical Metagenomic Testing against SARS-CoV-2**

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**Introduction:** Molecular testing for SARS-CoV-2 is a major public health priority worldwide. Current PCR-based tests have low false-negative rates and have limitations in epidemiological investigations without additional genome sequencing. Clinical metagenomics has the potential for greater sensitivity, with the significant advantage of providing detailed sequence data and unbiased screening for coinfections, but its clinical utility for the detection of SARS-CoV-2 has not been evaluated.

**Methods:** Twenty respiratory samples (nasal swab, aspirates, and sputum) from suspected COVID-19 cases, including 10 positive and 10 negative for SARS-CoV-2 by directed PCR targeting three viral genes (RdRp, E, and N) were tested using the Explify respiratory platform (DbyDNA) using shotgun and targeted enrichment clinical metagenomics in a pilot feasibility study. Raw results for SARS-CoV-2 positivity, read counts, and percent coverage were compared with PCR positivity and Ct values.

**Results:** Six of the 10 PCR-positive samples (60%) were also positive by shotgun clinical metagenomics when compared to a respiratory virus database containing the SARS-CoV-2 reference sequence, based on minimum genome coverage of 5%. After enrichment, the same 6 samples were positive based on a coverage threshold of 25%, but read number and sequencing depth were markedly increased relative to those obtained by a shotgun approach. Given the sensitive nature of the enrichment approach, level of detection studies were performed to further inform thresholds for positivity. Log transformation of read counts and the percent of genome coverage both showed strong inverse correlations with PCR Ct values. Additional microorganisms were also identified in samples, including potential coinfections with S. marcescens, M. catarrhalis, rhinoviruses A and B, and human adenovirus E.

**Conclusions:** A clinical metagenomic approach to SARS-CoV-2 diagnosis yielded agreement with directed PCR but was marginally less sensitive, especially in low positive (high Ct value) samples. Samples with low positivity for SARS-CoV-2 by PCR were missed by metagenomic next-generation sequencing (mNGS) at preset thresholds but identified with optimization of thresholds. Refinements in clinical metagenomic workflows could enable a metagenomic approach to match or exceed the sensitivities of current PCR-based tests for SARS-CoV-2. Sequence data also have the potential to enhance surveillance, identify coinfections – which may become critical factors in clinical management – and facilitate early detection of variants, which may have epidemiological and laboratory implications when using fixed primers, in addition to providing laboratory evidence of SARS-CoV-2 infection.

ID46. **A Systematic Review of the Genomic Diversity of SARS-CoV-2 Virus Detected in Dartmouth-Hitchcock Medical Center, Lebanon, NH.**

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**Introduction:** As the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) became a pandemic, sequencing initiatives such as Nextstrain demonstrated the global genomic diversity of the virus. There has also been documentation of intra-host viral mutations. The ability for a hospital laboratory to genotype SARS-CoV-2 and understand the diversity in its patient population has implications for tracking and controlling new outbreaks.

**Methods:** Archival total nucleic acid extracted from viral transport media or formalin-fixed, paraffin-embedded (FFPE) tissue from a pilot group of 45 cases and control viral RNA was subjected to real-time reverse transcriptase-polymerase chain reaction. Ion AmpliSeq SARS-CoV-2 Research Panel libraries were prepared on the Ion Chef Instrument using the Ion AmpliSeq Kit for Chef DL8. Base-calling and alignment were performed on Torrent Suite v5.12.0. Variant calling was performed on the Torrent Suite variantCaller plugin v5.12.0.2 using the Germ line – low stringency configuration. Variant calls were annotated by the COVID19AnnotateSnpeff v1.0.0.1 plugin, and IRRAMreport plugin v1.0.0 was used to generate assembled consensus FASTA for each sample. A multiple sequence alignment was produced, using MUSCLE, for samples that contained coverage across all targeted regions and several reference sequences. Sequence variants were called based on the reference sequence NC_045512.2.

**Results:** Of the first 45 samples sequenced, 31 had sufficient coverage. Of these, 20 contained the same six variations g.3037C>T, g.241C>T, g.23403A>G, g.14408C>T, g.25563G>T, and g.1059C>T, which corresponds to Nextstrain clade 20C.

**Discussion:** Additional nine more samples shared five of these variants but lacked g.1059C>T, which corresponds to Nextstrain clade 20A. Both of these clades are associated with a European origin. Most samples contained unique secondary mutations not seen in the global SARS-CoV-2 populations. The most frequent types of variants seen were missense followed by synonymous. Most variants were detected at ~100% variant allele fraction, but lower VAFs in some samples suggest the possibility of inter-host viral evolution. Despite the high degree of viral genome similarity in most samples, two contained none of the previously mentioned variants. These two viral genomes belong to a subset of clade 19A, which also localizes to Europe.

**Conclusions:** Resequencing residual SARS-CoV-2 samples has provided insight into the genomic diversity of infections in New Hampshire. This understanding may prove useful in tracking the origin of new nosocomial or community-acquired infections. Sequencing additional samples will inform on the clustering of viral strains in our cohort and enhance our understanding of the SARS-CoV-2 landscape in our patient population.

ID47. **Evaluation of Saliva as an Alternative Sample Type for SARS-CoV-2 Detection Using the Hologic Panther Aptima EUA Assay**


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**Introduction:** Coronavirus disease 2019 (COVID-19) due to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a worldwide pandemic. Diagnostic testing relying on SARS-CoV-2 RNA detection from primarily nasopharyngeal (NP) swabs is critical to effectively control the spread of the virus. Recently, researchers from...
Introduction: The current SARS-CoV-2 pandemic has imposed rapid development of testing to accurately diagnose severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2). The potential for false negative results necessitated versatile testing strategies, including routine repeat of reverse transcriptase-polymerase chain reaction (rt-PCR) testing at time periods from 12 to 72 h. Subsequently, the utility of repeat testing and the optimal time period for retesting were considered. Data from patients tested at least twice within a single hospital stay were analyzed for diagnostic accuracy impact and to determine the optimal repeat test interval.

Methods: Retrospective medical records from a quaternary care hospital were examined for hospitalizations between 03/18/2020 and 04/05/2020. All patients with more than one rt-PCR for SARS-CoV-2 ordered within a hospital stay were identified. The total number and percent of times that an initial result was followed by a discrepant result were determined as well as the average time between repeat tests.

Results: A total of 328 patients had repeat rt-PCR via nasopharyngeal swab for a total of 742 tests. Five patients out of 328 had eventual discrepant (“positive versus negative”) results (1.5%). The percent change from positive to negative was 0.9% (3/328). The percentage change from negative to positive was 0.6% (2/328). The time between each test showed an average of 69 h; however, the range was from <60 minutes to 421 h. Conclusions: These results show that conversion from negative to positive or vice versa on serial testing (1.5%). The relatively low frequency could be contributed to tenacious collection policies that decreased pre-analytical variation and the severity of illness in the hospitalized population at that time. Review did not identify any focused populations or clinical scenarios in which discrepancies clustered, favoring a random distribution. Examination of the five discrepancies showed 1 case that changed from negative to positive with resampling at 1 h, suggesting pre-analytical error. The other conversion from negative to positive was “discrepant” 10 days after initial negative (which was collected 10 days after symptom onset). This could be due to pre-analytical factors, viral shedding dynamics, or contamination during testing. Three conversions from positive to negative were most consistent with clearance of viral shedding. Review did not identify a specific testing interval more apt to be discrepant, supporting shorter time intervals as suggested (i.e., 12 to 24 h) for repeat testing. Given the relatively low conversion rate, these data would suggest repeat testing does not add significant changes in the rate of detection of SARS-CoV-2.
All negative samples from 3 random days of testing on the ePlex were included as well as samples in which infectious disease physicians questioned the negative results due to high clinical suspicion for SARS-CoV-2. Twenty NP swab samples with weak positive results (defined as Ct ≥33, which is <20 copies/μL based on LOD verification data) on LDP testing were subsequently tested on ePlex. Results were compared and correlated with SARS-CoV-2 testing history. Results: Fifty of 70 (100%) of samples negative on ePlex were also negative on LDP testing. Nine of 20 weak positives by LDP testing were also positive on ePlex. Four of 9 were from patients with no prior positive result. The average N1 target Ct value for those detected on both methods was 36.30 (SD = 2.0), and the average N2 target Ct was 36.67 (SD = 1.69). For those samples positive by LDP testing but negative on ePlex, the average N1 Ct value was 36.92 (SD = 1.36) and the average N2 Ct was 37.54 (SD = 1.19). Four of 11 were patients with no prior positive SARS-CoV-2 test result. A total of 100% of randomly selected negative NP specimens tested on the ePlex for SARS-CoV-2 were true negatives when compared to the LDP with a lower established limit of detection. A total of 55% of weak positives by LDP testing would have been missed if initially tested on ePlex. There was no difference in Ct values for those samples as compared to the ones that were detected by the ePlex test. Weak positive results were seen in both patients with a history of testing positive and those with a first positive, and history was not related to concordance between LDP and ePlex results. Supply shortages and testing demand limited the number of specimens that could be included in this comparison.

ID51. Evaluation of the SARS-CoV-2 Chromacode EUA Assay
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Introduction: The first SARS-CoV-2 EUA assay to obtain Emergency Use Authorization (EUA) through the FDA was useful for the detection of the novel coronavirus but included three different assays per sample, limiting the number of samples that could be run at a time. The HDP PCR SARS-CoV-2 Assay (Chromacode) allows for the detection of two SARS-CoV-2 N gene targets and RNaseP as an internal control multiplexed in a single RT-PCR reaction well. Additionally, run files can be uploaded to a secure cloud for automated analysis and interpretation using the need for manual review and interpretation of results. We evaluated this assay with patient specimens to assess its suitability for clinical testing. Methods: RNA was extracted from 80 nasopharyngeal swabs collected in viral transport medium using the EZ1 Viral Mini v2.0 Kit (QIAGEN). All 80 samples were previously tested for SARS-CoV-2 with either the CDC EUA assay or the Abbott m2000 RealTime EUA assay. The HDP PCR SARS-CoV-2 Assay (Chromacode) with FDA EUA status was run according to the manufacturers instructions for use only in a RT-PCR thermal cycler (Thermo Fisher). The 7500 run files were uploaded to the Chromacode Cloud server for automated analysis and interpretation. Results: Valid results were obtained for all 80 samples tested with the Chromacode SARS-CoV-2 assay. Positive results were obtained for 46 samples, and 34 samples were found to be negative for SARS-CoV-2. These results were 100% concordant with the previous results obtained with either the CDC assay or the Abbott RealTime assay. Conclusions: The HDP PCR SARS-CoV-2 Assay allows for flexible low- to high-throughput SARS-CoV-2 testing with single-well multiplexing and automated analysis and interpretation. This initial evaluation demonstrated accurate results when compared to other assays currently used in our laboratory.

ID52. Design and Optimization of Novel ITS2-28s rRNA Gene Primers for Fungal Species Detection from Formalin-Fixed, Paraffin-Embedded Tissues with a Targeted Next-Generation Sequencing Assay
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Introduction: Targeted next-generation sequencing (NGS) assays for ribosomal RNA gene fragments are currently used for pan-fungal species detection from fresh tissues and formalin-fixed, paraffin-embedded (FFPE) tissues. However, conventional primers amplify long DNA fragments that are not well suited for FFPE tissues, which limits assay specificity and sensitivity (75% to 90% and 56% to 91%, respectively). To minimize this problem, we designed a new primer set that targets a short DNA fragment within ITS2-28s rDNA and tested it in an FFPE panel containing 70 fungal species. Methods: An ITS2-28s rDNA region (300-nucleotide length) containing previously described potential primer sites was BLASTed against a database of 70 (100%) fungal species from Ascomycota, Basidiomycota, and Zygomycota phyla. Retrieved contigs were aligned (MAFT v6) and manually trimmed to a 132 to 144 nucleotide region with high diversity, flanked by conservative nucleotides with few mismatches. A degenerate primer pair was designed from the flanked regions and tested in silico using NCBI to determine coverage. Amplicon libraries were prepared using an Illumina two-step PCR-based protocol with modifications, followed by NGS using an Illumina MiSeq MICRO v2 kit for 300 cycles on a MiSeq sequencer. The sequencing dataset was analyzed with the Galaxy platform, and aligned sequencing reads with matching confidence scores ≥98% were queried using NCBI BLAST for species identification. Specificity of the primers was tested in two blinded studies, and sensitivity was tested in a non-blinded study. Results: In the first blinded study, sequencing data obtained from the DNA of seven fungal species and the G-blocks of 10 fungal species, covering different phyla, indicated that 100% of the species were identified without false positives. In the second blinded study, sequencing data obtained from human and animal FFPE tissues (n = 30) of histologically diagnosed clinical cases indicated that ≥96% of the fungal species identified by targeted NGS correlated with the results obtained from the histological examinations. In sensitivity study, mixes of five G-blocks with different concentrations (10 ng, 1 ng, 0.1 ng, 0.01 ng, and 0.001 ng) were sequenced three times. Sequencing reads were accurately detected in all concentrations without significant difference between runs for the same primer pair setting a small fragment of ITS2-28s rDNA exhibits high specificity and sensitivity in animal and human FFPE tissues. Further analytical and clinical validation will be performed to determine the utility of this targeted NGS assay for clinical services.

ID53. Non-invasive Microbial Cell-Free DNA Sequencing Detects Invasive Mold Infections in Immunocompromised Patients with Pneumonia
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Introduction: Non-invasive diagnostic options are limited for pulmonary invasive mold infections in patients immunocompromised with a history of invasive testing. We evaluated the performance of Karius microbial cell-free DNA sequencing of plasma (mcfDNA-Seq) for diagnosing IMI after hematopoietic cell transplant (HCT). Methods: In a retrospective cohort of 68 HCT recipients from 1999 to 2018 who underwent invasive testing (bronchoalveolar lavage (BAL) or lung biopsy) for pulmonary IMI, we compared the diagnostic performance of mcfDNA-Seq on plasma to the composite result of invasive diagnostic testing. Performance was also assessed in 19 control HCT recipients with non-IMI pneumonia. mcfDNA-Seq was performed by Karius (Redwood City, CA) on banked plasma obtained within 14 days of IMI diagnosis. DNA sequences were analyzed using a microorganism database including >300 fungi with a Research Use Only bioinformatics pipeline optimized for IMI detection. Organisms above a predefined significance threshold were reported along with the concentration of cell-free DNA in plasma for that organism. Results: A total of 163 diagnostic tests on BAL or biopsy samples identified an IMI etiology in 121 (74%) patients. mcfDNA-Seq non-invasively identified the same (or more specific) IMI etiology as composite invasive testing for 63% of these patients (47/75). Thirteen of 14 patients whose invasive tests were all negative also had negative mcfDNA-Seq results. Overall, mcfDNA-Seq results matched the composite result of all invasive testing for 69% of patients (47/68). In 5 patients, the mcfDNA-Seq identified additional molds that were not detected by invasive tests, all of which were consistent with the clinical context. The mcfDNA-Seq test had higher sensitivity for non-Aspergillus spp. (81%; 21/26) than for Aspergillus spp. (48%; 13/27). All 19 control subjects with non-IMI pneumonia were negative by mcfDNA-Seq.
suggesting high specificity for IMI in HCT recipients with pneumonia. **Conclusions:** Non-invasive sequencing of cfDNA in plasma samples identified the same pathogen as invasive diagnostic testing in the majority of participants with pulmonary IMI after HCT, with high specificity in a control population and additive diagnostic value in several samples.

ID54. A Comparative Study of qPCR to a NGS Metagenomics Assay to Detect and Quantify DNA Viruses in Pediatric Bone Marrow Transplant Patients

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**Introduction:** Viral infections are a significant cause of complications and death in solid organ and bone marrow transplantation (BMT). PCR assays are the gold standard for measuring the presence and quantity of targeted viruses. Test results are required to assess both viral presence and efficacy of treatment. Although these PCR-based assays are both sensitive and specific, they do require previous knowledge of targeted sequences, and are not, currently, designed for the detection/quantification of multiple viruses per reactions (i.e., multiplex). Metagenomic next-generation sequencing (mNGS), however, is a single reaction that agnostically identifies the presence and abundance of transplant-related viruses, yet our understanding of their sensitivity/specifcity and overall value, is less characterized. **Methods:** Twenty-two longitudinal plasma samples from 4 pediatric BMT patients were tested for 10 different BMT-related viruses using qPCR and/or mNGS. The primary objective was to assess the comparative value of identifying and quantifying these viruses by qPCR (ELITE MGB kits on the InGenius instrument [ELITech Group]) to mNGS (Galileo Pathogen Solution [ArcBio, LLC]). These viruses included: cytomegalovirus, human adenovirus, herpes simplex viruses 1/2, human herpesviruses 6a/6b, varicella-zoster virus, BK virus, JC virus, parvovirus B19, and torque teno virus. mNGS detection, but not quantification, of B19 and TVT was assessed; however, no qPCR was performed for these viruses. Extraction of samples was performed on the bioMerieux eMAG system. Prior to the sequencing of patient samples, quantification calibration samples were also extracted; however, an additional extraction step was required as internal controls and internal control material was not spiked into the calibrators prior to extraction, resulting in a deviation of protocol. **Results:** Qualitatively, the two assays exhibited 100% categorical agreement. Of the 7 positive CMV samples, quantitative viral load comparisons varied greatly. In addition, TTV was identified from all patient samples excluding 3 Patient #1 samples. **Conclusions:** Qualitatively, the two assays exhibited 100% categorical agreement, however, quantitative analysis varied greatly between the two methods, which could be a consequence of the additional extraction protocol on the calibration samples. Further analysis by additional reference materials may help resolve the discrepancy. Regarding workflow, the ELITech instrument’s capacity was 12 samples per run, resulting in 19.5 h runs. The ArcBio platform required less hands-on time; however, two overnight steps were required. Overall, further studies should be performed to assess whether a mNGS application is a viable diagnostic for BMT-related viruses, especially for those viruses that were not detected in our limited patient population.

ID55. Investigating Targeted Next-Generation Sequencing of 16S RNA as a Tool for Detecting Shiga Toxin-Producing E. coli and Salmonella in Ground Beef

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**Introduction:** Microbiome analysis is a critical quality control step in the assessment of ground beef and other food products. Next-generation sequencing (NGS) of bacterial 16S rRNA has emerged as a superior method for the unbiased quantification of microbes in food compared to traditional culture-based approaches. Ground beef contains a ubiquitous population of non-pathogenic microbes but may become contaminated with pathogenic bacteria during food manufacturing and processing. Here we assess conditions for detection of Shiga toxin-producing Escherichia coli (STEC) and Salmonella using targeted 16S RNA sequencing for quality control of ground beef. **Methods:** Raw ground beef was spiked with different strains of STEC and/or Salmonella Typhimurium. Tryptic soy broth (TSB) was added as an enrichment medium for incubation at 42°C. To determine the effectiveness of the enrichment medium, rate of pathogen growth, optimal length of incubation, and composition of background flora, samples were taken at 0, 10, 16, and 34 h. Total nucleic acid was extracted, then sequenced with the 16S rRNA gene sequencing assay of the Ion AmpliSeq Pan Bacterial Community Panel. After reverse transcription, library prep, templating, and sequencing were performed on the Ion GeneStudio System followed by Ion Reporter analysis to identify bacteria with a 48-h sample-to-answer turnaround. **Results:** We sequenced microbial 16S rRNA from ground beef over a 34-h time period after spiking in STEC and/or S. Typhimurium. We characterized enrichment effectiveness by 1) identification of bacterial genera and species, 2) calculation of abundance information, and 3) characterization of alpha diversity. At the 0-h time point, the majority of bacteria in the ground beef correlated with gut microflora such as lactic acid bacteria. Bacteria such as Leuconostoc, Pseudomonas, Carnobacterium, and E. coli that can cause discoloration, food spoilage, or disease were present at low levels considered to be acceptable. Samples spiked with STEC and/or S. Typhimurium peaked to 30% at 10 to 16 h. By 10 h, relative abundance changes resulted in dramatic changes in alpha diversity. **Conclusions:** These results show that STEC and S. Typhimurium became detectable by NGS in all cases after 10 h of enrichment with TSB. Enrichment of these pathogens leads to changes in the composition of the ground beef microbiome that are readily detectable by targeted 16S rRNA NGS. NGS-based microbiome analysis may serve as an informative food safety indicator for potential pathogens and a means for quality control assessment during food production. With the simplicity of the NGS protocol and rapid turnaround time, we anticipate this approach to become increasingly popular as a method for food product quality control.

ID56. Subtyping of Human Papillomavirus (HPV) Using Next-Generation Sequencing (NGS) Data in Cervical Cancer: A Feasibility Study with Comparison to Conventional Clinical Assays

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**Introduction:** Genomic profiling by next-generation sequencing (NGS) has been increasingly used in oncology to identify prognostic/predictive markers and to enroll patients into genotype-matched clinical trials. Analysis of data from clinical sequencing may uncover additional information such as integration of viral genome. Here we attempted to analyze NGS data from clinical sequencing of cervical cancer specimens to identify and subtype human papillomavirus (HPV) genome that integrated into human genome and compared the results with conventional clinical HPV assays. **Methods:** Primary cervical carcinoma cases that were subjected for clinical sequencing using our in-house developed NGS panel (paired-end 2x100 bp reads on the Illumina HiSeq 2500; covering 177 genes) from January 2017 to December 2019 were identified in the pathology database. The raw data were reanalyzed through a standard bioinformatic pipeline using commercially available toolkit bobio-nextgen, and reads were mapped to HPV viral genome reference to report the number of reads that match to different strains of HPV. Results of NGS-based HPV detection and subtyping were compared to those of conventional HPV testing, including Aptima high-risk HPV screening assay on contemporary cervical cytology specimen, as well as p16 immunohistochemistry in the tumor tissue. **Results:** We identified 42 cervical cancer specimens that were submitted for clinical NGS testing, including 32 squamous cell carcinoma, 7 adenocarcinoma, 1 clear cell carcinoma, 1 carcinoma of cervix, and 1 carcinoma of uterus. HPV genome was detected in 32 cases; 30 cases had high-risk HPV strains (HPV16, 18, 31, 33, 45, 56), and 5 had low-risk strains (HPV 42, 72, 77, 118). Three cases showed co-existence of two HPV stains (HPV45/77, HPV16/72, and HPV16/118). Among the cases with high-risk HPV detected by NGS, 29/30 (97%) showed p16 staining in the tumor, and 28/30 (93%) had positive Aptima test in the concurrent cervical cytology specimen. The two discordant cases are squamous cell cases, and both contained HPV51 by NGS-based subtyping. Focusing on the status of HPV16/18/45, there is 1 case with HPV16 identified in NGS data, but Aptima test reported negative HPV16 and positive HPV18/45.
Conclusions: Identification and subtyping of HPV genome in NGS-based clinical sequencing data are feasible and can be performed by commercially available toolkit. The results of NGS-based HPV identification and subtyping are highly concordant with conventional high-risk HPV testing methodologies including Aptima assay and p16 staining.

ID57. Analytical Performance Characteristics of Galileo ONE: An End-to-End Metagenomics Assay for the Unbiased Sequencing and Bioinformatics Analysis of Microbial DNA and RNA Directly from EDTA Plasma


Introduction: Metagenomics-based next-generation sequencing (mNGS) has the potential to revolutionize the identification of microbes. Here we describe the analytical performance of Galileo ONE using a series of dilutions of controls representing the range of microbes detected by this assay and used these to generate analytical data to act as a proxy for the close to 6,000 microbes detected. Methods: In brief, 20 intact bacterial, fungal, protozoal, and viral control stocks (Mycobacterium avium, Aspergillus fumigatus, Toxoplasma gondii, Streptococcus agalactiae, Bordetella pertussis, Escherichia coli, Staphylococcus aureus, Cryptococcus neoformans, HIV-1, parvovirus B19, HSV-1, HSV-2, VZV, CMV, EBV, HHV-6A, HHV-6B, ADV, JCV, and BKV (Arc Bio, LLC, Cambridge, MA) were diluted and combined to 1, 10, 100, 1,000, and 10,000 IU, copies or CFU/mL, as applicable, using pooled negative plasma. A total of 216 DNA and 144 RNA libraries were subsequently prepared and tested by 3 operators over 20 sequencing runs. All dilutions were spiked with assay internal controls, bead-beaten (DNA libraries only, Zymo Inc.) and extracted in duplicate (EZ1 Virus Kit v2.0, QIAGEN Inc) to allow the generation of ds/ssDNA and RNA libraries using Galileo ONE library preparation reagents. Paired-end sequencing was performed to a target of 30M paired end reads per library (NextSeq, Illumina Inc) and FASTQs were obtained after demultiplexing and analyzed with Galileo Analytics. Evidence of microbial sequences was analyzed alongside other QC parameters and linearity, precision, LOD, and LLOQ metrics were calculated. Results: A total of 360 libraries were sequenced and the resulting FASTQs were analyzed. Initial metrics at 30M paired end reads were estimated by analyzing low titer dropouts. Detection limits were estimated to be between 100 IU/CFU/copies/cp/mL (M. avium, S. aureus, S. agalactiae, HHV-6A, HHV-6B, and JCV) and 1,000 IU/CFU/copies/cp/mL (A. fumigatus, T. gondii, E. coli, C. neoformans) with the exception of parvovirus B19, an ssDNA virus with a comparatively small genome, at around 10,000 IU/mL. Final data analysis is ongoing and will be presented at AMP 2020. Conclusions: Here we report the analytical performance characteristics of an end-to-end mNGS assay and RNA libraries using Galileo ONE library preparation reagents. Paired-end sequencing was performed to a target of 30M paired end reads per library (NextSeq, Illumina Inc) and FASTQs were obtained after demultiplexing and analyzed with Galileo Analytics. Evidence of microbial sequences was analyzed alongside other QC parameters and linearity, precision, LOD, and LLOQ metrics were calculated.

ID58. Application of Whole-Genome Sequencing for Bacterial Strain Typing in Investigating Hospital Infections

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Introduction: Outbreaks of antibiotic resistant bacteria in health care institutions represent a major problem for patients. Understanding the relatedness of clinical isolates is important in distinguishing outbreak isolates from endemic and sporadic isolates, conducting epidemiological studies, and ultimately determining the rationale for controlling the outbreak. Suitable strain typing methods should be selected based on resolution, accuracy, and availability (speed). Although pulse field gel electrophoresis (PFGE) is considered a highly reliable typing method, its resolution is relatively low compared to more recent molecular strain typing methods provided by whole genome sequencing (WGS). Since only a few clonal lineages predominate in nosocomial infection, and therefore require relatively high resolution for isolate discrimination, WGS sequencing seems to be a more appropriate method for nosocomial infection investigation. This study evaluates various strain typing methods of WGS for their reliability and suitability in the investigation of an outbreak in a nosocomial infection setting. Methods: This study investigated epidemiological relatedness of multidrug resistant Acinetobacter baumannii isolated during an outbreak at Seoul National University Bundang Hospital. Core genome clustering, multilocus sequence typing (MLST), single nucleotide variant (SNV) analysis, and phylogenetic tree analysis were provided by the Wellcome Sanger Institute’s Pathogenwatch and the NIH’s PATRIC. Results: Core genome clustering, MLST, SNV, and phylogenetic tree analysis showed identical strain typing results that corresponded to the clinical report. Core genome clustering method was able to group isolates into several clusters. At the threshold of 1, isolates were clustered into two groups ([#2, #6, #11, #12, #5] and [#3, #8]). Isolates #4 and #9 were not clustered with any other isolates. At the threshold of 30, isolate #4 was clustered together with isolates #3 and #8. Isolate #9 remained separate from the other isolates. Conclusions: This is a study to examine and compare various strain typing methods for WGS application in clinical isolate typing. This study supports Pathogenwatch and PATRIC as reliable bioinformatic tools for strain typing.

ID59. Validation of ddPCR-Quantified Standards for Use in Viral Load Measurements by NGS

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Introduction: Molecular monitoring of disease burden depends on the precise quantification of disease markers. Unfortunately, next-generation sequencing (NGS) measurements of such analytes in plasma are reported in misleading units that are obfuscated by non-disease factors (e.g., allele fraction, copies per ng of DNA). In this study we developed two ddPCR assays targeting Epstein-Barr virus (EBV) DNA to create reference materials by which to calibrate our plasma NGS panel as part of an ultimate aim to accurately report disease markers in units of copies/mL after measurement by NGS. Methods: Primers and probes were designed targeting two conserved segments of the EBV genome (BMRF1, BALF4), and duplex ddPCR (Bio-Rad QX200) was used to quantify EBV in copies/mL. EBV+ cell line (Namalwa, Raji) DNAs and DNA extracted (LV kit, Promega RSC) from mock plasmas (Seraseq tested by NGS [modified LiquidPlex, ArcherDX, Exact Diagnostics] were used to demonstrate analytic performance. Results: EBV levels were similar in both EBV+ cell lines by both EBV ddPCRs, regardless of whether the DNA was intact or fragmented via sonication. In plasma-derived DNA, EBV loads by ddPCR varied depending on which of two nearly identical extraction instrument systems was used, with BMRF1 yielding consistently higher EBV loads than BALF4 ddPCRs despite similar amplicon lengths (67 and 75 bp, respectively). Two-dimensional (2D) plots of FAM and HEX droplets generated by duplex ddPCR revealed distinct cloud patterns in cell line versus mock plasma specimens. Specificity was 100% in EBV negative mock plasma and in no-template controls (elution buffer or water). Conclusions: Although ddPCR is often considered the gold standard for quantifying nucleic acid analytes, the two EBV ddPCR assays that we developed behaved differently from each other, and differently in cell line DNAs compared to plasma DNAs. These differences might reflect matrix effects (e.g., inhibitors), DNA fragmentation configurations, or polymorphisms affecting primer or probe binding. Thus, caution is advised when validating ddPCR assays, and clinical validation is required beyond analytic evaluation of assay performance. Future studies will evaluate serial clinical specimens using optimized ddPCR assays and NGS measurements of disease markers in concert with patient status and outcome.
ID60. Performance Evaluation of Abbott Alinity m System to Detect HBV, HCV, and HIV-1 Infections: Comparison with Hologic Panther Apta Assay System
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Introduction: Nucleic acid quantitative tests are important for detecting and monitoring response to anti-viral treatment in the HBV, HCV, and HIV-1 infections. The Abbott Alinity m system (Abbott Molecular Inc., Des Plaines, IL) is a fully integrated and automated quantitative assay, which utilizes real-time polymerase chain reaction (PCR) technology to amplify and detect each viral genomic sequence from human plasma or serum specimens. We evaluated the performance of Abbott Alinity m assay for HBV, HCV, and HIV-1 viral load measurement and also compared the results with Hologic Apta assay (Hologic, Inc., Marlborough, MA).
Methods: Assay performance of precision, limit of detection (LOD), and interference were evaluated using HBV, HCV, and HIV-1 at three different concentrations of quality control materials, respectively. Linearity and cross-reactivity for each assay on the Alinity m system were evaluated, using patient samples positive to each test. A comparative evaluation between Abbott Alinity m assay and Hologic Apta assay on the Panther system was also performed with clinical patient specimens. Results: The coefficients of variation (CV) of precision varied between 1.3% and 3.92% for Alinity HBV assay, 0.5% and 2.68% for HCV assay, and 0.62% and 2.05% for HIV-1 assay, respectively. The LOD results were 10 IU/mL, 12 IU/mL, and 20 copies/mL for HBV, HCV, and HCV-1 assays, respectively, as 100% replicates were detected. No interference was observed with high concentration of hemoglobin, bilirubin, and triglycerides for all three tests. Linearity for viral loads was excellent for assays (R^2>0.99 for HBV, HCV, and HIV-1). The specificity of all assays was 100%. When comparing the results of 150 HBV-, 44 HCV-, and 38 HIV-1-infected samples using both systems, viral load was strongly correlated (R = 0.96 to ~0.98).
Conclusions: The Alinity m system is designed for being a random access automated analyzer that can perform viral load tests in parallel with other Alinity m tests on the same equipment. From this study, we can show that the Abbott Alinity m system and Apta assay demonstrated excellent performance, and the results were also comparable with those of the Abbott Alinity m assay to detect HBV, HCV, and HIV-1 infections.

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Introduction: HIV, HBV, and HCV remain major infections around the world. Molecular assays have revolutionized infectious disease testing and therapeutic monitoring for determination of the prevalence of these infections for better diagnoses and treatment plans for patients. The AcroMetrix Infectious Controls are quality controls mimicking patient samples to monitor assay performance, providing additional confidence in routine test results, test methods troubleshooting, and system errors identification. We report here the characterization and evaluation of AcroMetrix HIV, HBV, and HCV quality controls for molecular diagnostic tests using the Cobas 6800 System. Methods: Series dilutions of each analyte (HIV, HBV, and HCV) were tested using Cobas 6800 assays to characterize the technical specifications such as LOD for each assay. Method comparisons were performed between Cobas 6800 System and Procleix Ulitro Assay using 15 positive samples above LOD and covering the analytical measurement range (AMR) of Cobas 6800 assays, and 15 negative samples below LOD. C_0 and C_95 statistical estimation were performed to determine the assay detection limit for 5% positive and 95% positive rate, respectively. Eleven AcroMetrix infectious controls containing high, medium, or low titer of HIV, HBV, and HCV were then tested during verification and validation study to evaluate the performance of the control products on Cobas 6800 assays. Results: Thirty samples were tested for each assay including contrived samples and AcroMetrix products. Positive samples with an assigned qualitative value from Roche Cobas 6800 were analyzed with statistical methods to determine mean, CV, percent of values above the cutoff by sample, 95% CI (based on F-statistic), and estimation of the C_0 and C_95 values. In the characterization study, all the products were tested side by side using Cobas 6800 System and Procleix Ulitro Assays. The results demonstrated that the two platforms are comparable to evaluate the products. For positive samples released from Procleix system, Cobas 6800 presents 100% agreement with Procleix for all three assays: HBV, HIV-1, and HCV. However, for negative samples, the difference of LOD for the two methods results in the overall agreement at 80% to 88% for the three assays, respectively. Conclusions: AcroMetrix infectious controls that mimic patient samples have been long used as independent molecular assay controls for the assessment of assay performance in clinical laboratories for HIV, HBV, and HCV. The characterization study using the Cobas 6800 System helps provide quantitative understanding for all the AcroMetrix infectious controls, and therefore may provide the potential for monitoring the HIV, HBV, and HCV clinical diagnostic assays with improved confidence in test results and assist in maintaining regulatory compliance.

ID62. WITHDRAWN

ID63. Evaluation of a Novel VZV Molecular Assay for Detection of VZV from CSF and Swabs
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Introduction: Primary infections of varicella-zoster virus (VZV), the causative agent of chickenpox, is usually self-limiting, but can manifest into encephalitis or pneumonia with increased risk for patients who are infected in adulthood. In addition, VZV infections can reactivate into herpes zoster in 10% to 20% of patients and cause severe complications including post-herpetic neuralgia, myelitis, and meningitis. Antivirals such as acyclovir are available for treatment as anti-viral agents are available for treatment as anti-viral agents are available for treatment, so rapid and accurate diagnostics is needed. In this study, we evaluated a molecular assay, Simplexa VZV Direct for detection of viral nucleic acid in both lesion (swab) and meningitis (cerebral spinal fluid, CSF) infections. Methods: Swab and CSF specimens enrolled for this study were residual de-identified specimens initially collected due to suspicion of VZV infection or possible meningitis. Specimens were tested on the direct assay and a reference method specific to the specimen type, which was performed at a different laboratory. Swab specimens were compared to direct staining immunofluorescence and culture and results were then validated with two PCR/bi-directional sequencing assays. CSF specimens were compared to two PCR/bi-directional sequencing only. Simplexa VZV Direct was performed following the package insert. Briefly, 50 µl of reaction mix was added to the reaction port of the disc. Then, after a vortex, 50 µl of specimen was transferred to the sample port. Results were reported in 1 hour and compared to the reference methods defined above to determine positive percent agreement (PPA) and negative percent agreement (NPA). Results: In total, 182 CSF specimens and 38 swab specimens were enrolled and tested in the study. CSF testing included 110 prospective, 2 retrospective, and 70 contrived specimens. Compared to the reference methods, PPA and NPA were 100% and 99.3%, respectively. Due to the limitation of positive specimens, 41/44 positives were contrived. The one specimen not detected by the Simplexa VZV Direct assay was one of the retrospective specimens. Only prospective specimens were used in evaluation of lesion infections. The reference method was positive for 13 specimens that were all detected by the Simplexa VZV Direct assay. No false positive specimens were detected resulting in a PPA and NPA of 100%. Conclusions: Overall, Simplexa VZV Direct assay is a sensitive and specific assay for detection of VZV from swab or CSF specimens. Hands-on time is minimal, and assay design allows testing of single specimens or small batches up to 8.

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ID64. A Multi-Lab Collaboration for Quantitative BK Virus Test Development on the Fully Automated Cobas 6800/8800 OMNI Channel
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Introduction: BK virus (BKV) is known as a significant cause of allograft failure in renal post-transplant patients. BK viral loads in urine and in blood are important measures used in the management of kidney transplant patients as well as other immune-compromised patients. Currently, there is no FDA-approved test for BKV. Three clinical laboratories collaborated with Roche on the development of an automated BKV LDT on the cobas omni Utility Channel of the Roche 6800/8800 instrument. The goal was to create a standardized and sharable omni protocol to use for the testing of patient BKV loads in diagnostic labs. Methods: Laboratory collaborators each submitted 2 to 10 primer-probe sets to the group for evaluation. All primers were assessed via the Agile Assay Design Tool of Roche’s Bioinformatics group to identify those with the best scores. A range of concentrations of primers and probes from chosen primer sets were tested in different laboratories; optimization of primer-probe sets using the omni channel reagents and cycling profile was performed on the cobas z480 light cycler instrument for easier manipulation of components. The optimized test parameters were then trialed on the cobas 6800 utilizing previously tested de-identified patient samples (36 plasma and 40 urine), as well as the EXACT diagnostics BKV panel. Test parameters assessed included accuracy, precision, reportable range, linearity, and assay linearity. Carry-over assessment and cross-reactivity were also explored. Results: Two primer-probe sets scored higher than the others via the Agile Assay Design Tool. These two sets were carried forward to the optimization portion of the study. Laboratories shared samples and results of primer-probe concentration trials to find those with the best performance. The top-scoring primer set was found to cross-react with JC virus (JCV); therefore, the second primer set was chosen for the omni channel validation. Using the commercial panel, >98% precision was observed with a reportable range between 2e2 and 2e7, with a linearity of R² = 0.9895. Using patient samples, 100% concordance of negative samples was observed. Concordance for positive samples was defined as less than 0.5 log difference between platforms. Concordance was 95% (2/21) for plasma and 27.6% (8/29) for urine samples. No carry-over was observed, and there was no cross-reactivity with JCV or EBV. Conclusions: We have identified a primer-probe set to develop and validate a quantitative BKV test using the omni utility channel on Roche’s cobas 6800/8800 system. Our initial assay validation data suggest further optimization of pre-analytic processing to address sensitivity, accuracy, and LLOD is necessary for the future implementation of the BKV assay in diagnostic labs.

ID65. Utilization of Digital PCR Assay for the Detection of HPV-16 in Cell-Free DNA in Patients with Head and Neck Cancer at an Oncology Center
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Introduction: Human papillomavirus (HPV) is a DNA oncovirus that is transmitted sexually and infects the skin and mucous membranes. HPV-16 is considered a high-risk genotype and is responsible for 90% of oropharyngeal squamous cell cancer (HPV-OSCC). Screening methods such as pelvic exams and Papanicolaou smears have been effective for early detection of HPV-associated cervical cancers; however, currently there are no sensitive methods for screening and monitoring of HPV-OSCC. The goal of this study is to evaluate the use of digital PCR (dPCR) to detect HPV-16 cell free DNA (cfDNA) in plasma of head and neck cancer (HNC) patients and HPV-negative donor plasma. Methods: A total of 91 samples from patients with HNC and 34 HPV-negative donor plasma samples (BioVIT, Hicksville, New York) were tested. Plasmids containing HPV-16 genome (Addgene) or glycerolaldehyde 3-phosphate dehydrogenase (GAPDH) were used for all experiments as positive and negative controls, respectively. Cell free DNA (cfDNA) was extracted using the QiaSymphony (Qiagen). An extraction control concentration of 0.7308 to 1.1456 ng/μL and samples with DNA fragments of 40 to 255 base pairs were considered acceptable for dPCR. The HPV dPCR assay was performed in duplicate on the QuantStudio 3D/Profilex (Applied Biosystems, Foster City, CA) using PCR master mix containing 5’ labeled fluorescent primers specific for HPV-16 (FAM) and GAPDH (VIC). The assay limit of detection (LOD) and lower limit of quantitation (LLOQ) were 1.3 copies/μL and 1.97 copies/μL, respectively. Results: Using HPV-negative donor plasma, 33/34 samples were negative by dPCR and 1 sample tested at the LOD cutoff (1.3 copies/μL), indicating a specificity of 97% (CI 84.67% to 99.93%). Preliminarily, 43.8% (14/32) of HPV-associated HNC patient samples were positive with viral loads ranging from 1.41 to 4.263.04 copies/μL with a median of 10.28 copies/μL. HPV cfDNA was detected in 57% (4/7) of HNC patients prior to tumor resection and 40% (10/25) of patients with metastatic disease. For patients who underwent tumor resection, HPV cfDNA was detected in 10.2% (6/59) of post-surgical samples. Conclusions: We performed a preliminary clinical validation of a digital PCR assay for absolute quantification of HPV-16 viral DNA in plasma samples from HNC patients. The assay was highly specific and able to detect low concentrations of HPV-16 cfDNA, which will be necessary to measure minimal residual disease which is used to monitor HPV-OSCC patients.

ID66. Detection of Adenovirus Serotype 7 in a Cancer Patient Population
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Introduction: Human adenoviruses (HAdV) are double stranded DNA that cause pneumonia, nephritis, hemorrhagic cystitis, hepatitis, and colitis. There are 7 species of HAdV (A through G) and more than 60 serotypes. The broad spectrum of disease is due to the many serotypes and their different tissue tropisms. HAdV enteritis is often caused by serotypes 40/41, but within our patient population, we have previously shown these two serotypes to be less common. Since HAdV is not a nationally reported disease, many outbreaks may go undetected or underreported. Sporadic outbreaks due to serotypes 3, 4, and 7 have been reported. In 2018, an HAdV type 7 (HAdV-7) outbreak was reported in the Northeast United States. HAdV7 is associated with severe disease and can be fatal among immunocompromised patients. Thus, we sought to determine the frequency of HAdV7 in our patients during the 2018 outbreak period. Methods: A total of 42 randomly selected HAdV positive remnant stool samples were included in this study. Stool filtrate was used to inoculate A549 cells (Quidel), and cell cultures were incubated for at least 96 h. Viral DNA extraction was performed on the EasyMag (BioMérieux), and amplification was performed using primers that targeted a specific, conserved, coding region of the HAdV hexon gene. Post-PCR product was purified using ExoSap-IT (Applied Biosystems) according to manufacturer’s instructions. Sanger Sequencing was performed on the MicroSeq 3500 (Applied Biosystems) with the Big Dye Terminator v3.1 reagents. Results: A total of 42 samples were tested representing 35 unique patients. Among these patients, 16 (45.7%) were female and 19 (54.3%) were male. The median age was 20 (age range: 1 to 68); 16/35 (45.7%) were pediatric patients. Sequencing results were obtained for 25/42 (59.5%) of the tested samples. From these, HAdV7 was detected in only 2/42 (4.76%) patients including 1 pediatric patient. From the remaining 23 samples, the most common types found were HAdV31 (n = 6) and HAdV41 (n = 5). Other types were present at lower frequency and included HAdV1 (n = 4), HAdV2 (n = 3), HAdV12 (n = 2), HAdV4 (n = 1), HAdV14 (n = 1), HAdV53 (n = 1), HAdV55 (n = 1), HAdV3 (n = 1), and 2 where a precise subtype could not be identified. Conclusions: Our preliminary results confirm that HAdV7 was present in our patient population during the outbreak period but at relatively low frequency. However, further analysis with a larger population is needed to better understand the frequency and the clinical impact of HAdV7 in our cancer patients.
ID67. Retrospective Review of Seasonality of Human Parainfluenza Virus Subtypes at an Oncology Center
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Introduction: Human parainfluenza viruses (HPIVs) are a group of common pathogens from the Paramyxoviridae family. They are single stranded RNA viruses and are divided into 4 subtypes. HPIVs are responsible for upper and lower respiratory infections in pediatric and adult hosts. HPIV subtype 3 (HPIV-3) causes more severe symptoms and illnesses. Oncology patients are at a higher risk of acquiring an HPIV infection and have a comparatively higher rate of mortality. According to previous reports, HPIV-1 infections peak during the fall of odd-numbered years, and HPIV-2 positives peak during fall of every year. HPIV-3 is the most common of all subtypes and peaks during spring and early summer, whereas HPIV-4 seasonality has not been well characterized. The goal of this project was to determine the incidence and assess the seasonality of all 4 subtypes among cancer patients at our institution. Methods: A retrospective analysis of 33,913 respiratory viral panels (RVP) performed at Memorial Sloan Kettering Cancer Center over a 2-year period (2018 to 2019) was performed. All RVP testing was conducted using the BioFire FilmArray Respiratory Panel (Biomerieux). Results: Over the course of 2 years, HPIVs made up 4% (1,320/33,913) of all positive RVPs, representing 978 unique patients which included 284 pediatric patients. Overall, HPIV-3 was the most common subtype identified, with a total of 857 positive cases, which peaked during spring and early summer months. For HPIV-4, 158 samples were positive, with the peak number of cases toward the end of the year for both 2018 and 2019. There was a 278.79% increase in the number of HPIV-1 positive cases between 2018 and 2019 (6.6% [95/1,440] versus 25% [186/716], respectively), with the highest number of positive samples in fall of 2019. HPIV-2 infection was the least common subtype observed, with 68 positive samples in 2018 and 24 positives in 2019. Due to the low numbers observed, no specific seasonality could be determined for this subtype. Conclusions: HPIV-3 was the most common subtype identified, and peaked during spring and summer of 2018 and 2019, whereas HPIV-1 peaked during fall of 2019. The seasonality observed in our oncology setting followed similar patterns as those seen in general populations.

ID68. Validation of the RealStar Adenovirus Reagents on Plasma and Stool Samples and Comparison to a Laboratory-Developed Test Using the MultiCode Adenovirus Reagents
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Introduction: Detection and quantification of adenovirus (AdV) is essential for the management of oncology patients. Our laboratory uses a laboratory developed test (LDT) for detecting different AdV serotypes in stool and plasma specimens. This LDT uses MultiCode AdV reagents, the MagNaPure Compact extraction instrument, and ABI 7500 fast for nucleic acid amplification. In this study, we developed a qualitative (for stool samples) and a quantitative (for plasma samples) real-time PCR test using the Altona RealStar kit, with extraction performed on the NucliSENS easyMAG instrument and the amplification on the ABI 7500 Fast, and compared its performance to that of the MultiCode AdV LDT. Methods: A total of 200 plasma samples (154 adenovirus positives and 46 adenovirus negatives) and 81 stool samples (69 adenovirus positive and 12 negatives) previously tested by the MultiCode AdV LDT were used to determine the Altona AdV LDT clinical sensitivity and specificity. Cross-reactivity with other pathogens was evaluated using a panel of 86 samples positive for viruses, bacteria, or fungi. Reproducibility and linearity was assessed using the Acometrix® AdV plasma panel for the quantitative PCR. The limit of detection (LOD) of the Altona AdV LDT was evaluated for both plasma and stool samples, and the lower (LLOQ) and upper limit of quantification (ULOQ) were also determined for plasma samples. Results: For plasma, the sensitivity was 87.3% (95% CI: 82.1 to 93.4) and specificity was 95.7% (95% CI: 85.2 to 99.5). Cross-reactivity with 1 sample positive for cytomegalovirus (CMV) was observed but not reproducible when other CMV positive samples were tested. For stool, the sensitivity was 100% (95% CI: 94.8 to 100) with a specificity of 91.7% (95% CI: 61.5 to 99.8). Overall, there was a total of 20/281 discrepant results between the MultiCode and Altona AdV LDTs. The LOD for the Altona AdV assay in plasma and stool was determined to be 174 copies/mL with an LLOQ and ULOQ of 201 copies/mL and >1.3E7 copies/mL, respectively, for plasma. Reproducibility was 100% for both specimen types. Conclusions: Although the Altona RealStar kit had overall good specificity, the analytical sensitivity was significantly lower than that of the MultiCode AdV LDT.

Informatics
I01. Assessment of RAS Dependency for BRAF Mutations Using Real-World Evidence Databases
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Introduction: BRAF is an important oncogene mutated in a variety of cancers with diagnostic, prognostic, and therapeutic utility. Class 3 BRAF mutations are different from class 1 (V600) and class 2 (non-V600 activating mutations) in that they are RAS-dependent with impaired kinase activity. However, there are contradictory findings regarding current BRAF classification in reported literature. Understanding RAS dependency and mechanisms of RAS activation in BRAF mutant cancer has important clinical implications. This is the first study to systematically assess RAS dependence of BRAF mutations with real-world evidence databases. Methods: This study is a retrospective data analysis of 119,538 non-redundant cancer samples using cancer genomics databases including GENIE (Genomics Evidence Neoplasia Information Exchange) and TCGA (The Cancer Genome Atlas). Frequencies of coexisting RAS (KRAS, NRAS, and HRAS) and RRGs (RAS regulating genes: NF1, PTPN11, and CBL) mutations were calculated for individual BRAF mutations, and compared according to the current BRAF mutation classification; cancer types where coexisting RS or RRGs mutations occur were also evaluated. We used COSMIC to keep only clinical-relevant mutations. Log2-based odds ratios were calculated from the 2×2 tables. Results: Class 1 BRAF mutations and BRAF fusions are mutually exclusive to RAS or RRGs mutations as expected, supporting their RAS independency. Class 2 and class 3 BRAF mutations, however, show variable and indistinguishable levels of enriched RAS (Figure 1), suggesting heterogeneity in RAS dependency and a need to revisit BRAF mutation classification. For RAS-dependent BRAF mutations, the coexisting mutations also involve RRGs and occur in a variety of cancer types, with certain coexisting mutations showing cancer specificity. In addition, RAS dependency of previously unclassified BRAF mutations was also assessed. Conclusions: In conclusion, we demonstrated the potential of using real-world evidence databases to validate existing variant classification. Current BRAF mutation classification based on in vitro assays does not accurately predict RAS dependency in vivo for non-V600 BRAF mutations. RAS-dependent BRAF mutant cancers with different mechanisms of RAS activation suggest the need of different treatment strategies.

I02. CarrierSeq, an Expanded Carrier Screening Product Using Next-Generation Sequencing Technology
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Introduction: Monogenic recessive disorders such as cystic fibrosis and Duchenne muscular dystrophy may severely impact quality of life and constitute a significant burden on healthcare resources. Historically, carrier screening methods for monogenic disorders have employed microarrays and individual probe tests for the detection of relevant genetic mutations. More recently, next-generation sequencing (NGS) has emerged as an efficient approach for detecting known and novel genetic alterations across an expanded set of targets. Here we describe CarrierSeq, an NGS-based panel that leverages AmpliSeq high multiplex PCR to detect known and novel mutations as well as copy number variations (CNVs) in 420 genes relevant to carrier screening for monogenic recessive disorders. Methods: We utilized AmpliSeq design principles to create a pool of approximately 14,000 multiplex primers that amplify the entire coding sequence (CDS) of 420 key carrier screening gene targets. Coverage of CDS regions are padded by at least 25 bases, and additional proximal targets are included to allow for CNV calling to single exon resolution in most cases. The design includes coverage of
triedly challenging targets having homologous or low complexity sequence such as the HBA1/2 and SMN1/2 genes. The new CarrierReporter software was developed to facilitate interpretation of point and copy number variation data to create customized carrier screening reports. Finally, we validated the panel on a set of more than 200 reference samples having variants that had been identified by 1000 Genomes and Sanger sequencing. Results: Testing of validation samples revealed a panel sensitivity and specificity of 99% for the detection of SNPs and indels. The panel also achieved 99% sensitivity and accuracy for CNV detection down to single exon resolution. The panel was able to accurately detect SNPs, indels, and CNVs in genes with homologs such as the HBA1/2 and SMN1/2 genes. Conclusions: We have generated a comprehensive and highly accurate panel for the detection of key SNVs, indels, and CNVs for carrier screening. The comprehensive nature of the panel allows for workflow simplification by replacing the output of several less comprehensive tests. We anticipate this assay to provide valuable information for carrier screening research.

I03. Evaluation of Roche NAVIFY Mutation Profiler for NGS Variant Annotation and Reporting
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Introduction: Bioinformatics pipelines guiding variant annotation and generation of comprehensive reports have become essential tools in molecular pathology. Through an early access program made available, we evaluated Roche NAVIFY Mutation Profiler® by comparing automated versus manual variant classification and reporting. Methods: FASTQ files from 15 tumor samples sequenced using the RainDance Thunderbolt 50-gene targeted panel were aligned to hg37 reference genome using NextGEne software. The tumors were marked as colorectal tumors. VCF data filtered to 5% minimum allele frequency were uploaded to NAVIFY Mutation Profiler, and the results were compared to those generated by manual curation. Mutations were classified according to the Association of Molecular Pathology guidelines (Tier I: variants with strong clinical significance; Tier II: variants with potential clinical significance; Tier III: variants of unknown significance; Tier IV: benign or likely benign variants). Results: Filtering VCF files at 5% allele frequency documented 6 mutations (median, range 4 to 14) per tumor. Manual curation with a cursory review of clinical trial data took approximately 1 h per case. In comparison, each case with complete clinical trial data was complete within 15 min using Roche NAVIFY Mutation Profiler. There was full concordance in the classification of variants in Tier I and Tier II (Tier I: 1A/1B, 100%: n = 6; and Tier II/ID: 100%: n = 23). Initially, there was lower concordance (77%, n = 13) in Tier III variants. However, upon review of supporting data provided by the software, the 3 discordant variants marked as Tier IV (benign) in the manual assessment were upgraded to Tier III (unknown significance). Eighteen variants not listed in either COSMIC or ClinVar databases were unclassified by NAVIFY Mutation Profiler. Several classifications of Tier I KRAS and BRAF variants were made by the tool based on presence of simultaneous mutations in other loci, providing additional treatment options not appreciated from manual curation. Conclusions: NAVIFY Mutation Profiler was useful in pinpointing potential outcomes based on the presence of secondary mutations in Tier IA variants. The software was also useful for the correct classification of Tier III variants. NAVIFY Mutation Profiler increased curation efficiency as evidenced by the clock time saved to prepare each case report. "This product is not for diagnostic purposes in the United States and is not commercially available in the United States.

I04. Optimizing the Detection of Insertions and Deletions Using Next-Generation Sequencing in the Clinical Laboratory
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Introduction: The affordability and efficiency of next-generation sequencing (NGS) has sparked widespread implementation of NGS-based approaches in clinical laboratories for cancer diagnostics. Given its applications for disease management and potential to inform patient care, the accuracy of NGS data is critical, yet these data are highly complex and can be challenging to interpret. Errors produced by sequencing and data analysis can generate erroneous artifacts, which can interfere with the detection of variants or lead to false interpretations. In this study, we have identified more than 40 clinical cases of solid or hematologic malignancies in which our current clinical NGS bioinformatics pipeline either undercalled the variant allele frequency (VAF) of an insertion or deletion (indel) by 1% up to 84%, incorrectly called a simultaneous single base substitution along with an indel, or did not report an indel that should have been detected (e.g., FLT3 internal tandem duplication [ITD]). To improve the ability of the pipeline to better detect and quantify indels, we utilized a software program called ABRA2 (Assembly-Based ReAligner), which uses a localized de novo assembly followed by global realignment to more accurately remap reads from NGS data to the reference sequence. Methods: DNA was extracted from formalin-fixed, paraffin-embedded tissue or peripheral blood, libraries prepared, and samples run on an HiSeq 2500 instrument. Output files were converted to FASTQ format and sequences were aligned to the reference genome using BWA-MEM. The resulting BAM files were either used for variant calling or processed through ABRA2 prior to variant calling. Software was run on a 10 node cluster with Dell R420 hardware. FLT3 ITDs were confirmed using capillary electrophoresis. Results: Prior to ABRA2, the detection of short indels (1 to 31 bases) or an FLT3 ITD (6 to 87 bases) by our standard bioinformatics pipeline would show 3% to 100% mismapped reads. After ABRA2, we observed correction of 50% to 100% of the mismatches for the short indels and 41% to 100% of the mismatches for the FLT3 ITDs. These changes led to improvements in the VAF by a difference of 1% to 30% for the short indels and 1% to 61% for the FLT3 ITDs. Moreover, 8 cases with single base substitution artifacts were corrected, and an FLT3 ITD was found in 6 cases that had only been previously detected by capillary electrophoresis. Conclusions: Based on these results, the use of ABRA2 will lead to improvements in the reported VAF, correction of miscalled single base substitutions concurrent with an indel, and the detection of previously missed indels such as FLT3 ITDs. However, compute power may be a limiting factor, as central processing unit (CPU) time in some cases was greater than one hour, whereas wall clock time was greater than 5 h.

I05. Optimizing Reference Mixture Samples for Bioinformatics Pipeline Assessment on Variant Calling Detection for Cancer Diagnostics and Treatment
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Introduction: Bioinformatics pipelines are an integral part of analyzing next-generation sequencing (NGS) data analysis, where the transformation of raw inputs into reliable mutations calls can have a significant impact on a patient’s health. Specifically, early detection of low-frequency somatic mutations is vital for diagnosis and pinpointing of targeted therapies for adaptive treatment options, but it can be challenging to reliably characterize such variants. To evaluate the performance of a bioinformatics pipeline in low-frequency variant detection, test samples that are complete, reliable, and possess a sufficient number of variants are required. Herein, we describe a strategy for generating an optimized set of reference samples containing maximal known mutations at defined variant allele frequencies through HapMap sample mixtures. Methods: Sequencing datasets from the publicly available HapMap project were mixed in silico to consider every possible permutation to the mixtures, and the optimal solution was defined as the mixture that yielded the maximal number of non-overlapping SNPs. Solutions were determined for multiple genomic contexts, including whole genome, exome only, or assay-specific target regions, as well as at variable detection sensitivity goals. Results: Ideal variants in paired samples mapped to SNPs were heterozygous in the first sample but absent in the second sample, and vice versa. As expected, samples from more different populations exhibited fewer variants in common, and thus were better candidates for selection. Different HapMap pairs were calculated depending on the genomic context, and significant variability in solutions was observed at the assay-specific target region level. Particularly, we identified an optimal reference mixed sample designed to have the largest set of variants within cancer related genes. Conclusions: The resulting mixture samples for each context yielded a higher number of confirmed variants than what is
available in commercial samples. Moreover, given that HapMap samples are commercially available at low cost, and have been extensively studied by different groups, the full set of true variants across the entire genome can be reliably classified. HapMap mixtures can be combined at any desired sensitivity level (e.g., 5%, 1%, 0.1%), permitting high levels of customization. Overall, the larger sets of variants available from HapMap mixtures can improve the calibration of NGS bioinformatics pipelines, yielding improved diagnostic tools for the patient’s benefit.

106. Use and Feasibility of Multi-Algorithmic Consensus-Based Bioinformatics Pipelines in the Detection of Fusions in FFPE Treated Samples

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Introduction: The number of biomarkers and biomarker types used in the management of cancer patients is rapidly expanding. Multiplexed assays utilizing next-generation sequencing (NGS) techniques can not only target several genes simultaneously; these types of assays can interrogate a wide range of biomarker classes. Among these classes, the accuracy at which larger complex structural variants such as fusions are detected remains variable, however, especially if a single analytical pipeline is utilized. Thus, multi-algorithmic consensus-based (MAC) approaches are needed to maximize test accuracy and provide for greater precision.

Methods: In this study, we examine the impact that deploying a MAC approach on the detection accuracy of fusions in formalin-fixed, paraffin-embedded (FFPE) samples evaluated with the Oncomine Comprehensive assay V3 (OCAV3). The OCAV3 is a multi-biomarker targeted assay (161 genes) focused on oncogenes, CNVs, and fusion events. The pipeline are reassembled using a Spades assembler (kmer sizes 55, 71, 99) and read size distribution. Our MAC pipeline employs an additional hybridization (FISH); the in silico

Conclusions: We have developed a highly sensitive and specific assay for robust characterization of gut microbiota. This highly multiplexed approach, with compatibility on both the GeneStudio S5 and the new Genexus Systems, enables an efficient and affordable means for conducting extensive analyses of the human microbiome having applications in the study of phenotypic variability, and the potential relationship to disease. For research use only. Not for use in diagnostic procedures.

108. Microhaploype Locus-Based Workflow for Sample Contamination Detection in Multiplexed Next-Generation Sequencing (NGS) Assays

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Introduction: Innovation in next-generation sequencing (NGS) technology has increased per-run data output and decreased per-base sequence cost. Multiplexed chemistry protocols with mixed nucleotide polymorphisms (SNPs) with a low recombination rate. Such tandem SNPs can serve as informative markers for evaluating sample mixtures. Ninety-six microhap SNPs in 26 conserved genomic loci were selected and included in a custom myeloid neoplasm NGS panel. Twenty-seven samples were captured with a DNA-based xGen Lockdown Probe Pool (IDT, Coralville, IA) and sequenced on a Miseq instrument (Illumina, San Diego). Sequencing reads were used to perform an in silico contamination assessment. Each sample was artifically mixed with another sample at various percentages (range 5% to 50%). The "contaminated" sample reads were aligned to genome reference GRCh37 using BWA-MEM. verifyBamID (University of Michigan, Ann Arbor) was used to analyze the aligned reads in the microhap sites for contamination assessment. True sample testing was performed with bone marrow transplant (BMT), trisomic, and mixed cell line samples. Results: A contamination estimation score (FREEMIX) from verifyBamID was derived for the in silico contaminated samples. A Pearson correlation of 0.994 was observed between the in silico contamination percentage and the
FREEMIX score, indicating the workflow is highly capable of detecting the various levels of contamination. Baseline FREEMIX scores for native samples were assessed and found to have a median value of 0.002. FREEMIX scores of 0.182, 0.124, and 0.456 in the BMT, cell line mix, and trisomic samples, respectively, were consistent with expected values. Using a FREEMIX score cutoff of 0.12, corresponding to a contamination percentage of 12%, we observed sensitivity of 100% and specificity of 94%. Conclusions: We describe a workflow that helps to identify contaminated samples using naturally occurring microhaps and verifyBamID. This approach in our workflow demonstrates versatility and accuracy in identifying contamination in NGS assays, with little added cost or need for exogenous controls.

109. Prediction of DDR and Other Mutation Signatures Using Panel-Based Sequencing

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Introduction: COSMIC signatures reflect mutational processes, either environmental or intrinsic. Such processes can lead to driver mutations and are considered the primary cause of tumorogenesis. Identification of DNA damage response (DDR) signatures or other mutation signatures in a sample enables research into the origin of cancer mutagenesis and potentially treatment optimization. Mutation signatures are generally mined from Whole Genome Sequencing or Whole Exome Sequencing data. We demonstrate that we can also identify mutation signatures using amplification-based targeted sequencing. This occurs because of the limited availability of DNA in formalin-fixed, paraffin-embedded (FFPE) samples and the higher success rates of targeted amplicon-based sequencing.

Methods: We used 1,500 FFPE samples from a pan-solid tumor cohort, sequenced on a targeted 1.7 Mb AmpliSeq panel on the Ion GeneStudio SS System, to develop and evaluate a method to identify mutation signatures. We filtered out germline mutations, removing variants at appreciable frequency in population databases, to generate a set of somatic SNVs. The single base change substitution (SBS) matrix for these somatic mutations was constructed. Cancer signatures listed in COSMIC are characterized across the entire genome, so identifying these signatures from targeted-sequencing data necessitates normalization of the input sample. This is achieved by adjusting the mutation frequencies of the sample using the ratio of trinucleotide counts in the genome and the panel. Then, the cosine similarity between the normalized sample and SBS COSMIC signatures is measured. Signatures with a strong match (>0.7) to the normalized sample are shortlisted. We also use an orthogonal approach to impute the signatures using a reduced candidate set, allowing us to understand the signature fit in the sample and reduce false positive signatures.

Results: Signatures were successfully detected with a cosine similarity (>0.7) for 38% of the samples. A single signature was detected in 10% of the samples. 2 were detected for 7%, and more than 2 signatures for 21% of the samples. A total of 13% of the samples showed UV signatures. MMR signatures (SBS6, SBS14, SBS15, SBS20, SBS21, SBS26, and SBS4) were seen at 0.31%, 0.13%, 0.06%, 0.63%, 0.19%, 0.63%, and 2.45% frequencies, respectively. We successfully identified the putative causal DDR mutation in a number of samples. For example, of 60 samples with NTH1 mutations, 59 were assigned NTH1 related signature SBS30. A sample with MUTHY mutations was assigned the SBS36 signature.

Conclusions: We have confirmed that we can identify diverse mutation signatures using panel-based sequencing, including methods robust to low input FFPE samples. For research use only. Not for use in diagnostic procedures.

110. Accurate Detection and Quantification of FLT3 Internal Tandem Duplications in Clinical Hybrid Capture Next-Generation Sequencing Data

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Introduction: FLT3 internal tandem duplications (ITDs) are found in approximately one-third of patients with acute myeloid leukemia (AML) and have important prognostic and therapeutic implications that have supported its assessment in routine clinical practice. Conventional methods for assessing FLT3 ITD status and allele burden have been primarily limited to PCR fragment analysis due to the inherent difficulty in detecting large ITD variants by next-generation sequencing (NGS). In this study, we assess the performance of publicly available bioinformatic tools for the detection and quantification of FLT3 ITDs in clinical hybridization-capture NGS data.

Methods: Fifty-five specimens from 40 patients diagnosed with AML and previously characterized by PCR fragment analysis and targeted NGS were selected for this study. Three bioinformatic tools were evaluated in the context of detecting and quantifying FLT3 ITDs from the NGS data: getITD, Pindel, and ITD Assembler. We also propose alternative methods for quantifying FLT3 ITD allele burden using soft-clipped reads and ITD junctional sequences.

Results: Pindel had the highest overall accuracy for detecting FLT3 ITDs and was able to detect all ITDs identified by PCR fragment analysis in addition to two cases that were below the limit of detection. FLT3 ITD allele burden was significantly underestimated by all bioinformatic tools evaluated. However, quantification of FLT3 ITD allelic ratio from the NGS data was significantly improved when mutant and wild-type reads were identified using soft-clipped reads and ITD junctional sequences.

Identifying mutant reads by previously identified junctional sequences also improved the sensitivity of detecting FLT3 ITDs in subsequent follow-up samples. Conclusions: Our results demonstrate that FLT3 ITDs can be reliably detected in clinical NGS data using available bioinformatic tools. We further describe a new method for quantifying the allelic ratio from NGS data that substantially improves the accuracy of the utilized bioinformatic tools. These described methods can be easily implemented into most clinical NGS workflows for routine assessment of FLT3 ITD status in patients with AML.

111. Identification of Large Deletions Affecting CTNNB1 Exon 3 in Solid Tumors

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Introduction: Large in-frame deletions spanning exon 3 of CTNNB1 is reported as an oncogenic event, which results in the truncation of the degradation-resistant β-catenin protein. This kind of deletion has been reported in colorectal carcinoma with a prevalence of 2.55%. However, detecting long INDELs (>50 bp) in next-generation sequencing (NGS) data has been challenging. Here we developed markSV, a robust structural variant caller, to detect CTNNB1 long INDELS in a Chinese cohort comprising 3,110 patients with solid tumors. Methods: DNA from 3,110 solid tumor samples was extracted and sequenced with a targeted panel of 520 genes called OncoScreen Plus. Sequencing data were processed by an in-house bioinformatics pipeline for read mapping (genome build hg19/GRCh37) and small variant calling. Large INDELS were identified by the in-house developed caller, markSV, which can identify DNA structural variants through analyzing split-read (SR) and paired-end (PE) signals. Candidates with at least 6 supporting unique fragments and containing SR signals were considered as positive.

Results: A total of 12 CTNNB1 exon 3 deletion deletions were identified in 3,110 patients. All 12 variants were in different positions with various lengths between 33 to 701 bp. Colorectal cancer was the most enriched cancer, with a prevalence rate of 2.5% (8/320), whereas the other two were 0.12% (LUAD, 3/2,593) and 0.51% (OV, 1/197). All these cases with CTNNB1 exon 3 deletion deletion were microsatellite stable. Of the 12 CTNNB1 exon 3 deletion variants, 4 cases spanned from intron 2 to intron 3, resulting in the deletion of the entire exon 3, 4 deletions spanned from exon 3 to exon 4, creating a joint exon 3-exon 4 with in-frame deletions in the middle; 2 cases spanned from intron 2 to exon 3, which may cause the
entire exon 3 to be lost due to alternative splicing; 1 case deleted 33 bp in exon 3 and inserted 3 bp, resulting in an in-frame codon change; 1 case spanned from intron 2 to exon 4, of which the function effect on transcription is unknown. Of the 8 colorectal cancer cases with CTNNB1 exon 3 deletion, 37.5% (n = 3) also carried driver mutations, such as BRAF V600E and KRAS G12H, G12V. Conclusions: In this study, we introduced a robust SV caller markSV, and analyzed the CTNNB1 exon 3 deletion incidence in Chinese solid tumor patients. The CTNNB1 exon 3 deletions were in various forms with the length between 30 to 1,000 bp, and were especially frequent in colorectal cancers.

112. Development of a Clinical Bioinformatics Pipeline for the Comprehensive Genomic Profiling of Patient-Derived Xenograft Tumors
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Introduction: Clinical trials using precision medicine for therapy selection require the use of comprehensive solid tumor panels in pathologic workup of solid tumors. Expanded testing can identify novel tumor mutations, which allows to refine treatment options for patients. Method: To evaluate the performance of a comprehensive bioinformatics pipeline for high-confidence calls and variant detection in patient-derived xenograft (PDX) models, we analyzed 15 PDX tumors, 6 human cell lines) 1 PDX tumor contained more than 3 mapped to the mouse genome. Of the remaining samples (15 PDX tumors, 6 human cell lines) 1 PDX tumor contained more than 3 overrepresented kmers, which all used to perform clinical variant assessment.

Methods: NGS was performed using the validated Oncomine Comprehensive Assay v3 panel, Ion S5 system, and Ion Reporter 5.10 (Thermo Fisher). A kmer-based (50,75-mers) overexpression analysis was performed to identify sources of contamination and relative balance between reads originating from mouse and human. Bam files were first randomly sampled (>100,000 reads) 3 times and mapped against a database of contaminants. To be considered a hit, kmers were required to align more than at least 20 bases with 1 mismatch allowed. Reads with hits highly expressed across all iterations were mapped using megablast (word size: 28, match/mismatch: 1, -2) to the nucleotide (nr/nt) database. Alignments with 100% identity (Evalue < 3e-16) were considered significant. Next. vcf files were filtered against a mouse model specific .vcf and contaminating variants flagged for review. Qagen Clinical Insight was used to perform an ortholog variant assessment. Our hope is that this will facilitate the adoption of the proposed method in downstream in vivo investigations.

113. Comprehensive Single-Nucleotide, Indel, Structural, and Copy-Number Variant Detection in Human Genomes with PacBio HiFi Reads
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Introduction: Long-read sequencing has revealed more than 20,000 structural variants spanning >12 Mb in a healthy human genome. Short-read sequencing fails to detect most structural variants but has remained the more effective approach for small variants due to 10% to 15% error rates in long reads, and copy-number variants (CNVs) due to lack of effective long-read variant callers. The development of PacBio highly accurate long reads (HiFi reads) with read lengths of 10 to 25 kb and quality >99% presents the opportunity to capture all classes of variation with one approach. Methods: We sequenced the Genome in a Bottle benchmark sample HG002 and an individual with a presumed Mendelian disease with HiFi reads. We called SNVs and indels with DeepVariant and extended the structural variant caller plsv to call CNVs using read depth and clipping signatures. Results: For 10-fold coverage with 13 kb HiFi reads, variant calling in HG002 achieves an F1 score of 99.7% for SNVs, 96.6% for indels, and 96.4% for structural variants. Additionally, we detect more than 300 CNVs spanning about 10 Mb. For the Mendelian disease case, HiFi reads revealed thousands of variants that were overlooked by short-read sequencing, including a candidate causative structural variant.

Conclusions: These results illustrate the ability of HiFi reads to comprehensively detect variants, including those associated with human disease.

114. Look before You Leap: A Toolkit for Moving Clinical Panels to GRCh38
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Introduction: One common reason a clinical diagnostics laboratory may be reluctant to move from GRCh37 (hg19) to GRCh38 (hg38) is not knowing how changing reference genomes will impact clinical pipelines and the interpretation of the variants they call. Although most genes are unaffected at the protein coding level, other than their genomic coordinates, many genes have new pseudogenes added to hg38, which can affect mapping quality; some genes have additional annotated transcripts in the new build; and some genes have flipped strand orientation, which can impact variant interpretation. To better understand how genetic regions of interest will be impacted by migrating to hg38, we have developed an R toolkit called Reference Genome Converter (ReGe) that identifies changes that could influence either the ability to map to a genomic region or the interpretation of genetic regions of interest for a supplied panel design. Methods: ReGe leverages the NCBi’s Remapping Service to extract information about hg38 positions, the introduction of insertions or deletions at the sequence level, and changes in strand orientation. It uses NCBi’s RefSeq to annotate gene and coding exon information for both hg19 and hg38. ReGe determines if one or more genes’ coding exons or specified intervals will have any of the following issues when they are mapped to hg38: 1) failure to map to the primary assembly, 2) mapping to multiple locations in the primary assembly, 3) preferential mapping to an alternative locus, 4) introduction of an insertion or deletion, or 5) change in strand orientation. In addition, our tool reports if any exons are missed or incompletely covered when mapping to hg38. Results: ReGe was applied to all coding exons for genes in an in-house medical exome panel (4,565 genes/62,685 unique exons). It created detailed and summary reports about the changes found, including resulting BED files and visualization showing the distribution of the identified issues across the genome, which made migration from hg19 to hg38 easy. Here, we demonstrate the results of this analysis and how it affected clinical decisions for assay validation. ReGe found that 95.9% of the exons mapped perfectly from hg19 to hg38. Out of the remaining exons, we found 12 exons (2 genes) that are no longer mapped to hg38 chromosomes, 123 exons (14 genes) with a flipped strand, and 18 genes that had new loci in hg38 affecting their data mapping quality.

Conclusions: Our hope is that ReGe will facilitate the adoption of GRCh38 as the reference genome for clinical and research labs by alleviating both the uncertainty and challenges that come with migrating to the newest version of the human reference genome.

115. A Novel Machine Learning Approach to Characterize Cancer Signatures for Improved Clinical Reporting
BioDiscovery, El Segundo, CA.
Introduction: It is well accepted that cancer is primarily a genetic disease that is caused by mutations in the genome. A number of genes and associated variants for small have been well characterized in relation to different cancer types. There exists a wealth of information with regard to these genes and small sequence variants (SNVs, indels, etc.) available from multiple knowledgebases online. In contrast, there are very few well-
defined CNV signatures that are associated with different cancers. Some of the well-studied signatures (like the 1p/19q co-deletion) can be used as prognostic markers for certain cancer types. However, no automated system has yet been developed to identify underlying cytogenetic signatures across many cancer types. **Methods:** In this study, we present a novel neural network algorithm based on self-organizing maps (SOM) that creates a 2-dimensional map of cancer. Each position on this map represents a particular cytogenetic signature (e.g., 1p/19q co-deletion), and the neighboring areas on this map have similar but slightly different signatures. We used more than 6,000 manually curated CNV profiles from more than 25 cancer types from the TCGA Project to train this neural network to automatically discover the hidden cytogenetic signatures. **Results:** Our neural network generated 225 unique signatures representing the cytogenetic landscape of cancer. Projecting each cancer sample onto our 2D lattice produced a mapping of cancer type clusters. We were able to generate well-differentiated survival statistics based on the specific pattern of this mapping. **Conclusions:** The map can be used as an orthogonal tool to the histology or tissue of origin classification of the tumors and provide additional diagnostic as well as prognostic power. We show how this map can effectively be used to classify new samples into unique clusters and provide prognostic estimates.  

**116. Pindel as a Back-up INDEL Caller to a GATK4 Mutect2-Based In-House Developed Somatic Variant Calling Pipeline for a Custom Clinical Cancer NGS Panel**  
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**Introduction:** Mutect2 is a versatile somatic variant caller that differs more than sensitive than, and roughly twice as fast as, HaplotypeCaller’s reference confidence mode. Mutect2 is very different from the early version that was included in GATK3. For example, it uses local assembly and re-alignment to detect SNVs and INDELs, and is based on several probabilistic models for genotyping and filtering. In this study, we evaluated GATK4 INDEL call performance with Mutect2 and compared to Pindel call. **Methods:** Data were generated from a 90-gene Agilent HaloPlexHS UMI-based paired-end hybrid-capture custom cancer sequencing panel run on Illumina MiSeqDx. Adapters were trimmed and molecular barcodes were processed using Agilent Genomics ToolKit (v4.0.1), sequence reads were aligned to the human reference genome (GRCh37/hg19) using Burrows-Wheeler Aligner (v0.7.17), SNV/INDEL variants were called using GATK4 Mutect2 (v4.1.0.0) in tumor-only mode, then Pindel (v0.2.59b), and normalized using vt (v0.57). Forty-five blood or marrow DNA samples were used to check for challenging INDELs within **v0.2.5b** (21 bps, 27 bps, 36 bps). Mutect2 failed to detect 1 FLT3 ITD, but successfully detected 11 FLT3 ITDs from which 10 were validated by Sanger sequencing. **Results:** FLT3 ITDs from which 10 were validated by Sanger sequencing.  

117. **Classification Methods for Germline and Somatic Single Nucleotide Variant (SNV) in Circulating Tumor DNA (ctDNA) of Small Cell (SCLC) and Non-small Cell Lung Cancer (NSCLC)**  
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**Introduction:** To leverage the potential of tumor-specific molecular profiling by next-generation sequencing (NGS), somatic variants need to be identified correctly. However, tumor or ctDNA samples may be sequenced without matched normal tissue or plasma depleted whole blood (PDWB). Finding accurate methods for somatic filtering is crucial in the absence of a matched normal sample. **Methods:** From the prospective, observational German Lung Cancer Multi-Marker Study, a total of 24 SCLC and 19 NSCLC late-stage patients were selected. All patients had matched PDWB and pre-treatment samples available, and additionally a median of 5 (range 0 to 19) post-treatment plasma samples were sequenced. The samples were analyzed with the AVENIO Surveillance Kits®, a 198-bp NGS panel targeting cancer genes. Mutations were reported by the AVENIO software v2.0®. The true set of tumor-derived somatic variants in pre-treatment plasma was determined by subtracting SNVs detected in the matched PDWB. To identify somatic mutations, database-informed filtering, filters based on allelic frequencies (AF), and an in-house developed machine learning algorithm (CSMutan) taking into consideration the variation in the AF of a variant were used. These classifiers were compared to the truth set to evaluate the performance of each method. **Results:** Database filtering based on evidence in ExAC, 1KGP, and dbSNP for germline variants and TCGA or COSMIC for somatic mutations resulted in >99.2% specificity (SP) but sensitivity (SE) <31.4% for both SCLC and NSCLC. Without the requirement for somatic counts in either TCGA or COSMIC, both SE and SP were >96.2%. To improve classification for variants that are not present in any of the databases, an approach using AF was explored, which can be particularly relevant if multiple samples are available. Assuming a response to treatment, the AF of somatic variants should decrease in post-treatment plasma. Classifying variants with an AF >20% in all plasma timepoints as germline resulted in an SE of 98.7% for both histologies. SP was 98.5% for NSCLC, but only 92.5% for SCLC. Using the machine learning classifier, which combines the coefficient of variation of a variant’s AF (CSMutan) and knowledge from databases, a SE of 100% and SP ≥99.4% was achieved. **Conclusions:** Sequencing of a normal sample is the most accurate approach to identify germline variants. For both SCLC and NSCLC, the evaluation of different methods demonstrated that it is feasible to identify somatic SNVs with high accuracy without sequencing a normal reference. A machine learning approach was identified as the best evaluated method for classifying SNVs as somatic versus germline. *For Research Use Only. Not for use in diagnostic procedures.*  

**118. Evaluating Machine Learning Methods for Accurate Variant Calling Detection on Acute Myeloid Mutation Analysis**  
Navigate BioPharma Services, Inc., a Novartis subsidiary, Carlsbad, CA.  
**Introduction:** Detection of low frequency DNA variations in emerging pathogenic sub-clones requires sensitive NGS techniques and manual review. Herein, we explored the utility of a large number of machine learning tools to determine an improved variant calling strategy for use on a targeted myeloid assay. **Methods:** A custom Archer sequencing assay, the Core Myeloid Plus (CMP), was developed to
identify mutations from a panel of 38 genes. A core dataset was established from 34 AML samples with previously identified true variants labeled appropriately. Raw reads from the CPM assay were processed using the ArcherDX pipeline to generate variant call files, followed by a pre-processing filtering step to remove low support calls. The final dataset was partitioned into training and testing regimes, and subjected to a 10-fold cross-validation protocol to determine optimal classifiers for variant calling. Results: Our findings show that from a number of more than 40 machine learning protocols, a significant number produced >98% accuracy with almost no false positives. These methods include Bayesian models, classification trees, neural networks, and lazy classifiers. Furthermore, data attributes such as allele frequency and total depth were appraised and ranked using several attribute evaluators to identify the most significant discriminators. Finally, unsupervised clustering analysis identified natural clusters in the data as a way to pick major clusters for true variants and false positives. Conclusions: Although traditional variant caller algorithms can often provide a discriminator to differentiate true calls from noise, such tools are often imperfect and thus require further investigation to generate accurate genetic data for true clinical use. Moreover, it is often difficult to discriminate true variants from noise using only a few parameters, or to determine the best parameters to use. With the advent of machine learning from AI, it is possible to test multiple machine learning methods using variable numbers of parameters on a large dataset to optimize predictions. Our solution provides a reliable method for variant calling that can continuously be improved as more data become available.

Introduction: As the importance of detecting low level biomarkers for early detection and monitoring of disease has become more apparent, the demand for ultra-sensitive next-generation sequencing (NGS) assays has grown. When searching for somatic mutations, whether to identify known or novel variants in a liquid biopsy or in major clusters for true variants and residual disease in leukemia patients, there exists a challenging requirement to look deep while casting a wide net. Sequencing errors introduced through random acts of chemistry make it difficult to separate true SNVs and INDELs from noise. Even with the integration of unique molecular identifiers (UMis) into NGS workflows to correct errors introduced during fragment amplification, errors due to platform-specific limitations and persistently problematic genomic regions may still remain. For comprehensive genevectors panels such as Illumina TSO500, manual filtering of these questionable detected variants becomes a daunting task that necessitates automated strategies that often use a limited number of variant features to differentiate true variants from the noise. Herein, we present a positional, variant-dependent noise reduction strategy for calling variants <5% VAF. Methods: Using sequencing data derived from a set of samples expected to be wild-type for somatic mutations, we created an error profile for SNVs and INDELs called at each position within the Illumina TSO500 panel. Raw reads were then processed using the TSO500 bioinformatics pipeline to generate variant call files. Next, a statistical model was built for each variant call based on the distribution of noise at that position, from which a p-value can be calculated that represents the probability that an observed or higher allele fraction for a particular variant came about due to error. Finally, for every variant call on a target sample, the p-value calculation is then used, along with other measures, to discriminate true variants from noise. Results: Given a set of 10 samples, the p-value of the aforementioned model was computed as an additional feature for classifying true SNV and INDEL variants, and a machine learning protocol was implemented to classify true variants from noise. Results show we were able to significantly improve the specificity of our variant caller without sacrificing sensitivity (>98%) as the limit of detection was lowered below 5% VAF, when compared to a static VAF filter. Conclusions: Evaluating detected variants against a background of systematic noise allows us to increase our confidence in calls made at lower levels of detection. Engineering specific features that estimated background noise at the position level, combined with traditional features such as allele frequency and depth, dramatically improves our ability to call low allele frequency variants within a large targeted panel.

I20. Clinical Cancer Genomics: Artificial Intelligence Assisted Data Re-analysis to Improve Detection of Potentially Actionable Mutations
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Introduction: Tumor next-generation sequencing (NGS) is now routinely used to identify therapeutic/prognostic markers in clinical oncology. Given the fast pace with which new drugs and clinical trials are developed, it is unknown if previous re-analyses of NGS data can identify additional actionable targets that can benefit patient treatment and management. We used a cognitive computing tool that incorporates continuously updated public databases to identify potentially actionable alterations at 2 time points in a retrospective mixed cancer cohort. Methods: A total of 2,232 solid organ malignancies that were clinically sequenced at Johns Hopkins (2017 to 2019) were identified. These comprised 33 cancer types with majority cohort (CRC), lung, and pancreatic (PDAC) cancer. All tumor samples were sequenced using a 400-gene targeted NGS panel. Alterations (i.e., SNV, indel, CNV, or TMB) were re-called using the same bioinformatics pipeline, and annotated by Watson for Genomics spring 2020 (baseline) and summer 2020 (re-analysis 1) to identify potentially actionable alterations. Results: At baseline, 70% (N = 1,565) of cases had ≥1 potentially actionable alteration corresponding to tiers 1 to 3 actionability scores that encompassed 103 drugs, most of which were TKIs (24%). A total of 24% (N = 350) of cases had alterations with tier 1 scores (FDA-approved/NCCN-endorsed biomarker-specific drugs for the tumor type in question). This was followed by 3% (N = 57) with alterations matching to tier 2 scores (FDA-approved biomarker-specific drugs for other tumor types). Lastly, 44% (N = 978) of cases only had alterations with tier 3 scores (biomarker-specific drugs in clinical trial). Certain tumor types (melanoma, CRC, lung, GIST) had a higher prevalence of tier 1 scores, whereas others (PDAC, CNS) had lower prevalence of tier 1 scores. Notably, 7% (N = 156) of cases had TMB status ≥20 mutations/Mb, which corresponded to a tier 1 score in 33% of TMB+ cases. At reanalysis 1, initial results show that six new cases now have ≥1 potentially actionable alteration. Conclusions: This study used AI assistance to identify potentially actionable alterations at 2 time points in a retrospective group of solid organ malignancies. At baseline, we found that 70% of cases have potentially actionable alterations, including ≥24% (24% of entire cohort) tier 1 alterations. This fraction is likely related to the enrichment for cancer types with targeted therapeutic options in our cohort. Whereas re-analysis 1 did not reveal significant improvements in newly actionable cases, future studies will determine the fractional increase in number of drugtrial options per case and explore re-analyses at longer intervals. We anticipate the yield of future re-analyses will be improved, as more pan-cancer drug approvals become available.

I21. Capturing and Visualizing Cancer Genomic Data with Category Variants in the JAX Clinical Knowledgebase (JAX-CKB)
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Introduction: Precision oncology aims to predict a patient’s response to a targeted therapy based on the tumor’s genomic profile and scientific evidence. Next-generation sequencing (NGS)-based sequencing tests can identify a vast number of specific genomic variations in tumor samples; however, evidence for therapeutic efficacy for specific or rare mutations is often lacking. Furthermore, published literature, drug labels, professional guidelines, and clinical trial recruitment criteria often utilize generic mutation terms such as “EGFR activating mutations,” “NTRK fusions,” and “PDGFRα exon 18 mutations.” This creates a challenge in interpretation, as patient-specific alterations must be associated with more generic categories to link patients to relevant therapeutic interventions. Methods: To bridge this divide, the JAX Clinical Knowledgebase (CKB) (https://ckbhome.jax.org/) devised the concept of category variants which
represent the more generic concepts such as exon or amino acid position, effects on protein activity, or structural changes often referred to in the primary sources. For example, “act mut” indicates the variant results in a gain-of-protein function, and “exon X” indicates a mutation in the specified exon. A rules-based engine was developed to associate all the specific variants in CKB with appropriate category variants, and the resulting hierarchical organization is displayed visually based on positional and functional effects, allowing the user to navigate from a specific variant to content for a category variant, and quickly interpret variant relationships. Additionally, powerful editing and quality control tools were introduced to ensure accuracy at scale. **Results:** In JAX-CKB, category variants were included in 16.8% (87/516) of the efficacy evidence (EE) lines associated with FDA approval. The 87 EE lines employed 36 category variants, which were associated with a total of 1,302 specific variants in CKB. A total of 54.1% (365/675) of treatment-related NCCN-guideline EE lines included 69 category variants, and enabled mapping of 4,030 specific variants in CKB to guideline-level evidence. Among the 625 active clinical trials with molecular requirements in their inclusion criteria, 50.2% (314/625) of trials were curated with 440 category variants from 224 genes. **Conclusions:** The strategy of using category variants allows for the extrapolation of evidence from generic mutation categories to specific variants and represents a novel approach to facilitate therapeutic decision-making in precision oncology, while maintaining data provenance and saving time.

122. **MPath STAR-QC: Automated Quality Control Application for Contamination and Sample Swap Detection Using Short Tandem Repeat Testing**

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**Introduction:** Short tandem repeat (STR) testing is often done as a quality control (QC) step before other clinical assays to help identify potential contamination or sample mix-ups. However, comparing paper-to-paper graph records of STR samples can be overwhelming when performed at a large scale. Every new sample needs to be compared with all prior samples from the patient in addition to every other sample in the concurrent batch. In high-throughput laboratories, performing this manual analysis for every sample can result in an enormous burden on laboratory staff. Each additional analyzed case results in an exponential increase in manual graph comparisons. Here, we present MPath STAR-QC, which alleviates this manual burden by automatically analyzing STR traces and flagging cases where contamination could be present or where patient results display historical inconsistencies indicative of a swap. **Methods:** STR traces are comprised of peaks with two main attributes: size and height. MPath STAR-QC represents these graphs digitally by converting them into a one-dimensional vector. These vectors are then utilized to quantitatively compare each sample to every other sample in our database using cosine similarity. This allows the application to “flag” samples where the profile is indicative of a QC issue. In the case of contamination, the sample will closely align to another sample from a different patient. In a swap, the sample will be dissimilar to prior samples from the same patient. The application visualizes this for the user by creating an adjacency matrix represented as a heatmap. MPath STAR-QC further allows users to select samples from the heatmap to build a side-by-side comparison of each sample’s STR trace profile, and highlight potential QC issues consisting of possible patient swaps and contaminations via a warning box. The clinical laboratory staff are also able to annotate at the batch/sample level and provide a QC status. **Results:** Since MPath STAR-QC is implemented, our clinical laboratory staff have registered 126 batches comprising 9,504 samples including baseline and post-transplant cases. From these registered samples, STAR-QC automatically flagged 187 possible swaps, and 230 possible contamination cases. Overall, MPath STAR-QC reduced number of cases that need manual review by 96%. **Conclusions:** MPath STAR-QC demonstrates that the integration of machine learning and information retrieval techniques, combined with feature-rich functional capabilities, has the potential to minimize the amount of paper records and manual review time needed for QC analysis in diagnostic molecular laboratories.

123. **Many NGS-Based Assays, One Platform: Ensuring a High-Quality Case Review and Sign-out Process with NGS Reporter (NGS)**


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**Introduction:** With an ever-expanding set of targeted treatments, next-generation sequencing (NGS) and molecular characterization of disease are rapidly becoming the standard of care. Clinical laboratories are implementing comprehensive NGS assays with increasingly complex infrastructure to meet the demand. Case review and reporting are two of the most challenging aspects for molecular pathologists (MPs), who need to evaluate each variant called by bioinformatics pipelines and report on their validity. This process is time consuming and can vary considerably across different assays. We built NGSR: a modular platform providing a unified environment to harmonize the review and reporting of variants. **Methods:** NGSR is an online, secure platform comprising interacting components to assist the review of NGS results. It supports data from different assays from targeted panels to whole exome sequencing. It includes: 1) a single page application as front end; 2) a RESTful API and microservices (e.g., a document generation service to generate templated reports); and 3) third-party integrations such as ClinVar and VarSome. NGSR’s modularity enables the rapid adaptation to different NGS assays by leveraging its APIs for data loading. We employed different technologies including Angular, ASP NET Core and node.js. **Results:** NGSR is integrated in the WCMNYC Clinical Genomics Laboratory infrastructure comprising: a LIMS system, automated bioinformatics solutions, and a communication system for submitting the results to downstream EMRs. Upon analyses completion, data from LIMS (test, patient, and sample information) and NGS results, including QC metrics from the bioinformatics pipelines are imported. Standardized interpretations of variants are fetched from the Precision Medicine Knowledge Base (PMKB, pmkb.weill.cornell.edu). MPs review all information about the case and its variants. They can evaluate evidence by viewing BAM files and add general or specific comments at both the report and variant level, as well as edit interpretations from PMKB. Detailed views of variants show data from external systems such as gnomAD. Importantly, variants excluded by bioinformatics pipelines can be “rescued” by providing proper justification. Similarly, variants can be filtered from the report. When all variants are reviewed, the MP can preview the PDF report and sign it out. The report, and the discrete variant calls, are then sent to the downstream systems. **Conclusions:** NGS tests are important tools in molecular pathology and indispensable in the expanding clinical testing landscape. Reviewing the results of different assays and platforms requires proficiency in different systems. NGSR facilitates this process by providing a unified framework to review results of diverse assays.

124. **Database for Managing Results of High-Throughput Sequencing Clonality Assays in Clinical Laboratories**

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**Introduction:** High-throughput sequencing of T-cell receptor (TCR) and immunoglobulin (ig) has been used for diagnosis and monitoring of lymphoid malignancies. Its ability to identify and track unique TCR and Ig sequences from multiple time-points and multiple specimens is especially powerful in comparing clonal processes spatially, temporally, and for minimal residual disease monitoring. However, the sheer amount of immune repertoire sequencing data requires bioinformatic tools, data management and analysis, and experienced pathologists for consistent and accurate characterization of clonotypes in a specimen. To fulfill these unmet needs, we developed a database application and pipeline for managing clonality testing and analysis in clinical laboratories. **Methods:** Specimens from patients with suspicion of lymphoid malignancies were tested with the commercially available LymphoTrack TRG, TRB, IGH (FR1, FR2, FR3, VLEADER), and/or iSK assays (Invivoscribe, Inc). Paired-end reads were sequenced on the Illumina MiSeq platform. Alignment and demultiplexing of targets were performed with LymphoTrack 2.4.3 (Invivoscribe, Inc). We developed an in-house database application and pipeline to manage and...
analyze the generated clonotype sequencing data. The data storage, display, and output functions were validated against the pre-existing workflow by uploading and comparing 52 specimens from 10 sequencing runs. Results: Patient and specimen details, and outputs generated by the LymphoTrack software, are automatically imported to the database. Results can then be viewed individually or organized into batches. Visualization tools are available for top 10 clonotypes, variable-diversity-joining (VDJ) gene frequencies, read-length histograms, and other quality control metrics for pathologist’s review. Tracking of a clonotype between runs enables patient-specific monitoring over time and across specimens. Alignment of individual clonotypes against reference sequences allows inspection of the alignment of full VDJ sequence and the complementary determining region 3 (CDR3). Comparison of identical clonotypes within a run provides quality assessment of index-switching and potential contamination events. Accuracy of the tools implemented in the database was validated by comparing to manually generated results. Conclusions: High-throughput sequencing enables objective and standardized interpretation of clonality testing in clinical laboratories. Enhancements in sequencing resolution require scalable data management and bioinformatics solutions. We developed and validated an in-house database application that integrates visualization tools, quality control, and result tracking that enables the efficient use of high-throughput TCR/lg assays for diagnosis and disease monitoring.

I25. MPath Lab QC: A Centralized Assay Agnostic Approach to Store, Review, and Finalize Laboratory QC for NGS-Based Genomic Clinical Tests

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Introduction: Quality control (QC) is an essential part of a laboratory test to ensure that the test is performing within the required analytic parameters. With increasing implementation of next-generation sequencing (NGS)-based genomic tests, there is an increasing need to electronically capture the laboratory QC workflows. Moreover, it is not uncommon for a laboratory to offer more than one NGS-based clinical test. Capturing QC metrics based on the Clinical Laboratory Improvement Amendments (CLIA) guidelines and the rich data output of NGS-based assays poses a complex problem. Here, we describe a common framework and an application for a centralized informatics infrastructure to electronically capture analytics and QC results, and enable QC review and approval. Methods: MPath Lab QC is designed based on n-tier architecture that includes data entry ports, backend application programming interfaces (APIs), and a database layer. In-house developed ReactJS component library is used for MPath QC frontend, backend APIs are developed in Python, and data are stored in MongoDB. MPath Lab QC queries laboratory and data management systems to capture NGS-specific run metrics, date timestamps, and other sequencer details. The system maintains a list of positive controls for each assay, and corresponding expected variants and their variant allele frequencies (VAFs). A role-based access control is implemented to track user authorization to assay specific QC portals and dictate their available actions. Results: We have implemented MPath Lab QC workflow for 5 of our NGS-based assays in our clinical laboratory, including assays for solid tumors and hematological malignancies. In addition to the sequencing QC metrics and sequencer details, expected VAFs from positive controls are automatically compared to the actual VAFs from analysis results. Discordant events are highlighted for easy and intuitive review. Additionally, MPath Lab QC workflow allows the analysts, lab supervisors, and pathologists to comment, upload corresponding documents, and approve the sequencing QC. Conclusions: Having a centralized system to facilitate Lab QC review of multiple NGS-based assays has enabled an efficient, transparent review and approval process. MPath QC facilitates consistent QC review workflow, assay-specific customizations, and intuitive visuals to ensure test performance while working on a wide array of NGS assays.

I26. MPath Results PCR: An Integrated Approach to Programmatically Load, Curate and Report Non-NGS Germline Results


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Introduction: Polymerase chain reaction (PCR) based molecular assays, such as Sanger or MLPA, are commonly utilized to identify genomic alterations. In the setting of germline testing, Sanger/MLPA can be used to test pre-specified familial variants or to confirm a variant identified in next-generation sequencing (NGS) based testing. Although advanced variant management systems have been developed for NGS based assays, Sanger/MLPA (and other non-NGS based assays) have traditionally been managed as standalone applications. Moreover, interpretation and clinical report generation of PCR based assays typically involve review of published articles and biological databases to categorize pathogenicity for cancer predisposition. An informatic solution that incorporates NGS and non-NGS is essential to ensure ease in curating/review of test results and consistency in reporting. Here, we present MPath Results PCR, an electronic platform to import, curate, and sign out germline non-NGS testing. Methods: We designed and developed a multi-tier client server architecture informatics solution to help curate, store, and report Sanger/MLPA assay results. The codebase is built on Python, Flask, Pandas, MySQL, MongoDB, and ReactJS. A total of 61 different Sanger/MLPA based tests are offered through Diagnostic Molecular Genetics (DMG) service. Results: MPath Results PCR consists of 3 main components: automated test registration, result ingestion and curation, and template driven report generation. The test registration pipeline routinely queries the laboratory information system to register newly ordered germline Sanger/MLPA tests. Test results are then populated into the database through a results uploader on the portal. This solution normalizes non-NGS data into a centralized variant database that also includes NGS germline reported variants. As a result, the new MPath Results portal serves as a central variant management system; it can access and associate the latest variant classifications, allele frequencies from population databases (gnomAD, ExAC, 1000 Genomes), and interpretation notes with the Sanger/MLPA results automatically, serving as a one-stop shop to curate and memorialize true positive events. The review portal also allows updates to the variant information and includes workflows to upload and review QC documents and Sanger/MLPA trace files. The last component comprises template based clinical report generation for consistency in clinical reporting. Conclusions: By creating a unified platform for curating NGS and non-NGS tests, we streamlined and enhanced the curation process for pathologists, first by giving them a common user interface and report format, and second, by auto-loading the previously reported variant information regardless of assay methodology.

I27. mrLab: Leveraging Mixed Reality in a Precision Medicine Laboratory to Increase Safety and Productivity of Healthcare Workers during the COVID-19 Pandemic


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Introduction: The COVID-19 pandemic has exposed limitations on how medicine is currently practiced. In biology laboratories, solutions like telehealth, social distancing, and staggered shifts have proven to be difficult in terms of productivity and safety. Advances in augmented and mixed reality (VR/AR/MR) in recent years have paved the way for real-world applications, with the biomedical field well poised to benefit from them. We report extending mrLab, an MR app for biology labs; adding remote assistance, voice recognition, hand, and eye tracking; and training modules to enhance productivity and enable contamination-free interactions enhancing safety. Methods: We developed mrLab: an MR application for Microsoft Hololens 2 connected to the Engländer Institute for Precision Medicine (EIPM) Laboratory Information Management (LIMS) at Weill Cornell Medicine, enabling biology lab technicians to overlay LIMS information to the real world. We extended mrLab to: 1) remotely assist on-site lab techs, overlaying teleconference
capabilities, i.e., video, 3D objects, screen, and document sharing to their reality; 2) train in mixed reality, overlaying SOPs of workflows in the form of text, images, videos, and 3D objects in their reality; and 3) reduce data entry and streamline navigation by automatically recording information into LIMS. mrLab leverages advances in spatial computing, gesture, eye tracking, and voice recognition to provide novel modality of interactions with equipment and team members. Results: We tested 2 main scenarios: 1) remote assistance for tissue culture of 3D organoids and troubleshooting equipment in the lab; and 2) training for DNA extraction and centrifugation equipment. Remote Assist allows users on-site wearing the device to interact with remote users via an integrated video-conferencing system while performing their task hands free. In the training case, a user wearing the device followed the step-by-step instructions shown via virtual text as well as virtual objects, such as 3D arrows indicating the location of controls on the equipment. Those virtual guides exploit spatial computing by “understanding” the location of the equipment. A supervisor can join remotely, providing further advice. Moreover, activities at the bench are automatically “recognized” and recorded in LIMS: no manual transcription required. Conclusions: The COVID-19 pandemic has taken its toll on clinical and research laboratories. To maintain safety in this setting, MR can be useful to enable comprehensive interactions among remote members of a team and improve training of personnel who cannot rely on the presence of expert technicians in the lab. Future versions will employ AI for real-time assisted training and auditing, preventing any errors during workflow execution.

I28. Building a Comprehensive Teaching Repository of Whole Slide Images
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Introduction: With the introduction of whole-slide imaging (WSI), pathologists have been able to examine tissue using digital images that can be shared amongst institutions and used for a myriad of purposes including diagnostics and teaching. Medical-grade scanners generate high-definition images, usually in SVS or TIFF format, tiled in a single-file pyramidal structure, with non-standard metadata and compression. The file sizes of images generated by slide scanners are large (~1 gigabyte or larger). In addition, the generation of these files cannot be easily managed (mainly due to size) and usually require a special viewer installed on the user’s computer to inspect the files. The desire to identify a solution for easier transmission and sharing led to exploration and implementation of deep zoom image (DZI) technology. Methods: The D2I format is XML based, and allows management of a collection of images while presenting these to the end-user as a single image. Tiles in the bottom layer of the pyramid are mapped by pixel to the original image. Tiles at each level are mapped at half the resolution of the level below. Once a DZI is compiled, capturing all the relevant sections of the original files. SVS (and related) files can be converted to DZI. The generated DZIs can be viewed using OpenSeadragon, an open-source, web-based viewer for high-resolution zoomable images, implemented in pure JavaScript, for desktop and mobile (https://openseadragon.github.io/). Doing so allows traversing of DZI images with ease. Results: We have developed a pipeline that takes the SVS images generated by the whole slide scanner and converts them to DZIs. We then take the generated DZIs and add them to our instructional material framework, allowing users to view high-resolution digital pathology images from any Internet-connected web interface including mobile devices. Unlike the previous paradigm where one had to download the SVS file and then open it with a special client application installed on the user’s computer, we are able to provide a resource that users can access from any part of the world to view and interpret high-resolution images within seconds of access to the resource. Conclusions: We believe this resource will be invaluable to the education and training of technicians in the lab. Future versions will employ AI for real-time assisted training and auditing, preventing any errors during workflow execution.

Other (e.g., Education)

OTH01. Effect of Implementation of a Medium-Sized NGS Panel and Organ-Specific Subpanels on Send-out Testing: Experiences of a Small, Hospital-Based Molecular Diagnostics Lab
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Introduction: Many large next-generation sequencing (NGS) panels are available, but only a small number of genes on these panels are currently relevant for patient management. Our institution implemented several tumor-specific subpanels of 20 to 36 genes that include all genes required by published guidelines. Methods: The TrueSight Tumor 170 panel (Illumina, San Diego, CA) is our base panel for tumor-specific subpanels for testing of formalin-fixed, paraffin-embedded (FFPE) samples. It analyzes tumor DNA for single nucleotide variants and insertions/deletions in 151 genes and for amplifications of 59 genes and analyzes tumor RNA for fusions and splice variants of 55 genes. The entire panel is run irrespective of histologic diagnosis, but for interpretation and reporting, only genes of tumor-specific subpanels are analyzed. Our full NGS panel and 6 subpanels (breast, colorectal, lung, melanoma, thyroid, upper GI) were launched in November 2019. Reference laboratory send-out data for the first 3 months after implementation (December 2019 to February 2020) were compared to the corresponding months in the previous year, focusing on non-small cell lung cancer (NSCLC) and colorectal cancer (CRC). Results: After implementation of in-house NGS, single gene testing orders decreased for surgical specimens and small biopsies of NSCLC (88%, 17 versus 2) and CRC (100%, 8 versus 0); however, panels of single gene tests continued to be ordered for cell blocks from fine needle aspirates of NSCLC, a specimen type not currently validated for our assay. Interestingly, in our analysis of larger, commercially available NGS panels decreased by 79% (14 versus 3) for NSCLC but increased for CRC (38%, 8 versus 11). In the same timeframe, there was a concomitant increase in ordering of our in-house NGS solid tumor testing, with NSCLC and CRC making up 30% and 40% of all in-house NGS panels ordered. Conclusions: Implementation of an in-house targeted solid tumor testing panel and smaller, organ-specific subpanels allows for more efficient testing and can be easily shared beyond the minimal number of genes required by guidelines. Preliminary but very limited data show successful implementation of NGS testing at our institution, replacing the vast majority of our send-out, single gene testing for NSCLC and CRC on surgical and small biopsy specimens. This is likely due to manual review of all requests for biomarker testing and education of ordering physicians regarding the benefits of in-house testing for certain specimens. Remaining send-out testing for larger NGS panels may reflect a desire to test for additional, commonly utilized genes, which are largely available on these panels.

OTH02. Establishment of a Multidisciplinary Precision Medicine Lymphoma Tumor Board Incorporating Results of Massively Parallel Sequencing
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Introduction: The impact of massively parallel sequencing (MPS) on the evaluation of lymphoma patients is of significant interest in precision oncology with implications affecting diagnosis, management, and outcomes. There are currently no established practice guidelines for testing, reporting, or outcome assessment. To address this gap, we established a multidisciplinary precision medicine lymphoma conference with the following goals: to learn about the role of MPS and genomic findings using our 40-gene panel in educating medical personnel in lymphoma diagnosis; to influence management decisions; and to create a database of cases with integrated genomic, pathological, and clinical results as a resource for patient management, clinical research, and education. Methods: The conference participants include faculty and trainees from various disciplines including pathology, genomics, medicine, nursing, and genetics. A clinic educationally interesting case is selected for presentation once a month as a part of an established tumor board. A PowerPoint template was created to contain the pertinent
clinical-pathologic information, including MPS findings, highlighting their impact on patient care and learning outcomes. The cases are presented by trainees with the supervising attending, and shared using remotely accessible teleconferencing platforms. Results: MPS testing provided diagnostic refinement for entities that may be morphologically similar but have distinct genetics based on age distribution. This forum permitted accurate identification of a pediatric type follicular lymphoma (FL) from FL in a young male supported by a MAP2K1 mutation, classification of lymphoplasmaicytic neoplasms, including an assessment of the presence of mutations in MYD88 (favoring lymphoplasmaicytic lymphoma) or in NOTCH2, TNFAIP3, and KLF2, and absence of an MYD88 mutation (favoring marginal zone lymphoma). Additionally, in a large B-cell lymphoma, detection of TNFAIP3 and GNA13 mutations favored a primary mediastinal large B-cell lymphoma. The diagnosis of a nodal marginal zone lymphoma dominated by T follicular helper (Tfh) cell expansion was facilitated by the detection of NOTCH2, CREBBP, KLF2 mutations, and absence of RHOA and IDH2 mutations. Conclusions: This conference is advantageous since it promotes remote participation by physicians from the satellite offices and non-academic centers. MPS resulted in refinement of the final diagnosis in a significant number of cases, thereby impacting clinical management. Our experience indicates that this tumor board provides an opportunity to develop practice guidelines for MPS testing, promote accurate diagnoses of lymphoproliferative disorders, educate multidisciplinary audiences on precision medicine principles, and develop opportunities for research.

OTH03. Educating in a Pandemic: Rapid Changes to Molecular Genetic Pathology Graduate Medical Education Training during COVID-19

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Introduction: The COVID-19 pandemic presented a unique challenge to graduate medical education (GME) as hospital systems rapidly reduced on-site training and emphasized remote and distance learning. We describe our strategy for the implementation of new educational models and teleconference tools to enable GME Molecular Genetic Pathology (MGP) curriculum along with evaluation data from trainees in our program impacted by the coronavirus pandemic. Methods: MGP learning goals, objectives, and Accreditation Council for Graduate Medical Education (ACGME) requirements were reviewed. A strategy was developed to ensure a consistent educational experience, including direct participation in clinical duties, migration of resources and teaching to remote formats, and creating new online educational activities. The Association for Molecular Pathology (AMP) suggested curriculum for this educational landscape was updated as a template to curate online content. We also utilized remote desktop connections and teleconference technology to facilitate teaching, case review, and sign-out. MGP trainees were actively involved in these changes and feedback on their experience was solicited and analyzed. Results: With changes to the curriculum, we were able to accommodate up to a 66% reduction of on-site trainees. A new online curriculum website was created to accommodate existing educational materials, improving accessibility. Secure remote desktop access was provided to each trainee and HIPAA-compliant teleconference technology was instituted to replace much of the in-person clinical sign-out and didactic sessions. One hundred percent of trainees responded favorably to the remote learning models. Specific comments included the ability to better visualize data during sign-out with a dedicated computer screen, increased usage and comfort with data analysis software providing a more in-depth understanding of the molecular assays, and increased time for discussion of individual interesting cases. Challenges included balancing on-site exposure to laboratory workflows, which were harder to appreciate remotely. Not surprisingly, there was a learning curve associated with new technologies and rare technical malfunctions (slow internet speed, microphone issues, etc.), which improved over the rotation. Conclusions: The COVID-19 pandemic provided new challenges as well as exciting opportunities for GME education in MGP. Despite reductions in on-site training and overall case volumes, adopting new online/remote education strategies allowed for a largely uninterrupted learning experience, providing maximum flexibility and safety for trainees and faculty. Based on the positive feedback received, implemented changes will likely persist and drive future curriculum innovation.

OTH04. Economic, Operational, and Clinical Considerations in Deploying Rapid NGS for Lung Cancer

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Introduction: As treatment decisions for oncology patients increasingly rely on molecular data, the ability of laboratories to provide these data in a timely manner can directly impact patient outcomes. To have timely access to these results, oncologists rely on a number of different testing modalities, including next-generation sequencing (NGS), which can take 14 days or longer for results. Rapid NGS testing (5 days or less) can quickly provide genomic information, but the effects on patient outcomes as well as laboratory operations and economics are yet to be described. This study assesses the anticipated results of a transition from our current lung cancer testing algorithm to rapid NGS testing. Methods: Molecular and clinical treatment data on lung cancer cases received in our lab during a representative month were collected. To compare the operational and economic impacts of our standard practice with a rapid NGS workflow, a maternal cost per case was determined for each, as was the number of hours needed to complete in-house tests. Lab hours were then assigned a dollar value reflecting both wages and benefits. For the purposes of this analysis, only the technical aspects are included; professional impact has not been assessed. Results: Incremental costs per lung cancer case utilizing testing through our current workflow would be reduced by 81.8% upon implementation of a rapid NGS solution. With 550 annual lung cases, this represents an annual savings of greater than $1 million in incremental costs. The number of technical hours required per work-up is predicted to decrease from 5.51 to 1.08 h, representing an aggregate savings of 2,432 h and more than $100,000 in labor costs (1.17 FTEs). Data from a representative month demonstrated that 87% of patients had a positive NGS result, and 31% had an actionable variant. Seven of the patients with actionable variants began treatment after the molecular results, and the treatment plan changed to include actionable therapy in two patients with a prior diagnosis of lung cancer. Conclusions: Our studies indicate that transition to a rapid NGS platform would eliminate redundant testing pathways and represent a significant reduction in direct operating expenses and work-hours. This labor savings is representative of enhanced efficiency, and can either reduce expenses or create capacity for volume growth without a commensurate impact on staffing levels depending on the needs of the laboratory. From the perspective of providing therapy based on molecular results, rapid NGS could allow for earlier targeted treatment. Although the results of this analysis are specific to our laboratory, it is hoped that this approach will be beneficial to other laboratories seeking to improve efficiencies.

OTH05. Study of the Critical Role Denials, Appeals, and Patient Engagement Play in the Financial Health of Pathology Practices and Molecular Laboratories

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Introduction: This study is presented to demonstrate the influence payer policies have on revenue, with specific focus on denials and impact on patient responsibility. Methods: Authors studied the shifts in denial rates and patient payments from 2016 to 2019, across 33,090,296 unique accessions. A comparative assessment was completed on the volume of impacted claims by payer, by denial reason code. Industry averages were used as the baseline for this study. Results: Medical Necessity continues to be among the fastest-growing denials, experiencing a 44.6% increase from 2016 to 2019 in pathology, along with Prior Authorization, increasing 118.8% since 2016. In molecular, Medical Necessity denials experienced a 6.3% reduction from 2016 to 2019. Prior Authorization saw an increase of 65.6%. Further research of denial trends reveals a large disparity in denials between pathology and molecular laboratories. Molecular saw average denial rates of 17% for BCBS; 26% for Humana; 24% for Aetna; 13% for UHC; and 17% for remaining commercial insurances. Cumulatively, 7% of anatomic pathology payments were denied in 2019.
 whereas molecular experienced 15%. Across all segments of the laboratory industry, appeal success rates for Medical Necessity averaged 33.2% on the first appeal, 12.1% on the second, and 12.3% on the third. Prior Authorization averaged 16.8% on the first appeal, 13.3% on the second, and 17.1% on the third. Overall, appeal success rates averaged 24.3% for a first appeal, 15.3% for a second appeal, and 17.5% for the third appeal. The 17.1% of total revenue generated by molecular laboratories comes from copays/eductibles and 1% from self-pay. Patient responsibility for molecular laboratories decreased by 12.6% from 2016. Average collections per accession increased during this same period, from $310.01 in 2016 to $362.14 in 2019. The average patient payment decreased from $359.89 per accession to $267.54. **Conclusions:** Appeals were responsible for 3.73% of total revenue generated in 2019 for molecular laboratories. A thorough and efficient appeals process is a critical part of capital and overall market adoption strategy. Patient payments are a considerable percentage of overall revenue and require thoughtful processes to engage those patients. Reduction in revenue assigned to patient responsibility is largely offset by better payments are a considerable percentage of overall revenue and require critical part of capital and overall market adoption strategy. Patient

**Solid Tumors**

**ST01. Clinical Application of oncoMonitor: A Simple cDNA Assay for Liquid Biopsy Monitoring of Treatment and Assessment of Therapy in Colorectal and Lung Cancers**

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**Introduction:** Recent advances in liquid biopsy methodologies have opened up a wide range of applications in clinical oncology ranging from initial diagnosis, tumor profiling, and therapy selection to assessment of prognosis and monitoring of treatment. With the growing demand for liquid-biopsy testing, several parameters will become key including the cost per assay, turnaround time, and ease of use, allowing a widespread adoption of these technologies. Here we present a fair simple method for targeted liquid-biopsy cDNA testing. We employed a tumor-informed approach by initial mutation scan of the tumor tissue and subsequent monitoring of the found mutation for minimal-residual disease (MRD) and therapy monitoring. We demonstrated the approach on advanced colorectal cancer and advanced non-small cell lung cancer.

**Methods:** We employed a heteroduplex-based assay in which the wild-type and mutant forms are physically separated by denaturing capillary electrophoresis using a standard capillary genetic analyzer, which was left over from Sanger-sequencing projects. The method is fundamentally based on a singleplex PCR, which dramatically lowers the demand for amounts of input cDNA. Following amplification the fragments undergo a heteroduplex forming process (denaturing and slow realnnealing) and are then directly introduced onto the genetic analyzer. We used the methodology to process a total of 214 plasma samples from 49 patients with advanced colorectal cancer and additional 88 plasma samples from 23 patients with NSCLC.

**Results:** We demonstrated amplification and separation conditions for a panel of frequently mutated genes including APC, TP53, EGFR, KRAS, BRAF, PIK3CA, and CCND1. Our results showed that a sensitivity of about 1% minor-allele fraction (MAF) can readily be obtained. We presented a longitudinal monitoring and early recurrence detection for colorectal cancer patients after liver resection and longitudinal chemotheraphy monitoring for non-small cell cancer patients.

**Conclusions:** Our oncoMonitor technology offers a simple and sensitive tool for cDNA liquid-biopsy testing. We have demonstrated its application in early detection of recurrence of metastatic colorectal cancer and therapy monitoring in advanced non-small cell lung cancer. The main advantage of our approach is separation of wild-type and mutant fragments, which enables to reveal low fractions (~1% MAF) required for the its clinical utility. Supported by Czech Ministry of Health project 17-30748A.

**ST02. Validation and Performance of Fusion Gene Panel for MI Family Translocation Renal Cell Carcinomas: Quality of RNA Is Important for Fusion Detection**

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**Introduction:** MI family translocation renal cell carcinoma (MIT-RCC) harbors translocations involving TFE3 or TFE6 gene. Accurate diagnosis of MIT-RCC is crucial for patient management because variable clinical behavior is observed with different fusion gene partners. Currently, fluorescent in situ hybridization (FISH) using break-apart probes for TFE3 and TFE6 is considered the gold standard for diagnosis. However, FISH cannot identify fusion partners and more importantly is unreliable for rare MIT-RCC cases that have small intrachromosomal gene inversions. Therefore, alternative assays are needed. The objective of this study is to validate the performance of the Archer FusionPlex assay for detection of MIT-RCC with translocations or intrachromosomal inversions.

**Methods:** MIT-RCCs with known TFE3 and TFE6 FISH status (N = 44), SeraCare RNA and formalin-fixed, paraffin-embedded (FFPE) fusion controls with 18 known fusions were used. After histologic review, RNA was extracted from FFPE patient tissue samples using a Covaris extraction kit. Target enriched cDNA libraries were prepared with a custom designed Archer FusionPlex panel (94 target genes) and sequenced on the Illumina NextSeq 550. Quality of RNA and sequencing was evaluated using preSeq value and Fusion QC score (RNA average unique start sites per GSP2 control). Fusion transcripts of TFE3 and TFE6 were analyzed using Archer Analysis software. **Results:** Fusion QC inversely correlated with PreSeq CT (cycle of threshold) values, suggesting RNA quality is important for good sequencing quality. SeraCare FFPE control was run 9 times, showing the results were 100% in agreement (i.e., all 18 fusions were detected) when Fusion QC was ≥100 (6/9 samples). However, the concordance dropped as QC values became lower. Quality of sequencing was also important for TFE3/TFE6 fusion detection. When Fusion QC value was ≥100, concordance to FISH results was 100% (14/14). When fusion QC value was less than 100, false negatives were observed and concordance was 71% (8/11). The assay successfully identified intrachromosomal inversions in 2 cases (TFE3-RBM10 and NONO-TFE3). Dilution study demonstrated fusion transcripts were detected with variant fraction down to 5% (1.20 dilution) for both TFE3 and TFE6.

**Conclusions:** Our validation study demonstrates Archer FusionPlex assay can successfully identify TFE3 and TFE6 fusions including intrachromosomal inversions. Our study also demonstrates that quality of RNA, determined by preSeq value, correlates with Fusion QC value and assay performance. False negatives may occur when the quality is lower (Fusion QC <100), supporting the importance of pre-analytical components.

**ST03. Testing for CDKN2A Loss in Infiltrating Gliomas Using Targeted Amplicon-Based Sequencing**

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**Introduction:** The tumor suppressor gene cyclin-dependent kinase inhibitor 2A (CDKN2A) encodes two proteins p14arf and p16ink4A that regulate cell cycle progression through inhibition of CDK4 and CDK6. Higher grade gliomas frequently harbor alterations in the retinoblastoma pathway, most commonly through deletion of the CDKN2A/CDKN2B locus. This finding has significant prognostic implications and is important to report as part of routine molecular profiling, as IDH1 mutated gliomas with homozygous CDKN2A/B deletions show worse overall survival.
Detection of copy number alterations can be inferred through read-depth analysis of targeted amplicon-based next-generation sequencing (NGS) assays. Whereas focal amplifications are relatively easily identified and are often reported by commercially available software, focal deletions and copy number losses are often more challenging. In this study, we assess the ability to detect CDKN2A deletions using our in-house amplicon-based targeted NGS assay. **Methods:** Twenty-seven infiltrating gliomas submitted for in-house clinical sequencing using the Oncomine Comprehensive Assay v2 (Thermo Fisher) within the past 2 years had also been sent out for concurrent FoundationOne CDx testing. A subset also underwent whole-exome sequencing. For Oncomine, read-depth analysis is used to identify copy number, which is normalized to tumor percentage as assessed by microscopic examination. Copy number results for CDKN2A were compared to FoundationOne CDx. Receiver operating characteristic analysis was used to determine optimal thresholds. **Results:** The 27 tumors examined in this study included 19 high-grade gliomas/glioblastomas, 2 oligodendrogliomas, 1 astrocytoma, and 5 infiltrating gliomas (NOS). Tumor content ranged from 10% to 85% (mean 66%). Seventeen tumors had CDKN2A/B loss reported by FoundationOne CDx, and 10 were CDKN2A/B wild-type. Whole-exome sequencing data were also available for 7 tumors, confirming FoundationOne CDx results in all cases. In the tumors showing CDKN2A loss, the raw copy number reported by Oncomine for CDKN2A locus ranged from 0.18 to 1.35 (mean = 0.63), whereas tumors without CDKN2A loss showed raw copy numbers ranging from 1.4 to 2.32 (mean = 1.99). The upper end of the 95% confidence interval for tumor content normalized copy number ranged from 0 to 1.22 for tumors with CDKN2A/B loss; no wild-type tumors had a value below 1.52. Using thresholds of 1.35 for raw copy number and 1.22 for the upper limit of the tumor-adjusted confidence interval resulted in 100% sensitivity and 100% specificity for the detection of CDKN2A loss in these tumors. **Conclusions:** CDKN2A deletion can be reliably identified with targeted amplicon-based sequencing by utilizing read-depth based approaches for determining copy number changes.

**ST04. Long Mononucleotide Repeat Markers Improve Detection of Microsatellite Instability in Non-colorectal Cancers**


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**Introduction:** Microsatellite instability (MSI) and mismatch repair deficiency (dMMR) are biomarkers that predict response to immune checkpoint inhibition in solid tumors. The current gold standard for polytymere chain reaction (PCR)-based MSI assays is the MSI Analysis System Version 1.2 (Promega), which consists of a small panel of mononucleotide repeat markers. These V1.2 markers display subtle electropherogram patterns that are difficult to interpret, observed among select non-colorectal cancers. Long mononucleotide repeat (LMR) markers that are more prone to replication errors may improve the sensitivity of MSI assays. In this study, we compared the performance of V1.2 and LMR markers. **Methods:** We studied 24 MMR proficient (pMMR) colorectal cancer (CRC), 24 dMMR CRC, 24 pMMR endometrial cancer (EC), and 42 dMMR EC samples, where MMR status was confirmed by immunohistochemistry (IHC). We also studied 22 MSI-high (MSI-H) samples of other tissue types and 12 MSI-low (MSI-L) samples, where MSI status was detected using the MSI Analysis System Version 1.2. Each sample was tested using 2 MSI panels: 1) the MSI Analysis System Version 1.2, which consists of 5 V1.2 markers, and 2) a prototype MSI LMR System (Promega), which consists of 4 V1.2 markers and 4 LMR markers. We defined a marker as unstable if a shift of at least 2 bases or a shoulder electropherogram pattern was observed in the tumor sample when compared to the matched normal sample. We defined a sample as microsatellite stable (MSS) if no markers were unstable, MSI-L if less than 30% of the markers were unstable, and MSI-H if at least 30% of the markers were unstable. **Results:** The specificity using the MSI V1.2 and MSI LMR panels were both 100% in pMMR CRC and dMMR EC. The sensitivity using the MSI V1.2 and MSI LMR panels were 100% versus 100% in dMMR CRC and 88% versus 98% in dMMR EC, respectively.

**ST05. Comprehensive Coverage of Lung Cancer Somatic Mutations by IntelliPlex Lung Cancer Panel**

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**Introduction:** Somatic mutations of several oncogenes play crucial roles in the progression of non-small cell lung cancer (NSCLC). The path to a successful cancer management relies on understanding the genetic alternation to select the best therapeutic option for each patient. Therefore, a time-saving and comprehensive molecular profiling method is desperately needed for precision medicine to improve the chance of having a successful outcome. **Methods:** IntelliPlex Lung Cancer Panel based on CODEX technology and PlexBio’s instrument platform is an in vitro qualitative assay intended for the identification of mutations utilizing DNA and RNA samples derived from formalin-fixed, paraffin-embedded (FFPE) tumor tissues from lung cancer patients. It targets 36 DNA mutations in the KRAS, NRAS, PIK3CA, BRF, and EGFR genes as well as 19 gene rearrangements of the ALK, ROS1, RET, NTRK1, and MET genes in less than 5 hours (nucleic acid extraction not included) and allows high-throughput screening of up to 94 samples in parallel with minimal hands-on time. The performance of IntelliPlex Lung Cancer Panel was evaluated on archived FFPE specimens by comparing the results with the results obtained by using next-generation sequencing (NGS) or commercially available Real-Time based PCR assays. All tests were conducted in a hospital laboratory located in Italy. **Results:** DNA was extracted and tested from 203 FFPE specimens and the overall agreement between IntelliPlex Lung Cancer Panel and the combination methods was 89.7% (182/203). The sensitivity and specificity were 91.8% (78/85) and 88.1% (104/118), respectively. On the other hand, 204 FFPE RNA samples were analyzed and the overall concordance rate compared with NGS or RT-PCR based assays was 94.1% (192/204). In addition, the sensitivity and specificity were 78.4% (40/51) and 99.3% (152/153), separately. **Conclusions:** PlexBio’s IntelliPlex Lung Cancer Panel, a 2-reaction multiplex detection panel, is able to support precision medicine in NSCLC treatment management. This panel offers a rapid and time-effective way to provide comprehensive coverage of somatic mutation analysis of lung cancer with high sensitivity and specificity.

**ST06. Assessment of Microsatellite Instability on a Multi-Racial Cohort of High Grade Prostate Cancer Using Idylla MSI Test**

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**Introduction:** High risk prostate cancer (HRPC) patients have been shown to be associated with somatic mismatch repair pathway defect (dMMR) and rarely with Lynch syndrome. African American (AA) patients and those with PCa crinifrom morphology or intraductal component are known to have a worse outcome. Herein, we set out to determine and compare microsatellite instability (MSI) status in a cohort of AA and Caucasian patients with high grade PCa (Grade Groups 3 to 5) with crinifrom pattern and/or intraductal component. **Methods:** Radical prostatectomy specimens obtained between 2013 and 2019 in our institution were reviewed by 2 genitourinary pathologists. Five micron thick formalin-fixed, paraffin-embedded (FFPE) tissue cores with crinifrom and/or intraductal...
morphology were cut and analyzed for MSI status. MSI status was determined using the Idylia MSI test. The automated, real-time PCR-based system performs melting curve analysis on amplified DNA and evaluates 7 novel proprietary biomarkers located in the ACRV2A, BTBD7, DIO1, MRE11, RYR3, SEC21A, and SULF2 genes. A tumor is designated MSI high (MSI-H) if ≥2/7 markers are positive. Tumors with ≤1/7 positive markers are considered microsatellite stable (MSS). Ten cases had known MSI status determined by immunohistochemical (IHC) markers for PMS2, MSH1, MSH2 and MLH1. Results: A total of 60 samples from 29 AA and 31 Caucasian patients were analyzed with the Idylia MSI Test. All cases were microsatellite stable (MSS). Three AA samples had ≥17 positive biomarkers (DIO1), whereas ≤57 samples had ≤0. Within available 10 MSI cases determined by IHC there was 100% concordance with Idylia MSI Test, including 2 of 3 DIO1 positive cases. Conclusions: The Idylia MSI test is reliable and fully automated option for MSI testing. To date, this is the largest cohort reported on high grade PCs with cribriform morphology and intraductal component. Further studies correlating with IHC are warranted.

ST07. CANTRK: A Canadian Multi-Centre NTRK Gene Fusion Testing Validation in Solid Tumors Project
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Introduction: Rearrangement involving one of the neurotrophic receptor tyrosine kinase (NTRK) genes (NTRK1, NTRK2, NTRK3) may generate fusion oncogenes in tumors of multiple organ sites. Although NTRK fusion is pathogenic for some tumors, e.g., breast secretory carcinoma (BSC), secretory carcinoma of salivary gland (SCSG), and infantile fibrosarcoma, it also occurs at low prevalence in many adult cancer types, e.g., lung, colorectal, etc. For the latter tumor types, a cost-effective testing strategy may include screening by an immunohistochemistry (IHC) protocol to detect tropomyosin receptor kinase (TRK) expression, followed by confirmation of NTRK rearrangement by fluorescent in situ hybridization (FISH) or next-generation sequencing (NGS). The clinical implementation of these laboratory developed tests (LDTs) requires validation. CANTRK is the largest ring study in Canada, involving pathologists and laboratory directors from 17 centres collaborating to validate these assays across multiple tumor types. Methods: Taking into account existing IHC and NGS platforms, each laboratory selects an IHC protocol and commercially available NGS panel for validation. Phase 1 collection of up to 20 NTRK fusion-positive (NTRK+) tumor samples including non-small cell lung cancer (NSCLC) and other solid tumors (BSC, SCSG, etc.), and 2) central development of LDT protocols using the Ventana or the Abcam's Anti-Pan-Trk antibody [EPR17341]. Phase 2 includes assay validation across participating laboratories, on their respective selected protocols/platforms, using the set of NTRK+ and NTRK negative tumor samples. Results: This project is ongoing. To date, 13 NTRK+ tumors have been identified by screening existing tissue microarrays (TMAs), combined with patient samples known to co-investigators from 5 institutions. The current sample collection includes 8 SCSG, 2 NSCLC, 1 BSC, 1 atypical Spitz nevus, and 1 sarcoma. These cases demonstrated either diffuse cytoplasmic or focal nuclear staining at different intensities. NGS completed on 8 of these tumors has confirmed the presence of NTRK gene fusions; the remaining cases are pending. The results from the multi-centre validation will be presented at the meeting. Conclusions: Availability of NTRK+ samples for validation of NTRK tests is challenging. CANTRK provides a framework for biomarker assay validation projects involving rare cancers. A multi-centre collaboration is instrumental in setting up the challenges faced by individual laboratories to source sufficient positive samples for independent validation and clinical implementation.

ST08. De-stained Cytology Smears Can Be Used for Detection of KRAS Mutations Using the Biocartis Idylla PCR-Based Molecular Diagnostic Assay
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Introduction: Molecular testing in lung adenocarcinomas is a part of the standard of care in oncology. Lung cancer patients with advanced stage disease face limited specimen availability for molecular testing. Core needle biopsies (CNB) or fine needle aspiration (FNA) may be the only available specimens, and the insufficient rate of cell blocks from FNA for molecular testing is reported from 6.4% to 57%. The goal of this study is to evaluate the Biocartis Idylla KRAS Mutation Test, a fully automated real-time PCR-based molecular assay, using de-stained cytology smears of lung non-small cell carcinoma (NSCLC) cases, as an alternative source to rescue limited quantity samples. Methods: The Idylla system has been optimized and validated for use with formalin-fixed, paraffin-embedded (FFPE) tissue sections. Thirty cytology smears of NSCLC cases with previously determined KRAS mutation status by next-generation sequencing (NGS) were selected, including 29 Diff-Quik stained and 1 Papanicolaou stained. Nineteen cases harbor a mutation in exon 2; 1 case harbors an exon 3 mutation, and 10 with wild-type. All mutations in these cases were covered in the Idylia assay. The cytology smears were reviewed for cellularity and percentage of tumor cells. The smear slides were scanned and de-stained before scraping the cellular material into the cartridges. For smears with cellularity ≥25% and tumor percentage ≥50%, half of the slide is used. Otherwise, the whole smear is used. The performance was compared to prior NGS test results. Results: Half of the slide was used in 25 out of 30 cases. For the remaining 5 cases the whole slide was used, including 4 hypocellular smears and 1 smear with moderate cellularity and 20% tumor cells. The Idylia KRAS mutation assay showed 30 of 30 valid results, and all of them agreed with NGS results on concurrent tissue. Although the limit of detection on cytology smears is difficult to assess, the Idylia assay detected KRAS mutations in 29/30 (96.7%) of the 29 previously determined wild-type cases. Three AA samples from 29 AA and 31 Caucasian patients were analyzed with the Idylla KRAS cartridge. Theoretically, it implies that the limit of detection on cytology smears is 600 tumor nuclei with a heterozygous genotype (2,000 pg/7 pg x 2 = 600). Conclusions: The Idylia KRAS mutation assay on de-stained cytology smears showed 100% concordance with previous NGS results when the mutations are included in the Idylia assay. Mutation analysis using de-stained cytology smears on the Idylia system can be a valuable, fast, alternative method to molecular testing on advanced NSCLC cases, especially when the sample is not sufficient for NGS. This principle can extend and apply to other Idylia mutation assays, such as EGFR or BRAF gene mutations.

ST09. A Next-Generation Sequencing Assay for Comprehensive Genomic Profiling and Identification of Microbial Signatures in Tumor Samples
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Introduction: The use of companion diagnostics based on next-generation sequencing (NGS) technology is revolutionizing cancer research, diagnosis, and therapy. We have developed NGS assays, OncoKey SL60 Plus and OncoKey SL 525 Plus which identify all relevant
DNA and RNA variants, as well as the microbial signatures in various tumor types. **Methods:** The OncoKey SL60 Plus and OncoKey SL525 Plus assays are DNA and RNA hybridization-based capture assay targeting 60 and 525, respectively, of the most clinically relevant genes for assessment of small variants, MSI, splice variants, CNV and fusions. In addition, the assay can identify 6 oncogenic viruses, namely EBV, HCV, HBV, MCVV, KSHV, and 30 subtypes of HPV, and 4 oncogenic bacteria, namely H. pylori, S. Typhi, S. gallolyticus and C. pneumoniae. The OncoKey SL assay also uses unique molecular indexing (UMI) to enhance sensitivity and accuracy. We validated the assay over 10 runs using commercially available reference standards (Horizon H7879, Vela customized reference standard, C3 from Horizon and Seraense D710-0496), customized 10 cell lines harboring oncogenic viruses and MSI, and 30 formalin-fixed, paraffin-embedded (FFPE) clinical samples with as low as 40 ng of DNA and RNA inputs. Sequencing was done on the Illumina MiSeq and NextSeq Systems. **Results:** While running OncoKey SL60 Plus on the MiSeq System and OncoKey SL 525 Plus on the NextSeq system, up to 8 samples can be batched. From as little as 40 ng of input, a mean coverage of >200x and a coverage uniformity of >90% with 50x coverage for DNA samples and >1 million mapped read counts for RNA samples can be achieved. The turnaround time is 5 days from sample to result, including sample analysis and interpretation with Sentosa's Reporter and Vela Analytics softwares, respectively. With the assay automated on the Sentosa SX101, 16 FFPE samples can be processed at once with only 5 hours of hands-on-time. The assay sensitivity and specificity at 5% Variant Frequency are ≥96.4% for OncoKey SL 60 Plus and ≥97.9% for OncoKey SL 525 Plus Assays. The assay also successfully detected HCV RNA spike-in and other targeted viruses in cell lines (2 to 100 copies/cell). Interestingly, we were able to identify the subtype of a known HPV+ clinical sample as HPV16, a high-risk subtype of HPV known to cause cervical cancer. **Conclusions:** Robust automation coupled with a sensitive and accurate UMI-based hybridization capture workflow, we demonstrate that the OncoKey SL60 and SL525 Plus assays can rapidly identify clinically relevant DNA and RNA variants in FFPE samples, as well as the microbial signatures in various tumor types.

**Introduction:** PD-1/PD-L1 inhibitors are being explored in gastric and gastrointestinal cancer as a clinical utility for PD-L1 expression in enrichment of response to immunotherapy in gastric cancer.

**Methods:** The Qiagen AllPrep DNA/RNA Formalin-Fixed, Paraffin-Embedded (FFPE) Kit Protocol on the QIAcube instrument. DNA was sheared using the Covaris ME220 Focused-ultrasonicator. Illumina TruSight Tumor 170 libraries were prepared using the Biomek NXi (Beckman Coulter) and sequenced using the NextSeq 500 System. Following sequencing, the TST170 Local App v1.0.1 (Illumina), housed in the Clinical Genomics Workspace (CGW) (Pierian Dx), was used to perform alignment and variant calling. Variants detected from paired DNA and RNA samples were combined into a single sample output and report in CGW. Variants were classified as IA to III according to the AMP/ASCO/CAP Guidelines. Interpretation and Reporting of Sequence Variants in cancer were compared to the targeted region of the Ion Ampliseq Cancer Hotspot Panel v2 (Thermo Fisher).

**Results:** We identified 336 variants (308 unique) in 119 genes including 2 fusions in the 28 CRC specimens that were sequenced. Among these, 78 variants in 15 genes fall into the categories IA to IIB. Class IA included a total of 20 variants in 9 genes including KRAS (12 variants), NRAS (1), BRAF (3), APC (2) and MSH2 (2). All of these variants would have been detected by the 50 gene panel except the 2 variants in MSH2. Class IB included single variants in each of 3 genes (BRAF, NRAS, and ERBB2). Copy number variation is a new feature to the TST170 panel and ERBB2 amplification would not have been detected by the smaller panel. Class IIC included variants in 6 genes: APC (18 variants), KRAS (1), MLH1 (2), TP53 (13), PIK3CA (2) and a STRNANIL fusion. Twenty-three of these variants were not included in the smaller panel. Class IID included 18 variants in 8 genes: APC (4), FBXW7 (3), TP53 (4), PIK3CA (3), PTEN (1), MYC (1), NF (1), and REXO1/AXL fusion. Eight variants would not have been detected by the CGW/2 panel. The Class III category included 258 variants in 113 genes. **Conclusions:** Although many of the detected gene variants would have been identified by our previous and smaller 50 gene panel, the expanded TST170 panel detected 34 potentially clinically actionable variants including 2 fusions and copy number variants not detected by the smaller panel.

**Introduction:** Loss-of-function mutations in tumor suppressor genes BRCA1 and BRCA2 predispose to breast and ovarian cancer. Research into these genes focuses on genetic variants including single nucleotide variants (SNVs), small insertion/deletions (indels) and copy-number variations (CNVs) defined as gain or loss of larger DNA fragments (usually >1 kb). For more than a decade, the gold standard for mutational screening of BRCA1/2 has been Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA), imposing significant time and cost burden. Advances in next-generation sequencing (NGS) now allow for the reliable detection of CNVs in addition to SNVs in a single assay. In this study, we test the capability of SureSeq myPanel Custom 7 gene BRCA CNV Panel to detect CNVs and indels as well as CNVs in research samples. **Methods:** We utilised the panel and associated SureSeq NGS Library Preparation kit to profile 16 breast cancer research samples with known CNVs as confirmed by MLPA. The panel allows detection of CNVs in addition to SNVs/indels in 7 key breast and ovarian cancer genes (ATM, BRCA1, BRCA2, TP53, CHEK2, PALB2, PTEN). We assessed the CNV detection concordance by comparing NGS calls to
events reported by an orthogonal technology (MLPA). Libraries were sequenced using the 2x150 bp read length protocol on an Illumina MiSeq. All NGS data were analysed using Interpret, Oxford Gene Technology’s (OGT) complimentary variant and CNV detection software. All NGS data were analysed using Interpret, Oxford Gene Technology’s sequencing protocol on an Illumina MiSeq. The high depth of coverage (≥1,000x) and excellent uniformity were achieved for all targeted genes, enabling confident detection of CNVs of various sizes in addition to the detection of gene specific SNVs and INDELs. The detected CNVs ranged from single exon to whole gene events in BRCA1, BRCA2, ATM, and TP53 genes. Confident detection of CNVs were reported at frequencies as low as 30% tumour content and CNV calls were 100% concordant with the MLPA data. Conclusions: We have demonstrated that the SureSeq myPanel Custom 7 gene BRCA CNV Panel in combination with OGT’s Interpret software can be used to reliably detect complex genomic rearrangements ranging from single exon to whole gene events. The high data quality enabled consistent SNV and indel detection at minor allele frequency (MAF) down to 1%. Our approach allows researchers to choose a single assay for comprehensive genomic profiling of BRCA1 and BRCA2 in breast, ovarian and other cancers.

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Introduction: An increasing number of clinical laboratories are seeking to add circulating tumor DNA (ctDNA) sequencing capabilities to their test menu to provide tumor biomarker testing support for cancer patients. Yet, commercially developed liquid biopsy platforms require time and resources beyond the capabilities of most clinical and academic laboratories. Liquid biopsy assays require a sophisticated and complex data analysis pipeline to call variants at low allele frequency (AF) with high confidence, positing additional barriers to entry. Commercially available ctDNA kits with integrated data analysis pipelines are a potential solution for laboratories seeking to incorporate liquid biopsy into their test menus. In the current study, we evaluated the analytical performance of the TSO500 ctDNA kit (ResearchDx, Illumina) in combination with a single laboratory’s NGS workflow with input DNA mass, target allele frequency (AF), and minimum depth of coverage (>1,000x). Genomic data analysis pipeline to call variants at low allele frequency (AF) with high confidence, positing additional barriers to entry. Commercially available ctDNA kits with integrated data analysis pipelines are a potential solution for laboratories seeking to incorporate liquid biopsy into their test menus. In the current study, we evaluated the analytical performance of the TSO500 ctDNA kit (ResearchDx, Illumina) in combination with a single laboratory’s NGS workflow with input DNA mass, target allele frequency (AF), and minimum depth of coverage (>1,000x).

Results: Thirty-six samples from 35 patients were evaluated (34 frozen tissue, 1 formalin-fixed tissue, and 1 bone marrow specimen). The analysis revealed that the sensitivity of NGS for detecting MYCN (100%), any SCA (93%), or any hSacA (86%) was excellent and moderately high for detecting any WCA (88%) or all hSacA (74%). In contrast, the sensitivity for detecting all SCAs (37%) and all WCA (38%) was low. Discordances were mainly due to methodologic differences leading to a higher detection limit for mosaicism (30% versus 10%) as well as lower coverage and resolution of NGS versus WGA. Two cases were discordant due to suboptimal sequence quality. The single discordant hSacA case was due to decreased resolution by NGS, leading to interpretation as a WCA rather than SCA. Importantly, none of these discordances affected patients' risk classification in our clinic.

Conclusions: Our NGS panel-based CNV analysis detects neuroblastoma relevant genomic alterations with high fidelity. The NGS panel alone would be sufficient for the detection of clinically relevant CNVs and sequence variants in the majority of neuroblastoma cases, despite its higher tumor percentage requirement compared to WGA.

ST15. Validation of a Comprehensive, Targeted Next-Generation Sequencing Panel for Solid Tumors
Virginia Commonwealth University, Richmond, VA.

Introduction: Effective, efficient genomic profiling genomic alterations from limited sample. Current diagnostic testing approaches require multiple methodologies across multiple platforms. Genomic data from solid tumor profiling allow detection of actionable and targetable mutations vital to providing diagnostic and/or prognostic data. It can also offer guidance on FDA-approved therapy options and/or clinical trial enrollment eligibility. This study describes the validation of a comprehensive, targeted next-generation sequencing (NGS) assay interrogating 161 genes relevant to solid malignant neoplasms. The Oncogenic Solid Tumor v2 can detect single nucleotide variants (SNVs), copy number variations (CNVs), gene fusions, and insertion and deletions (indels). Methods: The assay utilizes the Oncomine Comprehensive Assay v3 kit (OCAv3; Thermo Fisher Scientific) which is aligned with the NCI-Molecular Analysis for Therapy Choice (NCI-MATCH) trial. Both genomic DNA and RNA are extracted from the same formalin-fixed, paraffin-embedded (FFPE) specimens and used in a consolidated workflow involving a multiplex PCR amplification using Ion AmpliSeq primers and reagents. Amplions are sequenced on the Ion S5XL Sequencer and analyzed with Torrent Suite Software (version 5.10.1) and a custom pipeline. Seventy-three unique patient
samples were included in the analysis. There were a variety of different tumor types tested, with the highest numbers in lung, breast, brain, and colon (N = 16, 8, 8, and 8, respectively). **Results:** QC metrics for clinical implementation were established using these data. The minimum values for reliable variant calls for each sample were as follows: bases mapped, DNA: 486 million; RNA: 26 million; Q20 bases, DNA: 439 million; RNA: 26 million; and total reads, DNA: 4.5 million; RNA: 287,000. For each variant call, the coverage should be ≥190X, with a variant allele frequency ≥5%. Initial runs determined that due to low quality starting material from FFPE specimens, an additional processing step was required. Uracil-DNA Glycosylase (UDG) treatment was successful, demonstrated by a decreased deamination score and the removal of low-level artifacts. These samples were decreased deamination score and the removal of low-level artifacts.

**Conclusions:** Without loss of detection of expected variants. These samples were decreased deamination score and the removal of low-level artifacts.

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**ST16. MammaPrint and BluePrint Next-Generation Sequencing (NGS) Results Are Robust and Accurate for Patients with Early Stage Breast Cancer**


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**Introduction:** MammaPrint (MP) and BluePrint (BP) are gene expression assays that are utilized in early stage breast cancer (ESBC) patients to aid treatment planning. The MP 70-gene risk of recurrence and BP 80-gene molecular subtyping signatures were developed with microarray (MA) technology and have been commercially available since 2004 and 2010, respectively. In 2018, these tests were translated to next-generation sequencing (NGS) using targeted RNA-sequencing and commercialized (with CE mark) for use outside the US. Equivalence between MA and NGS has been shown previously for MP and BP in centralized and decentralized settings. Here we present experience with the NGS platform at various global sites and report new equivalence and robustness data for this approach. We also present data for a MP NGS test that has been developed for US use. **Methods:** Close to 1,000 samples have been studied with NGS and MA technologies in parallel. In this substudy, MP and BP results from MA and the CE marked NGS test were compared for 228 paired samples. The impact of different NGS reagent lots, operators and testing facilities on the consistency of test results was evaluated. Comparisons of test indices and result categories (e.g., MP Low or High Risk, and BP molecular subtype: Basal-, Luminal-, or HER2-type were conducted). Similar comparisons of MP indices and result categories will be performed for the NGS US test. **Results:** MP and BP indices generated at Agendia central labs showed high correlation between MA and CE marked NGS results (Pearson correlation coefficient (r) of 0.96 for MP, 0.89 for Basal BP, and perfect concordance in terms of BP molecular subtypes. Across 5 different lots of kits, operators and sites, there was high concordance of results across all samples with standard deviations less than 0.06 for MP and 0.07 for BP indices. Comparing NGS MP results obtained from different external sites with the centralized MA results yielded high correlation (r of 0.96) and concordance (PPA of 99.0% and NPA of 98.5%), and perfect concordance in terms of BP molecular subtypes. Across 5 different lots of kits, operators and sites, there was high concordance of results across all samples with standard deviations less than 0.06 for MP and 0.07 for BP indices. Comparing NGS MP results obtained from different external sites with the centralized MA results yielded high correlation (r of 0.96) and concordance (PPA of 99.0% and NPA of 98.5%). Similarly, high correlations for BP molecular subtype scores (r of 0.96 for Luminal-type, 0.88 for HER2-type and 0.98 for Basal-type) were obtained and there was perfect concordance for result categories. Correlation and concordance for the US MP NGS test and the centralized MA test will also be reported. **Conclusions:** Genomic profiling has become a critical part of treatment planning for patients with ESBC. The availability of a decentralized, robust, and accurate NGS version of MP and BP supports the trend towards localized healthcare, and provides additional benefits including retention of tissue, shorter turn-around time and improved patient outcomes.

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**ST17. Evaluation of Three RNA Quantification Methods for Next-Generation Sequencing of Formalin-Fixed, Paraffin-Embedded Tumor Samples**

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**Introduction:** With a rise in using combined DNA and RNA workflow for next-generation sequencing (NGS) testing of formalin-fixed, paraffin-embedded (FFPE) samples from solid tumors, it is essential to determine if the extracted nucleic acids are quantitatively sufficient and qualitatively acceptable for testing. Although Qubit fluorometric quantification has largely become the gold standard method to determine the quantity of FFPE DNA samples, there remains lack of consensus on the best method(s) for assessing FFPE RNA quantity and quality for NGS testing. **Methods:** RNA from 25 tumors samples (23 lung adenocarcinomas and 2 secretory carcinomas of salivary gland) were extracted using the Promega Maxwell RSC Instrument, with 18 biopsy specimens, 4 resections, and 3 cell blocks used. Of the 25 samples, 9 harbored known gene fusions (5 ALK, 2 NTRK, 2 ROS1), and there was 1 MET exon 14 skipping variant. The RNA quantities of 25 FFPE tumor samples were measured in duplicate by three different methods: Qubit 3.0, Quantus and GUSB-RTP-PCR. The Oncomine Focus Assay (QF) was performed with the Ion Chef for library preparations and the Ion Torrent S5 Prime for sequencing, on the 10 RNA samples with known variants, using 10 ng of RNA measured by each quantitation method. **Results:** Whenever RNA is detectable, Qubit typically gave higher readout of quantity compared to the other 2 methods (mean 39.0 versus 20.6 versus 32.9 ng/μL; p = 0.025). Qubit and Quantus failed to detect RNA in 6 and 1 small biopsy samples, respectively. Serial dilutions of 5 RNA samples confirmed that Qubit loses its sensitivity when the concentration of RNA is less than 3 to 4 ng/μL. Interestingly, there were no significant differences in QC metrics (total reads, mean read length and a percentage of fusion to total reads) when 10 ng of RNA from each method were subjected to NGS testing. **Conclusions:** For QF, the vendor recommends using GUSB-RTP-PCR to determine amplifiable RNA quantity. Based on our results, GUSB-RTP-PCR is able to provide a wide range of RNA measurements across various samples sizes; but it comes with associated labor and reagents costs. Considering wider range of RNA measurements, Quantus is an acceptable alternative to GUSB assay. Qubit loses sensitivity when measuring RNA from small biopsy specimens; but it has an advantage of using the least amount of sample for testing when the concentration of RNA is above its threshold of measurement. Optimizations of RNA quantification are required before implementation of RNA workflow to minimize the likelihood of rejecting specimens for NGS testing.

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**ST18. An Exome- and Transcriptome-Based NeXT Dx Test Enables Therapy Selection for Cancer Patients and Offers Insight into Emerging Composite Biomarkers for Immunotherapy**


1Personalis, Inc., Rancho Santa Fe, CA; 2Personalis, Inc., Menlo Park, CA.

**Introduction:** Diagnostic biomarkers that consistently predict patient response to immunotherapies have remained elusive in spite of increased use of these treatments. There is an unmet need for the development of integrative, composite biomarkers that can model the complex biology driving response and/or resistance to immunotherapy more effectively than existing single-analyte approaches. However, many of the current cancer diagnostic panels, with their focus on a small set of genes, have limited scope and variability in tumor mutational burden (TMB) assessments for certain cancer types, and limited utility to support emerging composite biomarkers. **Methods:** To address these limitations, we developed and validated NeXT Dx, a comprehensive whole exome and transcriptome based diagnostic test designed to simultaneously characterize tumor and immune genomics from a single limited formalin-fixed, paraffin-embedded (FFPE) sample. We developed an augmented exome NGS version of MP with uniformity of coverage across all ~20,000 genes, including boosted coverage of 248 cancer related genes. TMB was calculated using gold-standard whole exome data from non-synonymous variants (SNV's and indels). We validated this assay using tumor derived...
balanced assay in multiple driver genes to identify gene fusions in a third, we supplemented the panel with a complementary expression detect novel combinations between partner and driver genes in the panel.

Panel that approximately doubles the number of specific fusion isoforms describe FusionSync detection technology that combines 3 add, by supporting the detection of novel combinations between partners and drivers, we expanded the potential breadth of fusion isoform addition, by supporting the detection of novel combinations between fusion panel to target >1,300 fusion breakpoints in 49 driver genes. In Assay Plus development, we designed an expanded Ion AmpliSeq RNA Scientific, Carlsbad, CA; 3Thermo Fisher Scientific, Ann Arbor, MI.

ST19. A Comprehensive Approach for Detection of Known and Novel Gene Fusions with RNA Sequencing A. Marcomiz1, R. Gottumukkala1, G. Bee2, J. Kitzer3, X. Duan1, V. Mittal1, E. Wong-Ho1, C. Yang1, Y. Tseng1, S. Myrand3, P. Williams3, S. Roman2, S. Sadi2, F. Hyland1

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Introduction: Chromosomal rearrangements resulting in gene fusions play an important role in oncogenesis and precision medicine. Herein, we describe FusionSync detection technology that combines 3 complementary methods for broad detection of known and novel fusions. First, we developed an expanded Oncomine targeted RNA sequencing panel that approximately doubles the number of specific fusion isoforms for common driver genes. Second, we developed informatics methods to detect novel combinations between partner and driver genes in the panel. Third, we supplemented the panel with a complementary expression imbalance assay in multiple driver genes to identify gene fusions in a partner agnostic manner. Methods: As part of Oncomine Comprehensive Assay Plus development, we designed an expanded Ion AmpliSeq RNA fusion panel to target >1,300 fusion breakpoints in 49 driver genes. In addition, by supporting the detection of novel combinations between partners and drivers, we expanded the potential breadth of fusion isoform detection approximately 10-fold. To support partner agnostic fusion detection in ALK, FGFR2, NTRK1, 2,3, and RET, we tiled exonic junctions of these driver genes to generate expression signatures. To normalize the exon-tilling expression patterns across each gene we sequenced >100 formaldehyde-fixed, paraffin-embedded (FFPE) samples across dozens of different tissues and computed an imbalance baseline for each gene. Despite the expanded features of the fusion panel, input requirements remained at 10 ng per pool (20 ng total). Gene fusion algorithms were integrated into Ion Reporter Software, with supporting data visualizations and easily interpretable reports. Results: Hundreds of positive and negative fusion samples including FFPE clinical research samples, cell lines and commercial reference standards were sequenced on the Ion GeneStudio SS sequencer. We demonstrated accurate detection of all 31 fusion isoforms and 2 intragenic variants in SeraSeq control including after dilution of the standard to 10%. Complete concordance between targeted fusion detection and imbalance assay detection methods was observed in ALK, FGFR2, NTRK1, and RET cell lines down to 5% dilution. Combined, our results demonstrate a preliminary estimated sensitivity and positive predictive value >95% across all positive samples and a cohort of negative control FFPE samples. Conclusions: We demonstrated the advantages of FusionSync detection technology, which includes a targeted multiplexed RNA-seq panel combined with novel fusion detection methods. The FusionSync approach significantly expands the scope of targeted fusion detection with novel fusion and partner agnostic detection methods.


OmnisEq, Inc., Buffalo, NY.

Introduction: Cancer-testis antigens (CTAs) have restricted expression in normal adult tissues but have been found to be overexpressed in multiple tumors. This and their ability to elicit spontaneous cellular and humoral immune responses have rendered CTAs as good candidate targets for cancer immunotherapy. CTA overexpression is variable and influenced by factors such as tumor stage, grade, treatment and detection methods. Here we report the use of a targeted RNA sequencing panel that accurately detects CTAs in solid tumors as a screening tool for cancer vaccine and cell-based immunotherapies. Methods: Studies were designed to characterize the analytical performance of an RNA sequencing assay targeting expression of 6 CTAs which have shown potential as prognostic biomarkers and immunotherapeutic targets: CTAG1B (NY-ESO-1), CTAG2 (LAGE-1A), MAGEA1, MAGEA3, MAGEA4, and SSX2. Performance variables with respect to gene-specific amplicon specificity, linearity and limits of detection were estimated with various mixing studies and input RNA levels. The effects of the tumor micro-environment (adjacent normal tissue, necrosis) on CTA expression was evaluated by including these potential interferents in the assay. Analytical precision including intra-assay, inter-assay, and inter-operator reproducibility was measured by testing replicate RNA isolates. Accuracy was determined by comparing the CTA transcripts with those from established IHC, RT-PCR and whole transcriptome assays. CTA transcript stability in formalin-fixed, paraffin-embedded (FFPE) specimens was evaluated in serial sections from blocks with routine storage compared to originating matched fresh frozen specimens. Results: RNA stability was demonstrated by high degree of CTA expression correlation between matched frozen and FFPE samples. Analytic accuracy, sensitivity and specificity was demonstrated by high correlation between CTA RNA-Seq and RT-PCR results, and IHC where available. Reproducibility results show little variation between runs and operators. The studies established a baseline threshold of ≥20 reads per million (RPM) for the lower limit of gene expression detection and is the threshold used to interpret the CTA expression as “Positive” or “Negative.” Conclusions: The analytical performance of the RNA sequencing assay for reporting CTAs has been validated for clinical use using FFPE specimens from multiple tumors. With ability to process many samples within a single run and a 10 ng RNA input each, the assay is a robust method for identifying tumors that overexpress tumor-specific CTAs as potential targets for immunotherapies, including cancer vaccination and adoptive T-cell transfer with chimeric T-cell receptors.

ST21. WITHDRAWN

ST22. FGFR Gene Mutation Analysis in Urothelial Cancer Using the therascreen FGFR RQG Assay in FFPE Specimen Type L. Cai, S. Hood, A. Zimmer, D. Wang, W. Overman, L. Kam-Morgan, A. Chenn

Laboratory Corporation of America Holdings, Research Triangle Park, NC.

Introduction: The FDA approved therascreen FGFR RQG RT-PCR Test is a real-time PCR test for the qualitative detection of defined mutations of the FGFR3 gene in DNA derived from formalin-fixed, paraffin-embedded (FFPE) tumor tissue from urothelial cancer patients. The test is indicated for use as an aid in identifying patients with urothelial cancer (UC) which harbor these alterations and are therefore eligible for treatment with BALVERSA (erdafitinib). In this study, we have evaluated the clinical and analytical performance features of the assay. Methods: RNA was isolated from the tumor specimens using the RNeasy DSP FFPE Sample Preparation Kit. Reverse transcription was performed and the mutation detection was achieved through real-time PCR analysis on the RotorGene Q MxD instrument. RNA from urothelial tumor specimens was used to evaluate accuracy, repeatability and reproducibility of the assay.

Results: Of the specimen approach tested during validation, 8 FFPE specimens with known mutations and 2 FFPE specimens without mutations in FGFR3 had results that were 100% concordant. Repeatability was 100% concordant for FFPE specimens using 6 specimens with known mutations and 4 specimens without mutations. Reproducibility was 100% concordant
for FFPE specimens using 2 specimens with known mutations and 3 specimens without mutations. The thesrascreen FGFR RQG RT-PCR Test has been offered as a clinical test at LabCorp. Of the 305 FFPE specimens tested, 72.79% were negative, 24.92% were positive. The majority of positive specimens had 1 mutation. Two specimens had double mutations. The most common mutations and their frequencies were S249C (10.82%), Y373C (4.59%), R248C (3.61%), FGFR3- TACC3v1 fusion (3.61%), and FGFR3-TACC3v3 fusion (1.31%). Results could not be obtained in 5.1% specimens due to specimen degradation (1.97%) or low RNA yield (3.69%).

Conclusions: The thesrascreen FGFR RQG RT-PCR Test is a robust, reproducible and fast assay for molecular diagnostic utilization in urothelial cancer using FFPE specimen type.

ST23. Development and Validation of the OncoScreen RNA Panel for the Detection of Gene Fusions and Splice Variants in Tumors

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Introduction: The clinical utility of next-generation sequencing (NGS)-based cancer diagnostic tools has been proved for a diverse range of genomic alterations. With continuous discovery of clinically actionable targets, the demand for comprehensive analysis of both DNA and RNA is growing. To help solve the unmet needs, we developed OncoScreen RNA, a targeted RNA-sequencing assay, to support identification of gene fusions and alternative splicing events in 105 key cancer genes. Additionally, OncoScreen RNA also offers advantages by simultaneously measuring expression levels of 218 genes including 113 tumor microenvironment genes.

Methods: In brief, DNA and RNA were simultaneously isolated from formalin-fixed, paraffin-embedded (FFPE) tissue, and used to prepare NGS libraries that were sequenced on a NovaSeq 6000 system. Data processing and variant analysis were performed with an in-house developed bioinformatic pipeline. To ensure the quantification accuracy, unique molecular identifier (UMI) was incorporated to remove PCR duplicates and the strand information was retained to help identify potential RNA contamination. The analytical performance of OncoScreen RNA was first assessed using reference materials with defined ground truth (Seracare), then confirmed by digital PCR assays (Biorad).

Results: As a result, gene fusions and splice variants were generally detectable down to 3 copies of transcript per nanogram of total RNA input. In addition, no false positives were identified across the entire panel in negative samples, conferring a 100% specificity at the variant level. The assay was then further tested with 42 positive and 28 negative clinical samples across a variety of tissue and specimen types. In total, 95% of known RNA showed a specificity of 98% (95%CI: 86% to 100%) by identifying 30/30 gene fusions and 11/12 splice variants. Only 1 false-positive fusion was reported in 28 negative samples, giving a sample-base specificity of 96% (95%CI: 86% to 100%). Excellent reproducibility was observed with complete concordance for all samples performed in replicates.

Conclusions: In conclusion, OncoScreen RNA has demonstrated its potential as a complement to our DNA-based OncoScreen Plus panel to help patients benefit from both approved and investigational targeted therapies.

ST24. Benefits of Rapid Genotyping of KRAS Mutations versus NGS in Pancreatic Cyst Fluids

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Introduction: Pancreatic cancer necessitates early intervention to improve outcomes. Genotyping of the KRAS gene is sensitive and comprehensive but requires time and cost-intensive, with a 10 to 14 day turnaround time (TAT) for the moderate failure rate. A rapid test to identify common, actionable mutations in cyst fluid samples could allow faster diagnosis and initiation of treatment. Further, an orthogonal test could rescue samples that fail NGS. Here we assess the utility of rapid KRAS genotyping on cyst fluids to complement NGS.

Methods: Cyst fluids received between 10/12/19 to 12/18/19 were subjected to both rapid KRAS genotyping and comprehensive NGS. Rapid genotyping relied on Idylla ctkRAS Mutation Assay, which enables qualitative detection of 21 mutations in codons 12, 13, 59, 61, 117, and 146. Comprehensive genotyping was performed using target enrichment via anchored multiplex PCR in combination with NGS on an Illumina NextSeq. Our NGS panel detects mutations in KRAS in addition to 90 other hotspot-containing genes. Concordance was assessed by comparing KRAS mutations uncovered by rapid testing with those identified by NGS. Results: KRAS genotyping was performed in 32 pancreatic cyst fluid samples. NGS detected KRAS mutations in 21 samples and rapid testing in 20 samples. A total of 22/32 samples (68.8%) were concordant between rapid testing and NGS. Of the discordant results, 3 samples (9.4%) had differing KRAS SNVs detected by rapid genotyping versus NGS, and 1 sample (3.1%) had a KRAS SNV detected by rapid genotyping undetected by NGS. Further, although 4 samples failed rapid testing but passed NGS, 2 samples passed rapid testing that failed NGS. In conclusion, rapid testing of pancreatic cyst fluid samples enables same-day resulting, achieving sample to result in 2.5 hours versus 10 to 14 days for NGS.

ST25. PIK3CA Gene Mutation Analysis in Breast Cancer Using the thesrascreen PIK3CA RQG Assay in FFPE Specimen Type

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Introduction: The FDA approved thesrascreen PIK3CA RQG Test is a real-time PCR test for the qualitative detection of defined mutations of the PIK3CA gene in DNA derived from formalin-fixed, paraffin-embedded (FFPE) tumor tissue from breast cancer patients. The test is intended to aid clinicians in identifying breast cancer patients who may be eligible for treatment with PIKRAY (alpelisib). In this study, we have demonstrated the clinical and analytical performance features of the assay.

Methods: Genomic DNA was isolated from the tumor specimens using the QiAamp DSP DNA FFPE Tissue Kit. The mutation detection was achieved through real-time PCR analysis on the Rotor-Gene Q MDx instrument. DNA from breast cancer specimens were used to evaluate accuracy, repeatability and reproducibility of the assay.

Results: Of the FFPE (surgical resection and core needle biopsy) specimens and cell line specimens tested during validation, 15 specimens had known mutations and 3 specimens without mutations in PIK3CA had results that were 95.6% concordant. Repeatability and reproducibility were 90% concordant for FFPE specimens using 7 specimens with known mutations and 3 specimens without mutations. The thesrascreen PIK3CA RQG RT-PCR Test has been offered as a clinical test at LabCorp. Of the 640 FFPE specimens tested, 54.53% were negative, 41.56% were positive. The majority of positive specimens had 1 mutation. Nine specimens had double mutations and 2 specimens had triple mutations. The positive detection rates in resection tissue and core needle biopsy were similar with 44.50% in resection tissue and 40.05% in core needle biopsy. The most common mutations and their frequencies were H1047R (16.56%), E545K (9.53%), E542K (6.41%), H1047L (4.06%), Q546R (1.72%), and C420R (1.09%). Results could not be obtained in 3.91% specimens due to specimen degradation and limited amount of DNA. Core needle biopsy specimens had higher failure rate (4.98%) than resection tissue (1.83%).

Conclusions: The thesrascreen PIK3CA RQG RT-PCR Test is a robust, reproducible and fast assay for molecular diagnostic utilization in breast cancer using FFPE specimen type.

ST26. Detection of Microsatellite Instability Using Anchored Multiplex PCR and Next-Generation Sequencing

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Introduction: Microsatellites are short repetitive segments of the genome which are prone to instability from errors during DNA replication. Microsatellite instability (MSI) is particularly high in tumors harboring...
deficiencies in DNA mismatch repair genes. Patients with tumors categorized as MSI-High (MSI-H), can benefit from treatment with immunotherapies. Currently, patients are most often tested for MSI status using a PCR and fragment length analysis. The benefits of a next-generation sequencing (NGS) assay include the ability to investigate many more microsatellite loci simultaneously, as well as the ability to make MSI calls in parallel with tumor somatic variant detection. We developed a targeted NGS approach using Anchored Multiplex PCR (AMP) to determine MSI status. Here, we demonstrate the ability of our prototype assay to make MSI calls which correlate with MSI status as determined by PCR. This AMP based MSI assay can be used modularly, operating either as a stand-alone panel, or as a subset of a larger panel.

Methods: Our targeted NGS assay uses AMP chemistry to amplify select microsatellite loci. We then leverage molecular barcodes incorporated during AMP library preparation to determine the number of unique microsatellite tandem repeats at 133 microsatellite loci. The diversity of tandem repeats found at each microsatellite loci is compared to the diversity observed in a cohort of microsatellite stable (MSS) samples to determine whether each loci has increased diversity. The proportion of loci determined to have increased diversity is then used to categorize a sample as MSI-H or MSS relative to an empirically determined threshold of 20%. This NGS assay was assessed across DNA extracted from 98 formalin-fixed, paraffin-embedded (FFPE) samples which tested as MSI-H and 100 samples which tested as MSS by PCR. A rotating, out-of-sample validation technique was used to cross compare the stable and sample cohorts. Results: Analysis of our prototype MSI assay using these samples indicates excellent performance. The average percentage (mean ± SD) of microsatellite loci measured as unstable was 4 ± 4 for the samples measured as MSS by PCR, using our cross-validation method. The average percentage of loci tested as unstable was 65 ± 16 for samples measured as MSI-H by PCR. Using a threshold of >20%, microsatellites were unstable for calling a sample as MSI-H and ≤52% for MSS, we were able to call MSI-H status with a 95% binomial confidence interval of 91% to 99% sensitivity and 95% to 99% specificity. Conclusions: This prototype AMP NGS-based assay demonstrates high correlation with the PCR standard reference test for MSI status.

Methods:

- **Results:** We evaluated a cohort of 113 advanced renal cell carcinoma (RCC) patients who received matched tumor tissue-whole blood MSK-IMPACT targeted sequencing as part of their clinical management. Patient matched cfDNA-buffy coat MSK-IMPACT sequencing was performed on 112. One patient lacked a matched buffy coat and cfDNA was profiled unmatched. Incorporation of this patient’s whole blood sample in analysis allowed the identification and filtering of patient SNPs. Somatic mutations and copy number alterations (SCNA) from tumor tissue and cfDNA MSK-IMPACT were compared to determine the feasibility of using cfDNA to identify somatic alterations in RCC. We used the high sensitivity MSK-ACCESS assay to profile 30 cfDNA samples that were VHL WT by MSK-IMPACT, but mutated in their corresponding tumor tissue. De novo mutation calling and genotyping of variants previously detected for a given patient were performed with established clinical thresholds. Results: A total of 630 mutations were identified across all sequenced samples. VHL (87%), PBRM1 (48%), and SETD2 (35%) were the most frequently mutated genes. Of the 630 mutations: 578 (92%) were detected in tumor tissue only, 42 (7%) were identified by both cfDNA and tumor profiling by IMPACT, and 9 (1%) in cfDNA samples only. MSK-ACCESS identified 2 VHL mutations present in both tumor tissue and cfDNA samples and 9 mutations in cfDNA samples only. Of the variants novel to cfDNA samples, 4 were determined to be likely of hematopoietic origin (CH). Although 3p loss was observed in 79% of the tumor tissue samples profiled, cfDNA failed to show any SCNA, including 3p loss, likely due to low tumor purity. Conclusions: This study highlights the challenges and complexities of using cfDNA to profile advanced renal cell carcinoma. Mutation detection in cfDNA was enhanced using prior knowledge from tumor tissue profiling and cross-genotyping methods. The use of patient-matched samples greatly increased our ability to detect and filter patient SNPs and/or CH mutations from our tumor calls and demonstrates the importance of these controls in genomic profiling. Assay sensitivity, sample purity, and the level of tumor DNA in circulation must be considered in parallel when evaluating the clinical utility of cfDNA profiling with NGS panels.
Utilization of a Targeted Next-Generation Sequencing Assay for Assessment of Tumor Cellularity, and Genomic-Wide and Gene-Specific Loss of Heterozygosity (LOH)

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Introduction: In cancer, loss of heterozygosity (LOH) is a common occurrence where a chromosomal region containing a tumor suppressor gene (TSG) is lost. An important mechanism of tumor progression involves LOH followed by a second event that inactivates the remaining copy of the TSG. In certain cancer types, homozygous recombination deficiency (HRD) leads to an increase in LOH across the genome. Because HRD is an emerging biomarker for cisplatin and for inhibitors of poly ADP ribose polymerase (PARP1), understanding LOH in tumor samples is critical to support clinical research for the future of precision medicine.

Methods: We developed a large (500+ gene) targeted next-generation sequencing (NGS) assay (the Oncomine Comprehensive Assay Plus or OCA Plus) using Ion AmpliSeq library chemistry that requires only 20 ng tumor sample DNA as input and is compatible with formaldehyde-fixed, paraffin-embedded (FFPE) samples. To support LOH determination, we included high minor allele frequency (MAF) polymorphisms (SNPs) prevalent in different ethnicities and distributed across the genome. Copy number (CN) log-ratio profiles for each amplicon were generated by normalizing tumor sequence reads with a process matched informatics control. Log-odds for allele frequencies for SNPs were generated by variant calling. Both the CN log-ratios and allele frequency log-odds were used to segment the genome into homogeneous CN regions. Tumor cellularity and major and minor CNs of the homogeneous segments were estimated. All segments with major CN >0 and minor CN = 0 were included for assessment of genome-wide and gene-specific LOH.

Results: Performance was characterized in cell lines and FFPE samples. A titration series of 4 cancer cell lines with their respective matched normal was performed. The estimated tumor cellularity of the dilutions were concordant with the known truth for tumor cellularity and major and minor CN of the genomic segments in OCA Plus can be applied to evaluate other measures of genomic instability including assessment of large-scale CN changes and arm level aneuploidy.

Discussion: Evaluation of LOH using targeted NGS will provide reproducibility and low limits of detection of the ClearSEEK Panel.

Conclusions: Our preliminary results suggest that the analysis of PIK3CA mutations on the MassARRAY System using 10 ng DNA shows good sensitivity with a limit of detection between 1% and 2%. Using an NGS validated set of human FFPE samples, data presented here suggest that the MALDI-TOF-based assay could detect mutations with minimal single nucleotide variation (SNV) and low limits of detection of the ClearSEEK Panel.

ST31. Internal Validation and Performance Characteristics Using the Oncomine Precision Assay to Detect Multiple Variant Types from Solid and Liquid Biopsy Samples

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Introduction: Next-generation sequencing (NGS) sequencing platforms and assays have been evolving rapidly over the last decade with an emphasis on increased sample throughput and variant calling accuracy. These innovations have driven per sample cost savings and reduced time lower thus making NGS a very attractive method for characterizing sequence variants from oncological samples. One such assay/platform which exemplifies this evolution is the Oncomine Precision Assay run on the Ion Torrent Genexus Integrated Sequencer (Thermo Fisher Scientific). This pan-cancer assay allows for simultaneous detection of SNV/indel, CNV, and fusion variant classes from 50 key gene targets including PIK3CA, ERBB2, PIK3CA, and ERF. This assay is compatible with both FFPE and degraded DNA. Currently, the analysis of a cohort of 60 PIK3CA-positive and -negative samples is ongoing to validate the sensitivity, specificity, reproducibility, and limit of detection of the ClearSEEK PIK3CA panel.

Conclusions:

Our preliminary results suggest that the analysis of PIK3CA mutations on the MassARRAY System using 10 ng DNA shows good sensitivity with a limit of detection between 1% and 2%. Using an NGS validated set of human FFPE samples, data presented here suggest that the MALDI-TOF-based assay could detect mutations with minimal single nucleotide variation (SNV) and low limits of detection of the ClearSEEK Panel.
VAF ≥5% (n = 28). EGFR/MET CNV gain (3+ copies) and PTEN/CDKN2A CNV loss (0 copies) was detected with ≥98% sensitivity and PPV of 100% (n = 20) in FFPE CNV controls. 

Conclusions: Overall, this study demonstrates that the Oncomine Precision Assay for the Genexus system provides an automated and sensitive NGS solution for efficient and effective mutation assessment using both FFPE tissue and plasma samples.

ST32. Somatic Variant Analysis Using a Pan-Solid Tumor Expanded Gene Panel

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Introduction: Drug approval for targeted therapeutics requiring testing for multiple genes in multiple tumor types for selection of therapy has seen an uptick in recent years. In addition, for some tumor types diagnosis, classification and risk stratification are based on molecular features. Several tumor types were routinely sent out for sequencing due to lack of coverage for specific gene alterations. We identified a growing need to expand our next-generation sequencing (NGS) approach and bring testing in-house. Specific areas of need were detection of fusions, splice site variants, copy number variants, TERT promoter mutations and BRCA1/2 for PARP inhibitor eligibility. Here we present our data from implementation of the TruSight Tumor 170 gene panel. Methods: In February 2020 we implemented an expanded sequencing panel (TruSight Tumor 170, Illumina) to replace our prior 50 gene hotspot panel. Our workflow includes automation using the Biomek NX (Beckman Coulter) for library preparation followed by sequencing on the NextSeq 500 System. FASTq files are uploaded to the Clinical Genomics Workspace (CGW) (Perian Dx) which uses the TST170 Local App v1.0.1 (Illumina) for alignment and variant calling. Variants detected from paired DNA and RNA samples are combined into a single sample output and report in CGW which is interfaced with our electronic medical record. Results: In the first 19 weeks, we tested 255 samples from 248 patients. More than twenty tumor types have been tested with greater than 10 cases each for the following tumor types: non-small cell lung cancer (102), colorectal carcinoma (30), glioblastoma (23), breast cancer (16) and prostate (16). Whereas our prior panel was able to detect only SNVs and indels, the 170 panel detects SNVs, indels and CNVs. By adding a RNA component, we have fusion coverage in 55 genes, including detection of novel fusions, and splice site variants in MET, AR and EGFR. Beyond SNVs and indels, we’ve identified 23 TERT promoter mutations, 18 CNVs (gene amplifications), 33 fusions and 11 splice variants. Our send out testing volume decreased by ~75% since implementation and we have recouped 20% of our prior NGS volume.

Conclusions: Implementation of an expanded panel was well received by our institution and resulted in testing of more tumor types, detection of variants not covered by the prior assay and decreased send out testing.

ST33. Evaluation of a Mass Spectrometry-Based PIK3CA Mutation Assay for Predictive Breast Cancer Therapeutic Decision Making

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Introduction: Alterations of PI-3 kinases (PIK3) in cancer represent a novel drug-targetable biomarker for multiple solid tumors. One such drug, alpelisib, in combination with fulvestrant, has been found to increase clinical response to treatment and prolong progression-free interval in advanced breast cancer patients, whose tumours exhibit putative gain-of-function mutations in the major PIK3 gene, PIK3CA. Fourteen different PIK3CA mutations were evaluated in the SOLAR1 clinical trial exploring the treatment of breast cancer with alpelisib/fulvestrant; C420R, E542K, E545A, E545D, E545G, E545K, E545a, Q546E, Q546R, Q546X, H1047L, H1047R, H1047X, H1047Y. Identification of these variants is necessary for the selection of patients who might benefit from alpelisib/fulvestrant therapy. We describe the development and validation of a simple, highly sensitive assay for the identification of potential alpelisib-responsive cases of primary breast cancer. Methods: A novel assay was developed for use on the Agena Bioscience MassArray mass spectrometric analysis platform. The assay was optimized using commercial standards, and then was evaluated through testing of PIK3CA status-known formalin-fixed, paraffin-embedded (FFPE) clinical specimens on our MassArray instrument by standard protocols. Results: The ClearSEEK PIK3CA Panel for the MassArray platform evaluates 20 individual sequence variants encoding targetable PIK3CA mutations. Initial clinical validation shows an analytical sensitivity and specificity of 96.3% and 100%, respectively. Titrations of controls with known variant allele fractions (VAF) exhibit a lower limit of detection of 1.0% frequency with 10 ng input DNA. DNA titration exhibited a reliable variant detection with as little as 2.5 ng of input nucleic acid to a VAF of 2.0%. This novel assay showed 100% concordance with 23 previously identified PIK3CA positive FFPE clinical specimens. Conclusions: Our results show that the ClearSEEK PIK3CA Panel is a reliable, highly sensitive test for the 14 different amino acid variants of PIK3CA shown to be correlated with outcome to alpelisib/fulvestrant in the recent SOLAR1 clinical trial. Actionable variants can be detected with a little as 2.5 ng of input DNA, with a VAF of 1% to 2%. This corresponds to an initial tumor sample cellularity as little as 2% to 4%, demonstrating the ClearSEEK assay as an exquisitely sensitive and specific assay for detecting actionable PIK3CA mutations in FFPE tissue. This new PIK3CA assay is a robust, focused, and cost-effective testing option for breast cancer treatment and clinical decision support.

ST34. Single-Cell RNA Sequencing of Childhood Medulloblastoma

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Introduction: Medulloblastoma is an aggressive tumor of childhood comprising 4 major subgroups: WNT, SHH, G3P, and G4P, each with their own clinical outcomes, CNV, somatic variants, and transcriptional and methylation profiles. Large cohorts have been used to further divide these subgroups into finer subtypes with significantly different outcomes and molecular features. However, intra-tumoral cellular heterogeneity impedes a clearer understanding of MB biology, hindering development of more effective and less toxic therapies. Single-cell RNA sequencing (scRNA-seq) allows dissection of the cell diversity inherent in tumors, resulting in transformative findings in MB. We use droplet-based scRNA-seq to analyze primary tumor samples from 158 patients. Methods: Twenty-eight childhood MB (1 WNT, 9 SHH, 7 G3P, and 11 G4P) surgical samples were dissociated into single cells and analyzed using the Chromium scRNA-seq platform (10X Genomics, Pleasanton, CA). Gene expression data were analyzed using Cell Ranger, Seurat, Harmony and SCENIC bioinformatic workflows. Immunohistochemistry and deconvolution of bulk transcriptomic data were used to further explore identified subpopulations. Results: Neoplastic cells broadly clustered according to MB subgroup. After harmonization, each subgroup contained subpopulations exhibiting proliferation, undifferentiated, and neuronally differentiated transcript profiles, corroborating other recent MB scRNA-seq studies. We also identify new subpopulations including a photoreceptor-differentiated subpopulation, predominantly found in G3P MB. Deconvolution of transcriptomic data show that neoplastic subpopulations are associated with MB subgroups and finer subtypes. For example, photoreceptor subpopulation cells are more abundant in G3P-alpha subtype and the proportion of these cells was inversely proportional to MYC-associated progenitor subpopulations. Conclusions: Our findings build on recent single cell analyses by validating the presence of major conserved subpopulations within each MB sample and chart further neoplastic heterogeneity within these major subpopulations. Further understanding of this high-resolution biology may permit appropriate diagnosis, risk stratification, and treatment.

S68
ST35. Identifying Prognostic and Predictive Gene Alterations in Metastatic Prostate Cancer
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Introduction: Molecular testing of metastatic prostate cancer is important to help identify potential therapeutic targets. In February of 2020 our laboratory implemented an expanded next-generation sequencing (NGS) panel, the TruSight Tumor 170 (Illunia). The tumor-only panel offers more broad coverage than our prior assay and is able to detect clinically significant SNVs, indels, CNVs, fusions and splice site variants. In addition, the panel offers coverage of BRCA1 and BRCA2, comparable to a prior reference assay, for identifying metastatic prostate cancer patients eligible for PARP inhibitor therapy. Methods: DNA and RNA were isolated from formalin-fixed, paraffin-embedded (FFPE) tissues with the Qiagen AllPrep DNA/RNA FFPE Kit Protocol on the QiaCube instrument. Libraries were prepared for the Illumina TruSight Tumor 170 panel using the Biomek NX (Beckman Coulter) and sequenced using the NextSeq 500 System. Data analysis was performed using the TST170 Local App v1.0.1 (Illunia), housed in the Clinical Genomics Workspace (CGW) (Pierian Dx). Variants detected from paired DNA and RNA samples were report in CGW. Variants were classified as IA to III according to the AMP/ASCO/CAP Guidelines. Results: To date 16 samples from patients with metastatic prostate cancer were tested. Nine cases had fusions involving ETS transcription factors: TMPRSS2-ERG (8) and a novel STAT3-ETV1 (1). Three of these also had androgen receptor (AR) AR-V7 alternative transcript identified. One case harbored a TMPRSS2-5KOL fusion of uncertain significance. Two cases had no clinically significant variants identified. One had a CTNNB1 exon 3 variant and 1 a PTEN and TP53 variant. One of the TMPRSS2-ERG cases showed loss of MSH2/MSH6 by immunostain and MSH3 status. Upon sequencing an MSH2 variant at 24% variant allele fraction (VAF) and an MSH2 fusion were identified, suggesting double somatic MSH2 mutations as the mechanism for MSH-H. In 1 case AR and MYC amplification were identified and an AR SNV. In addition, the sequencing data suggested homozygous deletion of BRCA2 above the resolution of our assay suggesting HRD phenotype. Subsequent SNP array confirmed the BRCA2 homozygous deletion and this patient went on to receive PARP inhibitor therapy. In the final case, biallelic CDK12 variants were detected along with AR-V7, AR-V4 and a RBM33-KMT24 fusion. Recent studies suggest that tumors with biallelic CDK12 loss have a worse prognosis but may be more responsive to immunotherapy. Conclusions: In our initial cohort, 9/16 samples showed fusions involving ETS transcription factors. Five cases showed alterations in AR (splice variants, amplification and SNV). In addition, we identified mismatch repair deficient/MSH-H, BRCA2 homozygous deletion and biallelic CDK12 loss which all may have specific treatment implications.

ST36. Validation of an NGS Panel for Pancreatic Cyst Fluid Analysis
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Introduction: Pancreatic cysts have been increasingly diagnosed due to the expanded utilization of high-resolution radiological imaging such as CT and MRI. The clinical dilemma in managing these lesions are lack of accurate tools for evaluation of dysplasia and malignant transformation of the cysts. Methods: Genomic DNA was isolated from pancreatic cyst fluid samples, obtained by endoscopic ultrasound aspiration, using sonication, tissue homogenization by motorized tissue grinder, enzyme treatment, or homopolymer region. DNA was evaluated with 140 SNVs, 44 indels, 28 CNVs, and 63 fusion and splice variants previously tested by clinically validated NGS, Array, or fluorescence in situ hybridization (FISH) assays. Results: Our validation confirmed the pre-analytical, library and run level quality metrics established by the manufacturer. Our positive predictive values for all the variants groups are >96%. Our accuracy values for SNV, and indel are 99% and are above 97% for indels and fusions. The specificity and reproducibility was >99% for all variant types. Limit of detection studies successfully identified variants at 5% variant allele fraction (VAF). The sensitivity values for SNV was 98%, and CNV and fusion were 93%. Due to fluctuations in indel VAF near the LOD, sensitivity was approximately 90%. Conclusions: The TST-170 assay allows a comprehensive somatic mutation profile both at the DNA and RNA level across different sample and solid tumor types. Special care should be taken when reviewing indels especially if a variant is near the limit of detection (5%) or in a homopolymer region.

ST38. An RNA Sequencing Panel for Detection of Fusions and Splice Site Variants in Solid Tumors
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Introduction: The discovery of BCR-ABL1 rearrangement in CML ushered in an era of gene fusions as therapeutic targets. Gene fusions have become important therapeutic, prognostic and diagnostic markers in solid tumors with a variety of primary tissue types. Although fluorescence in situ hybridization (FISH) and reverse transcriptase-PCR are effective for detecting single known fusion events, RNA next-generation sequencing (NGS) panels have enabled a single test to detect known and novel fusions within multiple known genes. With recent approvals for drugs targeting ALK, ROS1, RET, NTRK fusions, and MET exon 14 skipping alterations, an NGS approach is ideal to test all of these targets
simultaneously. Implementation of TruSight Tumor 170 (Illumina) has allowed for the detection of fusion events involving 55 genes as well as splice site variants in MET, AR and EGFR. Methods: DNA and RNA were isolated from formalin-fixed, paraffin-embedded (FFPE) tissues with the Qiagen AllPrep DNA/RNA FFPE Kit Protocol on the QIAcube instrument. TruSight Tumor 170 (Illumina) libraries were prepared using on Biomek NX (Beckman Coulter) then sequenced on the NextSeq 500 System. The TST170 Local App v1.0.1 (Illumina), housed in Clinical Genomics Workspace (Pierian Dx), was used for FASTQ alignment and variant calling. All high confidence fusions were reviewed in Integrative Genomics Viewer to determine if they were in frame and to determine directionality.

Results: In the initial 19 weeks of clinical TST170 testing 255 samples were tested. In these samples, 34 gene fusions were detected in 32 tumors. Tumor types with fusions include prostate (11), lung (9), glioblastoma (7), and melanoma (1). Of these, 16 had potential therapeutic implications involving ALK (5), FGFR1,2,3,3 (3), ROS1 (2), NTRK1,2,3 (2), EGRF (2), RAF1 (1), and ESR1 (1). Eighteen additional fusions that were considered to have no or unclear therapeutic implications were identified, most commonly in prostate cancer (TAMRPS2-ERG (6), TAMRPS2-SKL, ST52-ETV1). Splice site variants were identified in lung cancer (MET exon 14 skipping (4)).

Conclusions: The fusion panel used has identified a large number of potential therapeutic implications. The ultra-rapid method is also more comprehensive than the PCR/CE-based method. The ultra-rapid workflow relies on the Biocartis Idylla assay, which provides same-day, comprehensive genotyping for expedient genomic characterization and initiation of treatment.

Methods: Our PCR-based rapid panel assesses L858R and exon 19 deletion after nucleic acid extraction from frozen, ethanol fixed samples via single base pair extension and sizing via capillary electrophoresis (CE), respectively. Our ultra-rapid workflow relies on the Biocept idylla EGFR assay and detects EGFR G719A, G719S, G719C in exon 19; del 9, del 12, del 15, del 18, del 21, and del 26 in exon 19; 1790M, 1796I, insG, insASV9, insASV11, insSSV and insH and p.250Efs in exon 20, and L858R and L861Q in exon 21. Frozen, ethanol fixed tissue is microdissected and placed directly into the assay cartridge for testing. Results: With PCR-based rapid testing, optimal turnaround time (TAT) was 2 days from biopsy to report, which is 80% faster than the 10 ± 4 day TAT for NGS. With the ultra-rapid workflow, optimal TAT is reduced to 2.5 ± 1 hours from receipt of tissue. Using frozen, ethanol-fixed tissue instead of formalin-fixed, paraffin-embedded (FFPE) enables same-day, comprehensive nucleic acid extraction further shortens in-lab TAT over PCR-based methods. The ultra-rapid workflow is a 90% improvement over optimal TAT for NGS and direct technical manipulation of samples in our lab was reduced by >99%.

Conclusions: The ultra-rapid method is also more comprehensive than the PCR/CE-based workflow; it allows for detection of 51 individual EGFR mutations, compared to only L858R point mutations and exon-19 deletions covered by PCR/CE. Conclusions: Overall TAT for the ultra-rapid workflow was reduced by ~50% compared to PCR/CE, and in most cases, results are delivered on the same day as tumor sampling. The lack of nucleic acid extraction and single-slide sample input enables targeted profiling of even scant tumor samples. Although both PCR/CE and the ultra-rapid workflow provide drastic improvement of TAT compared to NGS-based profiling, more comprehensive coverage of actionable EGFR mutations in the ultra-rapid workflow allows for faster and more definitive treatment of NSCLC.

S70. Rapid qPCR Testing in the NGS Era Enables Same-Day Resulting of EGFR Mutant NSCLC

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Introduction: Effective testing of advanced EGFR-mutant non-small-cell lung cancers (NSCLCs) is essential, as detecting actionable mutations. Next-generation sequencing (NGS) is the standard for delineating the EGFR mutation status but can take several weeks to provide these results. Our targeted, PCR-based molecular assays specific to common EGFR mutations can reduce turn-around time (TAT), but in many cases cannot provide same-day turnaround for rapid initiation of treatment. Here, we report our ultra-rapid workflow which aims to provide same-day, comprehensive EGFR genotyping for expedient genomic characterization and initiation of treatment.

Methods: Our PCR-based rapid panel assesses L858R and exon 19 deletion after nucleic acid extraction from frozen, ethanol fixed samples via single base pair extension and sizing via capillary electrophoresis (CE), respectively. Our ultra-rapid workflow relies on the Biocept idylla EGFR assay and detects EGFR G719A, G719S, G719C in exon 19; del 9, del 12, del 15, del 18, del 21, and del 26 in exon 19; 1790M, 1796I, insG, insASV9, insASV11, insSSV and insH and p.250Efs in exon 20, and L858R and L861Q in exon 21. Frozen, ethanol fixed tissue is microdissected and placed directly into the assay cartridge for testing. Results: With PCR-based rapid testing, optimal turnaround time (TAT) was 2 days from biopsy to report, which is 80% faster than the 10 ± 4 day TAT for NGS. With the ultra-rapid workflow, optimal TAT is reduced to 2.5 ± 1 hours from receipt of tissue. Using frozen, ethanol-fixed tissue instead of formalin-fixed, paraffin-embedded (FFPE) enables same-day, comprehensive nucleic acid extraction further shortens in-lab TAT over PCR-based methods. The ultra-rapid workflow is a 90% improvement over optimal TAT for NGS and direct technical manipulation of samples in our lab was reduced by >99%.

Conclusions: The ultra-rapid method is also more comprehensive than the PCR/CE-based workflow; it allows for detection of 51 individual EGFR mutations, compared to only L858R point mutations and exon-19 deletions covered by PCR/CE. Conclusions: Overall TAT for the ultra-rapid workflow was reduced by ~50% compared to PCR/CE, and in most cases, results are delivered on the same day as tumor sampling. The lack of nucleic acid extraction and single-slide sample input enables targeted profiling of even scant tumor samples. Although both PCR/CE and the ultra-rapid workflow provide drastic improvement of TAT compared to NGS-based profiling, more comprehensive coverage of actionable EGFR mutations in the ultra-rapid workflow allows for faster and more definitive treatment of NSCLC.

ST39. Uncovering Subsets of Non-Small Cell Lung Cancer (NSCLC) Enriched in Mutations in Cytoskeletal Dynamics and DNA Repair Genes: Additive Value of Large Gene Panels for Clinical Tumor Profiling

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Introduction: Next-generation sequencing (NGS) for molecular characterization of NSCLC is now routine. Smaller NGS panels focus on enriched in mutations in cytoskeletal dynamics and DNA repair genes, indicating their multistep inactivation defines a common NSCLC subgroup.

Methods: Samples were NSCLC from paraffin-embedded tissue or cell blocks with tumor of ≥20%. The referent methods included ALK, RET, and ROS1 FISH (Abbott) and a custom multiplex PCR (Cepheid). The L-NGS panel performed on the S5 Ion Torrent Sequencer. For comparison, a NSCLC-focused 542-gene panel covered by PCR/CE. Results: Samples were compared with next-generation sequencing (NGS) performed using the ultra-rapid workflow is a 90% improvement over optimal TAT for NGS and direct technical manipulation of samples in our lab was reduced by >99%. The ultra-rapid method is also more comprehensive than the PCR/CE-based workflow; it allows for detection of 51 individual EGFR mutations, compared to only L858R point mutations and exon-19 deletions covered by PCR/CE. Conclusions: Overall TAT for the ultra-rapid workflow was reduced by ~50% compared to PCR/CE, and in most cases, results are delivered on the same day as tumor sampling. The lack of nucleic acid extraction and single-slide sample input enables targeted profiling of even scant tumor samples. Although both PCR/CE and the ultra-rapid workflow provide drastic improvement of TAT compared to NGS-based profiling, more comprehensive coverage of actionable EGFR mutations in the ultra-rapid workflow allows for faster and more definitive treatment of NSCLC.

ST41. Rapid Assessment of Microsatellite Instability across a Spectrum of Tumor Types Using the Idylla System

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Introduction: Currently, MSI-IDylla is mainly utilized for testing colorectal carcinoma. This study evaluated the performance of this assay on other types of tumors. Methods: Tumors from various sites were evaluated using the MSI-IDylla assay to determine the microsatellite stability status as microsatellite stable (MSS) or microsatellite instability-high (MSI-H). Results were compared to 2 alternative assays. All results were compared with next-generation sequencing (NGS) performed using MSK-IMPACT (MSI-Sensor). A subset of results was compared with microsatellite immunochemistry (MMR-ICH). A total of 10% tumor content was the minimum cutoff for testing. Results: A total of 447 unique tumor samples, including endometrium (173), colorectal (32), prostate (25), small bowel (19), breast (19), and other (179) were studied. The overall time from initiation of the test until reporting of the results was <3
hours. Of these, 211 were MSI-H based on IHC testing with an average of 4.47 out of 7 unstable sites (SD: 1.47). From 357 samples with concurrent IMPACT results; 337 (94.4%) showed concordant results, whereas 20 were discordant (16 MSI sensor Instable/IHC MSS, 4 MSI-sensor stable/IHC MSI-H) (Pearson's chi-square: 0.00001); an additional 94 samples were MSI-sensor indeterminate, primarily related to low tumor content (28 of these were MSI-H and 56 MSS by IIdyla); MMR IHC was available on 45 of these samples, which showed concordant results in 41 samples. Mutual agreement suggested that IHC classification for 35 of out of 39 remaining samples was correct. The median TMB and mutation count for MSI-H tumors were 36 (SD: 58.5) and 41 (SD: 68.5), respectively, whereas for MSI-H tumors the median TMB and mutation count were 5.9 (SD: 91.4) and 6 (SD: 103.6), respectively. A subset (156 samples) had concurrent MMR IHC; 144 (92.31%) were concordant (78 MMR-D/MSS and 66 MMR-P/MSS) with IIdyla results, whereas 12 samples (7.69%) were discordant (10 MMR-D/MSS and 2 MMR-P/MSS-IIdyla). A total of 4/12 discordant samples had low tumor purity (<15% tumor content). A test of equivalence showed that the 2 assays are equivalent (p-value: 0.0315, P1-P2 = -0.0513). From 83 tumor samples with tumor purity <20%, MSI-Sensor was indeterminate in 46 (55.4%), whereas IIdyla was able to determine microsatellite instability in 39 of these indeterminate cases, highlighting the higher sensitivity of the assay.

Conclusions: MSI-IIdyla assay allows for rapid identification of microsatellite instability in various tumors. We have shown that this assay is equivalent to MMR evaluation by IHC and can complement MMR IHC for the evaluation of microsatellite instability. MSI assessment by IIdyla exhibited a higher sensitivity when compared to NGS MSI sensor and provided definitive results for numerous indeterminate cases.

ST42. DNA Methylation Profiling of DNA Extracted from Archived Stained Tissue Slides for Central Nervous System Tumor Diagnostics

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Introduction: Recently developed DNA methylation-based classification of Central Nervous System (CNS) tumors established itself as a robust and reliable tool to aid in pathologic diagnosis. Methylation profile can provide definitive evidence to complement and refine morphology-based diagnostics in tumors of the brain and spinal cord. Currently, one of the restraints for application of this testing for brain and spinal cord tumors is the relatively high DNA amount requirement and limited tissue availability. Tissue slides are used for hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining during surgery, and often a few unstained slides are left for molecular testing. The stained slides can be stored in the archives for years to decades and they are the largest available source of biospecimen and potentially useful for retrospective evaluation or prospective analysis for cases with limited amount of tissue. Stained tissue slides can be imaged and the tissue from these slides can potentially be used for molecular and methylation analysis. Methods: Six normal and 4 brain tumor cases were selected. Covariates were removed with xylene immersion of H&E slides or IHC-stained slides. Following rehydration, tissue was transferred into microtubes, treated by proteinase K and reverse crosslinking. DNA purification was done on QiAqube using AllPrep DNA/RNA mini kit. Each sample was subjected to bisulfite treatment using Zymo EZ DNA Methylation kit and methylation profiling by illumina MethylationEPIC kit. For 2 cases unstained slides were later submitted from outside facility. Stained slides were processed with respect to the regular protocol. Results: DNA extracted from normal brain tissue were demonstrated varied yields but were suitable for methylation profiling. Analysis of the tumor samples revealed that of the 2 different types of stained slides tested, the H&E stained slides yielded the highest amount of DNA with good purity compared to the IHC-stained slides. The DNA from touch-prep slides produced significantly higher calibrated scores for methylation classification compared to DNA from IHC slides. In addition, for 2 cases DNA for extraction was available from stained and unstained slides. Cases demonstrated high correlation of results obtained using DNA from stained and unstained results. Overall, using DNA extracted from archived stained slides resulted in high DNA yield and methylation-based classification with high confidence scores. Conclusions: We demonstrated that DNA extracted from archived H&E and IHC-stained tissue slides can be reliably used for further downstream methylation profiling, increasing the feasibility of the brain tumor methylation classifier when tissue is limiting.

ST43. WITHDRAWN

ST44. IDH1 and IDH2 Mutations in Colorectal Cancers

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Introduction: Pathogenesis of IDH gene mutations in colon cancer is unknown. This study aimed to elucidate clinicopathological and molecular characteristics of IDH1 and IDH2 (IDH1/2) mutations in colorectal cancers (CRCs). Methods: We evaluated IDH1/2 mutations in 1,623 CRCs using a next-generation sequencing assay. Results: IDH1/2 mutations, predominantly IDH1 p.R132C, were detected in 15 (0.9%) CRCs, 5 (3.0%) of 167 BRAF p.V600E mutated CRCs, and 3 inflammatory bowel disease-associated CRCs. They were significantly associated with old age, mucinous or signet ring cell adenocarcinoma, and high grade histomorphology. Mismatch repair deficiency was observed in 1 of 9 specimens examined. Concordance of variant allele frequency between IDH1/2 mutants and other tumor drivers of CRCs indicated IDH1/2 mutations could be tumors drivers suitable for targeted therapy. Multiregional analysis revealed IDH1 mutation in both adenoma and adenocarcinoma components. Conclusions: IDH1/2 mutations in CRCs were uncommon but enriched in BRAF p.V600E mutated CRCs. Accumulation of more IDH1/2 mutated CRCs are needed to further clarify their clinicopathological features and implications for targeted therapy with IDH1/2 inhibitors or PARP inhibitors.

ST45. Detection of Renal Cell Carcinoma with TFE3 Amplification Using Archer FusionPlex RNASeq Gene Expression Data

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Introduction: MIT family translocation renal cell carcinoma (MIT-RCC) harbors translocations involving TFE3 or TFE6 gene. Recently RCC with TFE3 amplification has been identified as a unique disease entity that often lack nuclear signature of RCC, which could be associated with aggressive behaviors. Accurate diagnosis of these tumors is crucial for patient management because variable clinical behavior is observed with different fusion gene partners. Archer FusionPlex assay can be used instead of fluorescent in situ hybridization (FISH), currently considered the gold standard for diagnosis. The fusion assay has the advantage of identifying the fusion partner and small intrachromosomal gene inversions; however, it does not detect rare cases with TFE3 amplification. The objective of this study is to determine whether TFE3 expression levels can predict RCC with TFE3 amplification. Methods: MIT-RCCs with known TFE3 and TFE6 FISH status (n = 29), RCC with no TFE3/TEF6 alteration (n = 5) and tumors from other sites (n = 19) were selected. RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue samples using a Covaris extraction kit. Target enriched cDNA libraries were prepared with a custom designed Archer FusionPlex panel (84 target genes) and sequenced on the Illumina NextSeq. Gene fusions and RNA expression values of all GSP2 primers of TFE3 and TFE6 genes were captured using Archer analysis software. Average of all mRNA values of GSP2 primers for TFE6 and TFE3 was calculated and compared between different tumor types. Results: TFE3 mRNA expression and TFE6/TFE3 mRNA expression ratio were significantly higher in RCCs with TFE6 fusion (3.16 ± 0.92 and 7.84 ± 2.45, respectively) as compared to RCCs with TFE3 fusion (0.18 ± 0.05 and 2.0 ± 0.02). RCCs with no fusion (0.13 ± 0.06 and 0.21 ± 0.06) or non-renal tumors (0.20 ± 0.03 and 0.17 ± 0.02). RCCs with TFE3 amplification had significantly higher TFE3 expression levels and TFE3/TEF6 ratio (0.58 ± 0.13 and 1.03 ± 0.39) as compared to...
cases without TFEB fusion (RCC with TFE3 fusion or no fusion/amplification). There was no statistically significant difference in TFE3 mRNA levels between the groups. **Conclusions:** We demonstrated TFB/FTE3 mRNA expression ratio was significantly higher in RCCs with TFE3 amplification as compared to tumors with TFE3 fusion or no TFE3 or TFE3 alteration. The results suggest that fusion negative RCCs with high TFB/FTE3 ratio could harbor TFE3 amplification. This assay could enable accurate molecular diagnosis of all MIT-RCCs in convenient single workflow assay. Furthermore, the same principle can be applied to identify other tumors with gene amplification, such as liposarcoma with MDM2 amplification when running Archer FusionPlex sarcoma panel.

**ST46. Identification of Novel Genomic Alterations in Pineal Parenchymal Tumors**

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**Introduction:** Tumors of the pineal region constitute approximately 1% of all brain tumors, and may be classified based on their cell of origin. Pineal parenchymal tumors are believed to originate from neuroendocrine cells that secrete melatonin. These tumors display a spectrum of differentiation from pineocytoma (grade I), pineal parenchymal tumors of intermediate differentiation (PPTID, grades II and III), and pineoblastoma (grade IV).

Mutations in rb and alterations in microRNA-processing enzymes like DICER1, DROSHA, and DGCR8 have been identified in pineoblastomas. A small in-frame insertion in KBTBD4 has been found only in PPTID, and none are known in pineocytoma. **Methods:** The surgical pathology records at Dartmouth-Hitchcock Medical Center were examined for cases of pineocytoma and tumors resected from 2008 to 2018. The available clinical records, imaging studies, operative reports, pathology reports and slides were reviewed. Hematoxylin and eosin-stained or immunostained formalin-fixed, paraffin-embedded (FFPE) tissue sections were examined by light microscopy. Hybrid capture-based next-generation sequencing was performed with the illumina TruSight Tumor 170 assay. Candidate variants were analyzed manually using the ENSEMBL Variant Effect Predictor Tool. In addition, chromosome microarray analysis was performed on the pineoblastoma using the Oncoscan FFPE Assay Kit and the Chromosome Analysis Suite. **Results:** Six pineal parenchymal tumors (3 pineocytomas, 2 PPTID, and 1 pineoblastoma) with available tissue were found in the pineoblastomas. In addition, the PPTIDs (3 pineocytomas, 2 PPTID, and 1 pineoblastoma) with available tissue were identified. Unique AKT2 R170W, MET T101I and PALB2 G989E variants were found in the pineoblastomas. In addition, the PPTIDs harbored unique TFRC D184Y and RAD51B K243R alterations. We found N-myc amplification in the 2 PPTID, which has not been previously described. Novel heterozygous deletions (5p14.3p13.3; 9p21.1p13.3; N-myc amplification, TFE3 fusion, TFE3 fusion, and NPM1. **Conclusions:** Pineal parenchymal tumors are rare and can be difficult to diagnose. Therefore, elucidation of characteristic genetic alterations will be useful for neuropathologists, especially for low grade pineal parenchymal tumors. We report here the first single nucleotide variants in pineocytoma, and novel genomic alterations in PPTID and pineoblastoma.

**ST47. Assessment of NTRK Alterations and TRK Inhibitor Therapy: A Single Center Experience**

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**Introduction:** Important information about neurotrophic receptor tyrosine kinase (NTRK) genes in malignancies was obtained through identification of activating Trk fusions across >20 tumor types. Although NTRK fusion-positive solid tumors are rare (~0.32% to 1%) in adults and children, they are targetable. Increasing interest in treating patients harboring NTRK fusions is due to their significant clinical response to pan-Trk inhibitors. This retrospective single-center case series highlights NTRK alterations noted in genomic profiles of 862 total tumor samples (including repeats) from both adult (564) and pediatric (298) patients over a period of ~5 years. **Methods:** Next-generation sequencing (NGS) was performed on hybridization-captured, adapter ligation-based libraries to analyze 324 genes (DNA) including rearrangements or up to 406 genes (DNA) and RNA of 265 genes utilizing DNA and/or RNA extracted from tumor samples at a CLIA-certified, CAP-accredited laboratory. **Results:** Five pediatric samples (from 3 males and 1 female) revealed the following NTRK alterations and diagnoses: 1 case of infantile fibrosarcoma with ET6V-NTRK3 fusion wherein it is essentially pathogenic in a male 2 months of age, treated with Trk inhibitor; 1 case of glioblastoma with CHTOp-NTRK1 fusion in a 15-year-old (yo) male; 1 case of pleomorphic xanthoastrocytoma in a 5 yo male with the TPR2-NTRK1 fusion and CDKN2A/B loss at initial diagnosis, resequencing 2 yrs later showed additional TP53 G24S mutation; 1 case of anaplastic astrocytoma in a 7 yo female showing NTRK3 R731W mutation. Also noted are 2 adults: a 70 yo male with colorectal carcinoma, NTRK1 H489Y (also KRAS G13D PIK3CA Q546R APC E1521* and TP53 R196*); an 89 yo male with the ETV6-NTRK3 fusion (along with NPM1 W288fs*12, DNMT3A F732del, and IDH2 R140Q) and a diagnosis of acute myeloid leukemia (AML) with mutated NPM1. **Conclusions:** Our cohort displayed occurrence of NTRK fusions in a spectrum ranging from rare solid tumors (infantile fibrosarcoma >75% incidence) to AML (<5% incidence). Demonstration of the remarkable efficacy of larotrectinib and entrectinib in clinical trials led to their accelerated tissue-agnostic US Food and Drug Administration (FDA) approval for adult and pediatric patients with NTRK gene fusion solid tumors. Thus, it appears beneficial to screen all solid tumors without any known oncogenic driver for NTRK fusions. Whereas multiplex IGR target next-generation sequencing (NGS) reads to the microbial genome within the tumor microbiome is not fully understood, particularly as it pertains to which bacteria are associated with specific oncogenic pathways with known oncogenic variants. These included: Bacteroides species were enriched within CRC and were associated with specific oncogenic pathways within pathways known to be oncogenic to the enrichment of specific bacterial species. **Methods:** The cohort consisted of stage I-III CRC with genomic characterization made by MSK-IMPACT. Pathogenic variants were identified by capture based NGS platform. Oncogenic variants were annotated with OncotkB and only considered a pathway altering mutation if the variant is considered oncogenic. Pathway classification performed according to Sanchez-Vega, et al., Cell 2018. Microbiome analysis performed using MSK-IMPACT sequencing data files with analysis of non-human DNA reads which were mapped to NT database with blastn algorithm. Taxonomic classification performed with crosswalk from NT to NCBI taxonomy with Kronotools. Bacterial species are considered present if >2 reads detected. This threshold is determined by orthogonal validation of microbial species. Enrichment calculated by odds ratio with correction for multiple hypotheses. Statistically enriched if Bonferroni adjusted 95% CI >1. **Results:** In this analysis, several bacterial species were enriched within CRC and were associated with specific signaling pathways with known oncogenic variants. These included: Streptococcus anginosus, HIPPO signaling pathway, Bacteroides xylanisolvens and Bacteroides fragilis, NOTCH signaling pathway, and Fusobacterium nucleatum, PI3K signaling pathway. **Conclusions:** This exploratory analysis showed that bacterial species are enriched within
ST49. **NKX2-1 Gene Variants in Solid Tumors: The Spectrum and Potential Impact in Surgical Pathology Diagnosis**

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**Introduction:** NKX2-1 encodes the thyroid transcription factor 1 (TTF-1) protein. Immunohistochemistry (IHC) for TTF-1 is important for establishing tumor origin in surgical pathology specimens, especially for suspected lung adenocarcinomas. Tumors that lack TTF-1 expression are often presumed to originate outside the lung; however, lung adenocarcinoma may lose TTF-1 expression or affinity to antibodies as a result of genomic changes affecting NKX2-1. This study examines the frequency and diagnostic significance of NKX2-1 genomic variants in solid tumors tested at a clinical genomics laboratory. **Methods:** Massively parallel sequencing (MPS) data from September 2016 to May 2020 at an academic clinical genomics laboratory were reviewed retrospectively. The MPS panel identifies single nucleotide variants and small indels in 152 genes with full coverage of NKX2-1 exons and exon-intron boundaries. Variants were categorized based on their predicted effect on TTF-1 expression or TTF-1 antibody binding (SPT24 clone, Leica). Concurrent surgical pathology reports were reviewed for the final diagnosis, IHC results, and clinico-pathologic data. **Results:** Sixty-three NKX2-1 variants were identified in 56 of 4,413 (1.3%) solid tumor cases sequenced. Thirty-three cases had concurrent TTF-1 IHC results, 18 of which were negative (54.5%, 18/33). Correlating with pathology, 6 of the TTF-1 IHC negative results could be attributed to non-carcinoma diagnosis, mucinous histology, or carcinoma of non-pulmonary origin. In the remaining twelve, pulmonary origin was suspected based on clinico-radiological findings and were enriched for NKX2-1 variants predicted to impact TTF-1 antibody binding (10/12). Variants comprised 7 frameshift variants, 2 nonsense variants, and 1 missense variant predicted to affect the putative antibody-targeted region (ATR). Ninety-three percent (14/15) of TTF-1 IHC positive cases were definitely classified as primary lung carcinoma in the pathology report. Interestingly, the majority of variants (10/15) were not predicted to impact TTF-1 antibody binding and clustered outside of the ATR (13/15), in contrast to the pattern observed in the TTF-1 IHC negative cases. **Conclusions:** NKX2-1 variants were observed in approximately 1% of solid tumor cases and were not restricted to lung tumors. Variants in TTF-1 IHC positive cases clustered outside of the ATR. Eighty-nine percent (16/18) of TTF-1 IHC negative cases were either from non-pulmonary origins or contained NKX2-1 variants predicted to impact the TTF-1 IHC results, a potential mechanism for the IHC negativity in these cases. NKX2-1 variants may help contextualize negative IHC results in cases where clinico-pathologic data strongly support a lung primary.

ST50. **Comprehensive Genomic Profiling of Different Subsets of Merkel Cell Carcinoma: Insights on Pathogenetic Pathways**

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**Introduction:** Merkel cell carcinoma (MCC) is a rare aggressive cutaneous neuroendocrine (NE) carcinoma. It can arise from incorporation of Merkel cell polyomavirus (MCPyV) DNA into the genome of a host cell or from ultraviolet light-induced genetic damage. Tumors in the latter group include those with a "pure" small cell NE phenotype and those with "combined" NE and other morphological elements, most often foci of squamous cell carcinoma (SCC). We performed comprehensive genomic profiling of MCPyV+ and MCPyV- (pure and combined) tumors to better understand the mutational profiles of the different subsets and to shed light on their pathogenesis. **Methods:** The study protocol was approved by the hospital research ethics board. The cohort was comprised of 51 MCCs, consisting of 21 MCPyV+ tumors, 13 MCPyV- pure tumors, and 17 MCPyV- combined tumors which contained invasive or in situ SCC. Isolation and separate sequencing of the SCC component was attempted in 12 cases. All samples underwent comprehensive genomic profiling using the Illumina TSO500 DNA next-generation sequencing (NGS) panel. The panel interrogates all exons of 523 cancer-related genes, identifying small mutations in all genes, copy number gains in a subset of oncogenes, and global parameters of tumor mutation burden (TMB) and microsatellite instability (MSI). Data were processed using an in-house bioinformatics analysis pipeline, which included the CNVkit algorithm to provide copy number gains and losses for all 523 genes. Results: After eliminating samples failing to meet the minimum threshold for median read depth (>100x unique reads), 35 tumors remained (14 MCPyV+, 8 pure MCPyV-, 13 combined MCPyV-). The SCC component was successfully sequenced in 5 combined tumors. TMB was lower in MCPyV+ tumors than in MCPyV- ones (mean 1.66 versus 29.9/Mb, p <0.01). In combined tumors, no significant difference was observed in TMB between the NE and SCC components (41.6 versus 39.9 Mb, p = 0.90). MCPyV+ tumors featured frequent mutations in TP53 (95%), RB1 (81%), and NOTCH family genes (90%). Mutations in MCPyV+ tumors were not consistently identified in any specific genes. The frequency of overlapping mutations in the NE and SCC components of combined tumors, ranged from 3.3% (S515) to 99% (S812). None of the tumors were MSI-high. **Conclusions:** Our results support existing evidence that MCPyV+ and MCPyV- MCCs are fundamentally distinct entities with different mutational profiles. Combined tumors with genetically related SCC and NE elements may arise via high grade transformation of the former, or due to a shared tumor stem cell precursor. The consistently recurrent mutations common to both MCPyV- subsets suggest that these pure and combined tumors arise via similar pathways, albeit with ultimate dominant expression of the NE phenotype in the former.

ST51. **Correlation between MMR IHC and MSI Testing for Detection of MSI-High Solid Tumors**

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**Introduction:** MSI (microsatellite instability) is a predictive biomarker of response to immunotherapy irrespective of tumor site. Either immunohistochemistry (IHC) of MMR (mismatch repair) proteins or MSI testing by PCR are acceptable methods for detection of MMR deficient (dMMR/MSI-High (MSI-H)) tumors which may benefit from checkpoint inhibitors. IHC is inexpensive and widely available but prone to technical and interobserver variability; whereas, MSI testing is highly reproducible but more labor intensive with the need to enrich for tumor cells. Here, we evaluate the correlation between IHC and MSI testing results across a wide range of cancers in clinical practice. **Methods:** Following discussion with oncology, both MMR and MSI testing was instituted on all invasive gastrointestinal tumors and all metastatic tumors, regardless of type. A total of 613 cases were tested over a period of 1.5 years. Cases were reviewed to determine the concordance rate between these methods. Discordant cases were re-reviewed by 2 pathologists, blindly and independently. **Results:** IHC and MSI results were concordant in 585 (95.0%), discordant in 17 (2.8%), and inconclusive in 13 (2.1%) cases due to suboptimal IHC. The IHC was available for review in 15/17 discordant cases. Review of 14 (82.4%) of dMMR/MSI-H discrepant cases highlighted false dMMR interpretations due to heterogeneity in staining in all of these cases. Three (17.6%) cases were pMMR/MSI-H discrepant. Review of 2 available cases showed 1 case of false-pMMR interpretation (on slide review). lymphocytes within the tumor were positive, whereas tumor nuclei were negative) and 1 true discordance case (patient with recurrent colon adenocarcinoma with several specimens showing true-pMMR and MSI-H (5/5 markers). Records showed a family history of colon cancer and prophylactic hysterectomy in several family members, strongly suggestive of Lynch syndrome; however, further genetic testing was not performed. **Conclusions:** Concordance between MMR-IHC and MSI testing is high.
and both IHC and MSI testing are independently reliable and sensitive for detection of MSI-H tumors. IHC quality and tumor staining heterogeneity may lead to inconsistencies in interpretation that account for the majority of discrepant cases. Tumors with marked decrease or focal patchy IHC staining should be designated "inconclusive" with additional MSI testing performed.

ST52. Circulating Tumor DNA Genomic and Methylation Profiling in Advanced Non-small Cell Lung Cancer Patients
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Introduction: Circulating tumor DNA (ctDNA), which harbors tumor-specific genetic and epigenomic information, can be applied for early tumor screening. Increasing evidence supports that DNA methylation is the most promising marker for the early detection of cancer due to its high sensitivity, stability and being easily detected qualitatively and quantitatively. Methods: In total, 45 advanced non-small lung cancer (NSCLC) patients (8 stage IIIB and 37 stage IV) were enrolled in our study. Parallel captured-based targeted sequencing for somatic mutation profiling, which consisting of 520 cancer related genes, was performed with plasma samples. DNA methylation detection, covering 100,100 CpG sites, was performed on the same samples. Results: Somatic mutations were detected in 36 patients (80%, 36/45), though the aberrant methylation signals were detected in 41 patients (91.1%, 41/45). ctDNA methylation signals showed higher sensitivity than somatic mutation, however, it was not statistically significant (P = 0.229). Classic NSCLC oncogenic genes (including EGFR, ALK, BRAF, ERBB2, KRAS, MET, RET, and ROS) were merely detected in 29 patients (64.4%, 30/47), which appeared significantly lower than methylation signals (P = 0.002). A positive correlation between maximum allele fraction of detectable mutations and methylation signals (P = 0.006). Three patients were found negative in both somatic mutation and methylation profiling, which might be due to a rare ctDNA release in plasma. What’s more, elderly, smoking patients showed a trend toward higher methylation detection rate than young, never smoking patients, however, the difference did not reach statistical significance. Conclusions: ctDNA methylation can be used as a diagnosis biomarker in NSCLC patients. More importantly, methylation exhibits a higher sensitivity than detectable somatic mutation in plasma. However, a further study focus on early-stage NSCLC patients is needed to validate the application of cancer early detection.

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Introduction: Fluorescence in situ hybridization (FISH) is a molecular-genetic technique used to evaluate chromosomal abnormalities including gene amplification and rearrangements. In contrast to next-generation sequencing (NGS), FISH can be perceived as "outdated," even though payors continue to regard some procedures as "investigational." In an effort to demonstrate enduring clinical utility, we reviewed utilization in our setting to demonstrate the clinical utility of FISH testing in conjunction with NGS testing. Methods: Clinically reported FISH results from 2013 to 2020 were classified into 4 categories: diagnostic (1p/19q, EWSR1, SYT, CHOP/DDIT3, FKHR); prognostic (PIK3CA, MYC, BCL2, BCL6); predictive (MET, EGFR, HER2, FGFR1, ALK, ROS1, PDGFRα, RET, KRAS, CDKN2A); and "orthogonal" for use as NGS confirmation (FGFR3, CDKN2A, BRAF, FGFR2); HER2 testing in breast cancer was excluded. Failure and failure rates were defined as cases having insufficient material for testing and division by total, respectively. Results were classified as abnormal, borderline/equivocal, or normal. Results: The 14,327 FISH cases had an overall failure rate of 1.76%, compared with an overall failure rate of 4.21% (1,260/29,927) for NGS. Categorical breakdown of FISH tests revealed that 5.6% were diagnostic, 9.6% were prognostic, 84.4% were predictive, and 0.4% were orthogonal tests. The most common FISH tests done in our institution were MET and EGFR amplification. Among reportable FISH cases, we found: overall abnormal rate was 15.9%, borderline/equivocal rate was 1.0%, and normal rate was 83.1%. For diagnostic tests, the abnormal/borderline/equivocal/normal rate was 42.9%/0.8%-56.3%, whereas prognostic tests 22.4%-10.0%/76.5%, predictive tests 13.2%-1.1%/85.7%, and orthogonal tests 37.5%/1.8%/60.7%. Although the number of FISH tests performed yearly was inversely correlated with NGS, declining by 2.3-fold from 2013 to 2019, the directly actionable result rates identified by FISH increased over time by 2.1-fold from 2013 to 2019. Conclusions: In our practice, we applied >90.0% of FISH testing for diagnostic or predictive purposes. Despite reduction in overall FISH test volume due to NGS testing, the fraction of actionable results increased over time. The overall frequency of 13.2% actionable results in the predictive category highlights that FISH remains a cost-effective diagnostic tool.

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Introduction: Genomic profiling has been increasingly used in oncology to identify prognostic/predictive markers and to enroll patients into genotype-matched clinical trials. Previous study showed that genomic profiling in metastatic breast cancer patients affected clinical decision making in 9% of the patients. In light of recent approval of targeted therapy and more understanding of therapy resistance, we evaluated the impact of commercial tissue-based genomic profiling on breast cancer clinical decision making at our institute. Methods: Invasive or metastatic breast cancer cases diagnosed between January 2014 and December 2019 with a genomic profiling test performed were identified in the pathology database of our tertiary medical center. Per practice of medical oncology, 17.2% of patients received testing for diagnostic or predictive purposes. At least 3 common sites include liver (31%), breast (23%), and lymph node (15%). Sequencing was successfully performed in 140 (93%) specimens, including most of EDTA-decalcified bone biopsies (14/15, 93%). Actionable targets including germline alterations predictive to FDA-approved therapeutic were observed in 10 cases (6.4% of 159, 5% of 160). Clinical impact was observed in only 12% of the cases; however, a further study focus on early-stage NSCLC patients is needed to validate the application of cancer early detection.
The Journal of Molecular Diagnostics

ST55. Comprehensive Genomic Profiling in Patients with Advanced Cancer in a Large US Healthcare System
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Introduction: The rapid evolution of precision medicine treatment options in cancer care has warranted broad molecular characterization of solid tumors. We are assessing the impact of implementing comprehensive genomic profiling (CGP) on a broad scale in a large community-based healthcare system on the management and outcomes of oncology patients. The present study describes genomic testing results and actionableability in a large real-world cohort.

Methods: Patients diagnosed with advanced cancer were tested for genomic aberrations using small panel tests (2015 to 2017) or CGP tests (2018 to 2020) across multiple sites in the Providence St. Joseph Health system. Mutation data as well as deidentified electronic medical record data were abstracted for use in this study. Biomarker actionability was assessed based on current FDA and NCCN guidelines, OncoKB, clinicaltrials.gov and natural language processing (NLP)-based large-scale curation of investigational targets from the literature. Results: This study included 4,244 genomics tests on 3,821 advanced cancer patients tested with CGP (n = 2,724) or small panel tests (n = 1,520). The median age of patients was 66 years; more than half of the patients were female (53%) and white (73%). The most frequently tested tumor type was lung (32%), followed by colorectal (13%), and melanoma (6%). More than half of the tumor specimens (51%) tested with CGP and 27% of specimens tested with small panels harbored a biomarker associated with an FDA-approved or standard-of-care therapy (NCCN-recommended, OncoKB levels 1 or 2, AMP/CAP/ASCO levels A or B). In addition, 72% of CGP-tested and 30% of small-panel-tested tumors had 1 or more biomarkers associated with clinical trial eligibility. NLP-based literature curation also revealed 64% of tumors tested by CGP and 52% by small panels had a biomarker associated with an investigational therapy. The number of variants of unknown significance (VUS) was 5 per report for CGP and 1 per report via small panel testing. CGP also assessed 19 different signatures associated with tumor type-agnostic therapies. 2.5% of patients had high microsatellite instability and 10.4% exhibited a high tumor mutational burden (≥10 mut/Mb).

Conclusions: The present study demonstrates that CGP testing increases the proportion of patients with potentially actionable biomarkers across tumor types. In ongoing work, we are investigating the impact of CGP on biomarker-informed treatment decisions and patient outcomes.

ST56. Development of Quality Control Reference Materials for Microsatellite Instability (MSI) Testing
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Introduction: Microsatellite Instability (MSI) occurs in cancer cells when there is deficient DNA mismatch repair. Historically, MSI has been used to screen for Lynch syndrome, but has renewed importance as a biomarker for immunotherapeutic response, making MSI testing critical for immunoncology. Five mononucleotide repeat sequences are frequently monitored for instability using PCR and capillary electrophoresis fragment length analysis. Increasingly, next-generation sequencing (NGS)-based MSI testing is offered because these assays can evaluate thousands of MSI loci, rather than being limited to 5 loci. Regardless of the testing method, clinical testing for MSI has challenges, and assay design, validation and monitoring requires stable, reproducibly manufactured reference samples. SeraCare has recently developed MSI reference materials to support precise measurements of MSI in cancer patient tests.

Methods: An MSI-High reference material was constructed from extracted genomic DNA from an MSI-high cell line and characterized by a targeted NGS panel as well as by the Promega MSI Analysis System, v1.2. Two plasmid-based MSI controls containing biosynthetic constructs with shortened repeat lengths of BAT-25, BAT-26, NR-21, NR-24 and MONO-27 were constructed and blended with normal genomic DNA from GM24385 reference human cell line to AF5% and AF20%. Blending is guided by digital PCR so that the allelic frequency for each altered MSI marker is precisely 5% or 20%. QC testing was performed using the BioRad ddPCR Microsatellite Instability (MSI) Assay as well as the Promega MSI Analysis System, v1.2.

Results: NGS analysis data for the gDNA MSI-High cell line reference material were compared to measurements from known MSI-High (MSI-H) and MSI-Stable (MSS) cell lines to verify MSI-high status, and confirmed by the Promega MSI Analysis v1.2 assay. The plasmid-based MSI controls at AF5% and AF20% blends showed detection of all 5 MSI markers detected in the ddPCR-based MSI Assay (Bio-Rad), and the measured AFs were concordant to the target AFs. MSI measurements by qPCR and fragment analysis detected all 5 MSI markers at AF20%, but not at 5% AF, presumably because this AF value was below the limit of detection for these assays.

Conclusions: We have developed new MSI reference materials from human cell lines (gDNA MSI-H) as well as biosynthetic constructs (gDNA MSI Panel AF5% and AF20%) to aid in the development, validation and monitoring of NGS-based MSI assays. These MSI reference materials also help benchmark NGS assay performance, as well as support MSI assay development and monitoring test performance.

ST57. Mutated Allele Frequency and NRAS Mutational Status Are Significantly Associated with High-Risk Prognosis by 31-Gene Expression Profile
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Introduction: Mutations in BRAF, NRAS, and KIT are frequently seen in cutaneous melanoma (CM). Knowing the mutational profile of a tumor through next-generation sequencing (NGS) can provide patients and their healthcare providers with targeted treatment options for patients with specific mutations. A 31-gene expression profile (GEP) has been validated as a prognostic tool for 5-year metastatic risk. The test classifies patients as low (Class 1A), intermediate (1B and 2A), and high (2B) risk. Our objective was to determine if BRAF, NRAS, or KIT mutation status and allele frequency are associated with prognostic 31-GEP test results for CM. Methods: DNA and RNA were extracted from formalin-fixed paraffin-embedded (FFPE) primary CM tumors for targeted sequencing and gene expression profiling. The 31-GEP assay was performed in a CAP-accredited/CLIA-certified laboratory using a high-throughput RT-qPCR assay as previously described. An NGS panel including BRAF, NRAS, and KIT was performed on an Ion GeneStudio SS Prime. TMP (TorrentSuite) software was used to align reads to the reference human sequence (GRCh37), whereas Ion Reporter (Thermo Fisher) software was used to detect and annotate variants. A minimum of 200,000 mapped reads, 50× mean depth, 85% on target, and 80% uniformity were required for clinical reporting. For confirmation of each reportable mutation with an allele frequency greater than 5%, Sanger sequencing is required for the first ten instances. Allele frequency was normalized to tumor percentage in sample. Results: Similar to previously reported mutation rates for CM, 46.1% (407/883) of cases had mutations in BRAF, 18.9% (167/883) in NRAS, and 4.4% (39/883) in KIT. A total of 75.3% (113/150) of Class 2B cases had a mutation of any type compared to 72.6% (122/168) of Class 1B/2A and 64.8% (366/565) of Class 1A cases. Although BRAF/Mutation status did not correlate with 31-GEP results, KIT and NRAS mutations were significantly associated with GEP results (p = 0.026 and 0.001, respectively). More so, NRAS mutations were significantly associated with high-risk 31-GEP results (Class 2B versus 1A, padj = 0.012). Additionally, overall mutant allele frequency of BRAF, NRAS, and KIT was positively correlated with GEP results, with higher mutant allele frequencies being significantly associated with Class 2B, the highest-risk GEP results (0.4 versus 0.1 Class 1A; P <0.001).

Conclusions: Although mutational status has not been shown to impact prognosis, our results suggest that overall mutant allele frequency and NRAS mutations have a significant, positive correlation with 31-GEP Class 2B test results. Further studies are needed to assess whether combination of GEP and mutational
status can improve prognostic stratification and/or selection of targeted therapy.

ST58. Genomic Profiling Uncovers Mutation Signatures That Differentiate Pediatric Rhabdomyosarcoma (RMS) Subgroups and Predict Clinical Outcomes

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Introduction: RMS is the most common soft-tissue sarcoma in childhood, and is a heterogeneous disease with variable clinical outcomes. We assessed the clinical utility of genomic profiling in informing diagnosis and prognosis of RMS.

Methods: Fifty primary RMS tumors from 50 pediatric patients were subjected to next-generation sequencing testing using the CHOP comprehensive solid tumor panel. The panel interrogates 238 cancer genes for single nucleotide variants (SNVs), indels, and copy number variations, and 110 fusion gene partners for more than 600 fusions. Variants were classified according to the AMP/ASCO/CAP guidelines. Genomic changes of each subgroup were summarized, and their impact on overall (OS) and event-free survival (EFS) were evaluated by Kaplan-Meier curve with logrank test.

Results: The cohort consists of 11 alveolar RMS (ARMS), 30 embryonal RMS (ERMS), 5 spindle/sclerosing RMS (SRMS), and 4 unspecified RMS (NOS) patients. All ARMS tumors were positive for either PAX3-FOXO1 or PAX7-FOXO1 fusion. Whereas SNVs were rare in ARMS, we observed amplification of MYCN in 3 tumors, gain of MYCN in 2 tumors, and gain of ALK or GNAS each in 3 tumors. ARMS patients with gain/amplification of MYCN had significantly worse OS (p = 0.01) and EFS (p = 0.02), and those with gain of GNAS had worse OS (p = 0.01). Loss of heterozygosity of 11p15 was found in 17 ERMS patients, and co-amplification of FGFR1 and MYC in 11, but neither alteration affected OS or EFS (p > 0.05). Clinically significant mutations of Dicer1 (8), NF1 (8), TP53 (6), BCOR (5), FBXW7 (3), ARID1A (3) or CTNNB1 (3) were frequently found in ERMS. Dicer1 and CTNNB1 mutations were only found in non-anaplastic ERMS. Although NF1 mutations were dominantly (7 out of 8) seen in non-anaplastic ERMS, deletions of NF1 were often observed in anaplastic ERMS. Mutations and deletions of TP53 were largely associated with anaplastic ERMS. Ten ERMS with impaired cell cycle/TP53 pathway (TP53, CDKN2A/2B, MDM2/1, MDM4, CDK4/1) showed significantly worse OS (p < 0.01) and EFS (p < 0.001); 4 with biallelic NF1 alterations and 5 with defects in WNT pathway (CTNNB1/3, CDC73/2) showed worse OS (p < 0.03 and p < 0.01, respectively). Three SRMS harbored a MYOD1 p.L122R mutation, and the other 2 had an infantile SRMS-associated fusion, FUS-ETOR2 or VGLL2-CTD2. Germline pathogenic variants in TP53, Dicer1, NF1 or PTTCH1 were confirmed in 5 ERMS and 1 NOS patients, and were associated with worse OS (p = 0.0001) in ERMS.

Conclusions: Gain of MYCN or GNAS in ARMS, impaired cell cycle/TP53 pathways, biallelic loss of NF1, and WNT pathway defects in ERMS, as well as germline mutations are associated with poor survival in patients with RMS. Our data show that genomic alterations should be evaluated for incorporation into risk classifiers for pediatric RMS.

ST59. Detection of Actionable Alterations in Breast and Ovarian Tumor Tissues by Testing with a 50-gene NGS Panel

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Introduction: Breast and ovarian cancers are the most common invasive cancers among women worldwide. Staining patterns are used to guide treatment options. Next-generation sequencing (NGS) is the preferred method to enable simultaneous characterization of multiple relevant genes in cancer specimens. However, NGS testing in clinical settings remains limited. Here, we report on the clinical utility of a 50-gene NGS panel used for detecting actionable genetic alterations in breast and ovarian tissues submitted for testing at a national reference laboratory.

Methods: We retrospectively analyzed de-identified results from 238 consecutive formalin-fixed, paraffin-embedded (FFPE) tissues submitted for testing with a 50-gene NGS panel. This assay uses targeted exon capture and NGS to detect single nucleotide variants (SNVs), insertion/deletions (indels), copy number variants (CNVs), tumor mutation burden (TMB), and microsatellite instability (MSI) in 50 of the most commonly altered genes in solid tumors. Specimens were from patients with breast cancer (n = 123, median age 54), ovarian cancer (n = 115, median age 63), or both (n = 1, age 48). Clinical annotation was performed by IBM Watson for Genomics, version 44-225. Results: In total, 302 and 188 pathogenic (P) or likely pathogenic (LP) variants were identified in 91.5% (114/124) of breast cancer and 95.7% (111/116) of ovarian cancer tissues (median = 2 and 1 to 5 [median = 1]) P/L variants per positive patient, respectively.

Most frequently altered genes were TP53 (56% of patients), PIK3CA (36%), MYC (24%), ERBB2 (13%), FGFR1 (17%), and ESR1 (14%) in breast cancer patients, compared with TP53 (78%), KRAS (14%), PIK3CA (11%), BRCA1 (10%), and MYC (5%) in ovarian cancer patients. Clinically actionable alterations (levels 1 to 3) were identified in both breast and ovarian cancer patients; 237 alterations in 86.3% (107/124) of breast cancer patients and 165 alterations in 95.7% (111/116) of ovarian cancer patients. Conclusions: This 50-gene NGS solid tumor panel identified clinically actionable alterations in 86.3% of breast cancer and 95.7% of ovarian cancer patients. This assay is suitable for detecting clinically actionable gene alterations in clinical settings.

ST60. Microsatellite Instability Testing for Lynch Syndrome Screening in Colorectal Adenomas

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Introduction: Lynch syndrome (LS) is the most common cause of hereditary colorectal cancer. Patients with LS carry a germline variant in one of the DNA mismatch repair protein (MMR) genes, which results in microsatellite instability (MSI). Universal screen MMR/MSI status in colorectal cancer (CRC) helps to identify patients with LS, which is imperative for treatment and surveillance. Studies showed that MSI high (MSI-H) is detected in some colorectal adenomatous polyps. If MSI-H can be detected in adenomas from LS patients, early detection could prevent cancers related to development of the LS patients and their family members. Currently, MMR/MSI testing in adenomas is not used as a screening test to identify patients with LS. We evaluate the feasibility of MSI testing for colorectal adenomas to identify patients with LS.

Methods: A retrospective review of our pathology database was performed. We selected 42 colorectal adenomas (tubular adenoma/tubulovillous adenoma) from 21 patients with history of LS who had undergone colonoscopy screening, 16 adenomas from 12 patient with sporadic CRC (SCRC) carrying BRAF mutation or MLH1 promoter methylation, and 20 adenomas from 16 patients who underwent colonoscopy screening (CS). Germline mutations in LS patients and SCRC/Lynch syndrome (LS) carrying MLH1 mutation or MLH1 promoter methylation in patient with SCRC was previously confirmed by molecular tests. Archived formalin-fixed, paraffin-embedded (FFPE) adenomatous tissue sections were used for MSI testing. The MSI status of adenomas was detected using Idylla MSI assay. Patients’ demographics, location, percentage of the adenoma and MSI results were analyzed. Patients’ clinical characteristics were recorded by reviewing the electronic medical records.

Results: The mean age for the patient group with LS is 55.3 years and 80.5 and 62.7 years for the groups with SCRC and CS, respectively. The frequency of MSI-H adenomas was significantly higher in the patients with LS (19%, 8/42) compared with patients with SCRC (6%, 1/16) and patients who underwent CS (0/20). Majority of the adenomas were tubular adenoma (76 tubular adenoma, 1 villous adenoma, and 1 tubular adenoma). The MSI-H adenomas include 8 tubular adenoma (7 from patients with LS and 1 from a patient with SCRC [MLH1 showed complete absence of nuclear staining in the tumor cells]), and 1 villous adenoma (from a patient with LS). Conclusions: The frequency of MSI-H adenoma is much higher in patients with LS than non-LS patient. Our results indicate that MSI testing for colorectal adenomas can help to identify patients with LS at an early, premalignant stage, potentially preventing cancer development in LS patient and facilitate an earlier surveillance for the patients and their family members.
ST61. Detection and Interpretation of Canonical and Cryptic Splice Sites in Solid Tumors and Their Relevance to FDA Approved Therapies and Clinical Trials

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Introduction: Splice variants are a common mechanism to inactivate or truncate genes. Our laboratory has performed clinical sequencing of more than 3,000 formalin-fixed, paraffin-embedded (FFPE)/fine-needle aspiration (FNA) tumor specimens using Thermo Fisher Scientific’s Oncomine Comprehensive Assay v3 and we observed that Ion Reporter may not display canonical and cryptic splice variants. With the advent of broader approval of genes associated with DNA damage repair and their relevance to PARP inhibitors, it should be noted that many of these genes could be inactivated by splicing variants. Furthermore, based on a recent assessment of germline splice variants by Landrith et al., we chose to investigate whether available splice prediction tools provided utility in reporting cryptic and/or canonical splice variants in the context of tumor sequencing. Methods: As previously described, our variant analysis incorporates our custom analysis workflow with the Ion Reporter output. From our knowledgebase, we retrieved 2,901 unique tumor specimens with at least 1 reported clinically significant variant (CSV) or a variant of uncertain significance (VUS). We identified 415 CSV (309 unique) and 91 VUS (86 unique), potentially resulting in abnormal splicing. We also assessed available treatment recommendations for these variants. Results: We reported 13,247 variants, 7,629 (57.6%) CSV and 5,618 (42.4%) VUS in 2,901 unique tumors. Of these, 415 (5.4%) CSV and 91 (1.6%) VUS were splice variants occurring in 380 (13.1%) unique FFPE specimens. Of the 309 unique clinically significant splice variants, 261 (84.4%) affected the canonical donor (127) or acceptor (134) sites, 26 (8.4%) were deletions (5 to 37 bp), 8 (2.6%) were intronic and 12 (3.9%) were cryptic variants. SpliceAI and Alamut predictions were performed on 56 unique variants, 8 of which had literature evidence, or internal RNAseq data to support the aberrant splicing. Whole transcriptome RNAseq is being performed for an additional 16 concordant and 4 discordant targets to further assess the accuracy of SpliceAI predictions. Of the 380 unique specimens with clinically significant splice variants, FDA approved therapies were available for 18 cases (4.7%) and clinical trials were recommended for 302 (10%) cases. Of the 17 cryptic and intronic VUS variants that were predicted by SpliceAI to alter splicing, these impacted ATM, BRCA1, MLH1, NFI, PTEN, RAD51B/C, and RBP1 genes. All of these genes had therapy recommendations. Conclusions: Detection of synonymous or intronic splice variants that lie beyond canonical splice acceptor or donor sites can be challenging. As the detection of these variants may impact treatment recommendations, the importance of accurate reporting is critical. Incorporating supportive prediction tools like SpliceAI may provide utility with confirmatory RNAseq.

ST62. Co-occurrence of PTEN and TERT Mutations Predicts Poor Prognosis in Glioblastomas


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Introduction: Glioblastoma is the most common and malignant brain tumor in adults. Mutations of the PTEN gene and the TERT gene promoter region are found in 15% to 40% and ~50% of glioblastomas, respectively. Although these 2 genes are frequently mutated in glioblastoma, there is controversy in their prognostic impact in glioblastomas. Therefore, we investigated the mutational status of PTEN and TERT in correlation to the prognostic outcome of the glioblastomas.

Methods: A clinically validated 161-gene next-generation sequencing panel was performed on 105 patients with a diagnosis of glioblastoma from 2016 to 2020. The panel detects mutations, fusions, copy number variations, in addition to analyses of MGMT methylation, microsatellite instability (MSI), and tumor mutation burden (TMB). The Log-rank test and report...
more aggressive phenotype than classical LCIS (CLCIS). By using whole transcriptome sequencing we aimed to characterize the expression profile of PCLCIS in comparison to CLCIS and HGDCIS. Methods: Seven cases of PCLCIS, 10 of CLCIS and 10 of HGDCIS were selected from our case files. Diagnoses were confirmed on all cases by 3 pathologists using immunohistochemical staining for e-cadherin. Microdissection was performed on formalin-fixed, paraffin-embedded (FFPE) tissue sections to isolate the carcinoma in situ. RNA extraction was performed using the AllPrep FFPE kit (Qiagen, Germantown, MD). Whole transcriptome library preparation was with the TruSeq RNA Exome kit and sequencing was performed on a NextSeq 550 (Illumina, San Diego, CA). Sequencing reads were mapped to the human reference genome (hg19) with RSEM and DESeq2 used to identify differentially expressed genes for each group of comparison. False discovery rate was controlled by the Benjamini-Hochberg method, and genes that had more than twofold changes with FDR <0.05 were selected for analysis. Results: Principal component analysis revealed a separation of expression profile for PCLCIS compared with HGDCIS and PCLCIS. Samples from HGDCIS and PCLCIS clustered together, suggesting similarities between these 2 groups. Gene differential analysis resulted in significant differential expression of 579 genes for PCLCIS versus HGDCIS, 256 genes for CLCIS versus HGDCIS and 99 genes for HGDCIS versus PCLCIS. One gene, FOS, was differentially expressed between all 3 groups. FOS is a component of the transcription factor AP-1 and its role breast carcinogenesis is, at this time, unknown. FOS expression was highest in the LCIS group, lowest in the PCLCIS group, and expressed moderately in the HGDCIS group (FDR = 4.57e-08, 0.033, 0.015). As expected, CDH1 expression was similar in the PCLCIS and CLCIS groups and significantly higher in the HGDCIS group. Conclusion: In contrast to prior copy number studies in frank carcinoma of breast, our data show that although different from both HGDCIS and CLCIS, more than 2.5 times as many genes were differentially expressed between PCLCIS and CLCIS than between HGDCIS and PCLCIS. Thus we conclude that PCLCIS is significantly closer at the expression level to HGDCIS than to CLCIS.

ST65. Aberrant PAX3 (Paired Box Gene 3) RNA Splicing Is a Potential Marker for Diagnosis of Melanoma
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Introduction: Melanoma is an aggressive malignancy arising from a neural crest-derived melanin-producing cell lineage known as melanocyte. Gene expression is regulated by alternative RNA splicing (AS), a post-transcriptional modification that has been implicated in carcinogenesis. The objective of this study was to identify melanoma specific aberrant RNA splicing signatures in our clinical data set comprising multiple solid tumors. To that end, we have identified a recurrent PAX3 RNA splice variant in melanoma samples that underwent comprehensive genomic profiling (CGP) as part of routine clinical cancer care. Methods: Targeted next-generation sequencing (NGS) was performed in a Clinical Laboratory Improvement Amendments (CLIA) certified, College of American Pathologists (CAP)-accredited laboratory (Morsani Advanced Diagnostics laboratory at the H. Lee Moffitt Cancer Center & Research Institute, Tampa, Florida). The Moffitt STAR NGS panel is based on a targeted NGS platform (Illumina TruSight Tumor 170 (TST170) panel), which is designed to detect genetic alterations in 170 genes associated with potential clinical utility in solid tumors, including 148 genes for substitution and insertion/deletion (indel) detection, 55 genes for fusion and splice variant detection, and 39 genes for copy number alteration (CNA) detection. Data analysis were performed using the Illumina BaseSpace Enterprise TST170 app v1.0 and a customized analysis pipeline within the Clinical Genomics Workspace software platform from PierianDx. Retrospective analysis of aberrant RNA splicing was performed on a clinical cohort of 1,255 solid tumors and 397 melanomas from TCGA skin cutaneous melanoma (SKCM) data set. Results: Our clinical cohort consisted of 24% (298/1,255) melanoma samples. A novel PAX3 intron 4 aberrant splicing alteration was identified in 59% (n = 1,762/298) and 31% (n = 124/397) of melanomas in our institutional clinical cohort and TCGA (SKCM) data sets, respectively. Across all solid tumors in our clinical cohort, only 0.6% (n = 8/1,255) of non-melanomas were positive compared to 14% (176/1,255) of melanomas. Five different splice forms involving intron 4 were observed. PAX3 intron 4 aberrant splicing is predicted to result in a shorter transcript due to premature early termination leading to subsequent loss of downstream DNA binding domain (DBD) and Transcription activation domain (TAD). Conclusions: PAX3 intron 4 splice variant occurs frequently and is specific to patients with melanomas. This recurrent aberrant splicing alteration can serve as a helpful genomic signature to resolve the classification of immunohistochemical challenging cases such as cancers of unknown primary.

ST66. Biomarker Testing for Patients with Advanced/Metastatic Non-Small Cell Lung Cancer (NSCLC) in Academic and Community-Based Practices in the United States (US)
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Introduction: NSCLC is a solid tumor with a growing number of actionable biomarkers that may determine treatment options. Current National Comprehensive Cancer Network (NCCN) guidelines (2020) recommend a broad panel approach to be taken to identify actionable markers, preferably by next-generation sequencing (NGS). This retrospective observational study was designed to evaluate the utilization of various testing modalities over time in academic and community-based practices. Methods: This study utilized the Flatiron Health electronic health records (EHR)-derived database, which includes de-identified data from more than 280 cancer clinics representing more than 2.4 million cancer patients in the US. Patients diagnosed with advanced/metastatic NSCLC on or after January 1, 2014, were eligible for analysis who received at least one line of systemic therapy after diagnosis. Only one test per patient was conducted, testing modalities, and time from sample submission to test results are entered by health care providers at the point of care. Descriptive statistics evaluated these data for academic and community-based practices. Results: A total of 21,738 patients were eligible with 82,079 genetic biomarker tests recorded for analysis from 2014 through 2020. Most patients (n = 20,167, 92.8%) were from community-based practices. Median age was 69 years and 55.4% were male. The proportion of patients tested was 69.5% in 2015 and 73.3% in 2019; with NGS-based testing among 33.0% and 68.9% of patients diagnosed in 2015 and 2019, respectively. The rate of NGS testing had a similar pattern for academic (33.6% and 79.0%) and community practices (32.9% and 68.1%) for these years. However, academic institutions performed biomarker tests within their own labs (64.1% of all tests), whereas for community practices this was only 5.7% of all tests. The proportion of tests with inconclusive or unsuccessful results was 5% for NGS, 0.5% for polymerase chain reaction (PCR), 1.9% for fluorescence in situ hybridization (FISH), 0.07% for immunohistochemistry (IHC) and 0.3% for other sequencing methods. The time to receive test results was an average (standard deviation) of 10.4 (11.5) days; 5.9 (9.4) days for PCR, 9.6 (13.2) days for FISH, 11.5 (10.2) days for IHC and 11.9 (10.1) for NGS-based tests. Conclusions: These real-world data suggest the increased adoption of testing, specifically NGS, across both academic and community-based practices in the care of patients with NSCLC. This study is limited by the low rate of academic practices included in the Flatiron Health EMR. These real-world data were not collected for research purposes and may contain errors.

ST67. Initial Tertiary Reporting Results from Personalize My Treatment (PMT); A Pan-Canadian Initiative Integrating Precision Oncology across Canada: PMT-001 Pilot Project
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Introduction: Identifying and enrolling rare cancer patient sub-populations into clinical trials presents an enormous challenge to both pharmaceutical companies and clinicians. Furthermore, in Canada, the lack of accessible harmonized standard of care molecular testing for cancer patients results in a discordance in the number of genes tested across Hospital sites. Identifying patients with biomarkers of interest across Canada is essential to attract precision oncology clinical trials to
Canada and to offer cancer patients better therapeutic options and improved outcomes. Founded in 2014, Exactis Innovation is a non-profit Academic Research Organization which aims to integrate precision oncology across Canada. Exactis has consolidated a pan-Canadian network of 13 cancer care centre sites and 4 federated laboratories. Network sites host an REB-approved molecular cancer patient registry (PMT), through which patients can be profiled, identified as carrying a biomarker, followed throughout their disease trajectory and be re-contacted if their molecular profile matches a clinical trial or an approved therapy. Here we report the results of Exactis’ first profiling initiative (PMT-001), performed on 420 tumors specimens from 4 cancer types across 8 Exactis Network sites within 3 Canadian provinces in a 4 months’ time period.

Methods: Breast, ovarian, lung and colorectal tumor specimens were profiled using the Oncomine Comprehensive Assay v3 (OCAv3) panel. VCF files were uploaded to NAVIFY Mutation Profiler, a cloud-based software that provides curation, annotation, interpretation and reporting of somatic variants identified by next-generation sequencing (NGS). The software reports a tiered classification based on consensus recommendations from AMP, ASCO, CAP and ACMG. The reports were automatically tailored to the Canadian region (e.g., drug approvals by Health Canada).

Results: At least 1 aberration was identified in 346 participants. As expected, the top mutated genes across the 4 cohorts were TP53, PIK3CA, KRAS, and BRCA1/2. At the fusion level, we identified EML4-ALK, KIF5B-RET, and MET exon 14 skipping in lung cancer participants and fusions in RSP02/3 genes in colorectal cancer participants. A clinical trial matching exercise was performed on 103 patients from 1 site based on the presence of a biomarker only and revealed that 62% of the cases matched to clinical trials open in Canada.

Conclusions: The profiling pilot of the PMT initiative shows how strong foundations within the Exactis pan-Canadian network and streamlined NGS testing and reporting are resulting in fast, high quality and meaningful molecular data for Canadian cancer patients.

ST68. Neurotrophic Tyrosine Receptor Kinase (NTRK) Gene Fusion Testing in Clinical Trials of Larotrectinib
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Introduction: NTRK gene fusions are oncogenic drivers in a variety of tumor types with a high prevalence (>80%) in certain rare cancers and a lower prevalence (generally <5%) in common cancers. NTRK gene fusions may be detected by fluorescence in situ hybridization (FISH), reverse transcriptase polymerase chain reaction (RT-PCR), or next-generation sequencing (NGS). Larotrectinib, a highly selective, CNS-active tropomyosin receptor kinase (TRK) inhibitor, had an objective response rate of 79% and a median duration of response of 35.2 months in 159 patients (pts) across a variety of non-primary CNS cancers. We report details of the NTRK gene fusions and testing methods in the patient (pt) population.

Methods: Data were pooled from 3 clinical trials of larotrectinib in pts with TRK fusion cancer (NCT02122913, NCT02576431, NCT02637687). NTRK gene status was determined by local molecular testing at each site. Data cutoff: February 2019. Results: The analysis included 177 pts with 18 different tumor types. The most common tumor types (by investigator assessment) were soft tissue sarcomas (STS; n = 65) including infantile fibrosarcoma (IFS; n = 29) and other STS (n = 36); bone sarcoma (50%) and melanoma (14%). The most common fusions were ETV6-NTRK3 (n = 79, 45%), TPM3-A TRK (n = 30, 17%), and LMNA-NTRK1 (n = 13, 7%). There were 39 different fusion partners identified, of which 28 occurred in only 1 pt. NGS was the most common testing method (DNA in 49 pts, RNA in 70, in both in 38). FISH (n = 11), PCR (n = 8), and NanoString (n = 1) were used on a limited basis in specific tumor types (breast, IFS, salivary).

Conclusions: These results demonstrate that NTRK fusions occur with many partners, supporting the need for validated and reliable testing methodologies. NGS was the most commonly used testing method used in identifying NTRK fusions in the 3 clinical trials.

ST69. Biomarker Testing and Overall Survival among Patients Diagnosed with Advanced or Metastatic Non-small Cell Lung Cancer
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Introduction: Biomarker testing is the foundation for evidence-based care of patients diagnosed with advanced or metastatic non-small cell lung cancer (NSCLC). Multiple actionable biomarkers are included in treatment guidelines. This retrospective observational study was designed to characterize the outcomes of patients with actionable biomarkers in a real-world practice setting. Methods: This study utilized the Flatiron Health electronic health records (EHR) database. Patients diagnosed with advanced/metastatic NSCLC on or after 1/1/2011 were eligible for analysis who had a positive biomarker result (EGFR, KRAS, ALK, BRAF, or ROS1) recorded in the EHR database at any time after the eligible diagnosis. Patients were eligible regardless of PD-L1 expression status. Overall survival was analyzed using Kaplan Meier method; living patients were censored at the end of the database. Data were available through 12/31/2019 for analysis. Results: Out of 60,025 patients in the database, a total of 10,534 patients met eligibility criteria, and were included in the analyses: 4,551 EGFR+; 3,986 KRAS+; 850 ALK+; 575 BRAF+; 182 ROS1+; and 390 with ≥2 co-occurring biomarkers. Mean age was 66.8 yrs (standard deviation, SD 10.6), 59.9% were female and 31.1% had no history of smoking. Mean days from advanced/metastatic diagnosis to first positive biomarker test was 55.8 days (SD = 337.4), with an average of 5.4 (SD = 3.6) biomarker tests per patient. Next-generation sequencing (NGS)-based testing was performed among 54.5% of patients with biomarker-positive disease; however, this was only 19.6% of patients diagnosed in 2011 versus 79.6% of patients diagnosed in 2019. The mean time from receipt of the specimen to a positive test result was <15 days for all biomarkers and testing methods, with the exception of BRAF as measured by fluorescence in situ hybridization (FISH), (n = 23, 25.1 days, SD = 49.0), and ALK, BRAF, EGFR, KRAS, and ROS1 tests where the method was not recorded (all ≥20 days). Median overall survival for each cohort from the start of first-line therapy was 29.3 (ALK+), 23.4 (EGFR+), 24.8 (ROS1+), 18.5 (BRAF+), and 13.5 months (KRAS+).

Conclusions: NGS-based testing among patients diagnosed with advanced or metastatic NSCLC has grown over time. Survival outcomes remain poor for patients with KRAS+ disease, as there is no approved treatment that targets this mutation in NSCLC. The time to receive test results is relatively short for all modalities (<15 days except for the few FISH tests); suggesting that biomarker testing may not lead to delays for treatment initiation. However, the standard deviations are relatively large, suggesting high variability. This study was limited by the non-research nature of data collection and could include data entry errors.
ST70. A Predictive Model of the Diagnostic Value of Next-Generation Sequencing-Based Genomics Testing in Patients with Advanced or Metastatic Non-small Cell Lung Cancer in the United States

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Introduction: The National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines for non-small cell lung cancer (NSCLC) version 6.2020 recommend use of next-generation sequencing (NGS) for the detection of actionable genomic biomarkers. This modeling study was designed to understand the potential improvement of biomarker detection in US patients with advanced or metastatic NSCLC when using NGS versus combinations of single-gene tests (SGTs). Methods: A dynamic decision tree-based model was developed to compare NGS (including comprehensive and small NGS panels) with various combinations of common SGTs. NGS included ALK, EGFR, ROS-1, BRAF, KRAS, RET, MET, and NTRK1/2/3. SGTs covered all but RET, MET, and NTRK1/2/3. All strategies included PD-L1. Input data were obtained from the published literature and from Ipsos Healthcare. The likelihood of correctly diagnosing patients was based on prevalence of actionable biomarkers, testing strategy, and sensitivity and specificity of tests. Treatment was assigned per NCCN guidelines for patients with actionable biomarkers. True and false negative results assigned patients to platinum-doublet chemotherapy. Percent improvement in diagnosis outcomes was reported by a relative percent change from SGT- to NGS-based approaches. Results: The model predicted that the use of NGS-based diagnostic strategy in patients with advanced or metastatic NSCLC can improve detection of actionable biomarkers by 23.8% and increase the proportion of patients receiving guideline-recommended therapies by 9.0% compared to SGT strategies. Patients initially receiving suboptimal first-line treatment due to incorrect biomarker test results decreased by 34.9%. Conclusions: The current analysis reaffirmed testing strategies with NGS are more comprehensive in the detection of actionable biomarkers and can improve the proportion of patients receiving NCCN-recommended therapies. This study was based on 100% of patients being tested, and results will vary depending on rate of NGS adoption.

ST71. Clinical Characteristics of RET- and NTRK-Rearranged Tumors in a Single Tertiary Cancer Center

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Introduction: Gene rearrangements have assumed critical roles in tumor pathogenesis. A small subset of tumors harbor RET or NTRK gene rearrangements that drive tumorigenesis. Importantly, studies have shown that targeted gene-rearranged tumors with tyrosine kinase inhibitors (TKIs) generates durable therapeutic responses. Herein, we aim to review the frequency of RET or NTRK-rearranged tumors in adult patients in our institution and compare it to public genomic datasets. Methods: We performed a retrospective analysis of tumors harboring RET and NTRK fusions of cases tested using a customized clinically validated targeted RNA sequencing panel (NYU-FUSIONSEQer) (n = 580; cohort 1). Tumor types, metastatic disease and presence of additional primary tumors were recorded when available. Results were compared to TCGA datasets, in addition to the MSK-IMPACT study set (n = 4,372 cases; cohort 2). Results: A total of 21 RET (n = 10; 1.7% of NTRK and RET tumors) and 13 NTRK (n = 7; 1.2%) fusions were identified. Patients with RET-NTRK rearranged tumors were younger than those in the NTRK-rearranged group (mean = 44.2 versus 61; p = 0.02). RET-fusions were associated with lung adenocarcinoma (LUAD) in cohort 1, in contrast to cohort 2 where thyroid carcinomas ranked first (n = 38/57; 66%) and LUAD ranked second (LUAD n = 6/10; 60%, versus n = 18/57; 33%, p = 0.03; RET fusion partners included KIF5B (n = 6), ETV6, EML4, NCOA4, and CCDC6 (n = 1, each). NTRK1-fusions were associated with thyroid and soft tissue tumors in both cohort 1 and 2 (n = 2/4; 50%, versus n = 9/12; 75%, p = 0.5). NTRK1-fusions showed strong association with mammary analogue of secretory carcinoma (MASC) in cohort 1 in contrast with cohort 2 which showed a predominant association with thyroid carcinomas (MASC n = 4/17; 24%; versus n = 0/7, 0%). NTRK1 was fused with PDIA6, MUSK, TPM3 or KIF5B (n = 1, each), and NTRK3 with ETV6 (n = 6) or EML4 (n = 1). Conclusions: Although the prevalence of RET and NTRK fusions in our patients is similar to public datasets, the distribution is different with higher incidence in LUAD and MASC, respectively. Both fusions seem to confer an aggressive course mostly in LUAD further highlighting the urge for accurate identification and traging these patients for TKIs.

ST72. Incidence of T790M Mutation by ddPCR in Patients Progressing on First- and Second-Generation TKIs and Clinical Outcomes on Osimertinib

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Introduction: EGFR TKIs are standard of care for advanced NSCLC patients harbouring an EGFR mutation. In developing countries, first- and second-generation TKIs are still the treatment of choice as osimertinib may not be a feasible option owing to availability or cost constraints. Nearly 50% of patients who progress on first- and second-generation drugs develop a secondary targetable T790M mutation. Many of these patients are unable to undergo a biopsy after progression or may have insufficient tissue for further molecular testing and the role of liquid biopsy becomes important in this setting. Data regarding incidence of T790M mutations and outcomes on osimertinib in patients who have a positive T790M mutation detected by ddPCR were scarce, especially in Indian patients. Methods: Retrospective data of patients with EGFR mutant advanced NSCLC who received first- and second-generation EGFR TKIs from 2015 till 2018 were evaluated. The development of T790M mutation on progression was determined by liquid biopsy (testing cell free DNA) by droplet digital PCR. Progression free survival and overall survival of those patients who received further osimertinib were analysed. Results: A total of 251 patients developed progression on first- and second-generation TKIs, and 131 (52.19%) underwent testing for T790M mutation by ddPCR. Out of this cohort, 65 patients (49.61%) were found to have a secondary T790M mutation. There was no significant factor like age/sex/TKI used/library of mutation which could predict the development of T790M Mutation. Fifty-eight patients received subsequent osimertinib and median overall survival (from initiation of osimertinib) and progression free survival was 9.33 months and 6.9 months, respectively. The drug was well tolerated. However, 6 patients developed symptomatic venous thrombosis on osimertinib. Conclusions: ddPCR is an important tool in detection of secondary T790M mutation. It has advantages of being highly sensitive and non-invasive. The incidence of detection of T790M mutation by ddPCR in our study is lesser than reported in AURA trial. However, this is dependent on various techniques like stage of disease, tumour load and various pre and post analytic variables. Further large studies are needed to determine if there are differences in eastern and western population regarding incidence of T790M mutation.

ST73. Frequency of EGFR Mutations and ALK Expression in NSCLC in the North of México

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Introduction: Lung cancer is the most common cause of cancer-related deaths; every year, more than 8,000 people die of lung cancer in Mexico. The identification of the epidermal growth factor receptor (EGFR) mutations and expression of ALK (4F5) implicates in the clinical behavior of the disease and the potential value for targeted therapies. Our study aims to analyze the frequency of EGFR mutations and ALK expression in patients from northern Mexico with pulmonary adenocarcinoma. Methods: This retrospective analysis studied cases of pulmonary neoplasias sent for EGFR analysis with real-time PCR (ARMS) and ALK (4F5)
immunohistochemistry from northern Mexico from a 5-year period. Every case was reviewed morphologically and subjected to immunohistochemical analyses for TTF-1 previous to the molecular analyses. Mutational analyses were performed using 1 platform. We registered the age and gender of every patient. The mutations’ results were correlated with the age of the patient, sex, and histological varieties.

**Results:** We received 1,008 samples from patients with histologically confirmed lung carcinoma for mutational analyses, from which only 885 met the criteria for inclusion in the study. Of the 885 samples, the histological patterns were 619 (69.94%) solid predominant. The patients’ median age included in the study was 64 (range, 28 to 91), and 424 (47.91%) of patients were female. The frequency of EGFR mutations was 30.05% and was significantly more frequent in female patients, with 57.6%. The exon 19 deletion was detected in 54.8%, followed by L858R point mutation in exon 21 in 34.8%, representing 89.6% of all the ALK (4FS) analysis was performed in 833 cases. ALK expression was identified in a total of 114 patients (13.7%). In 18 patients (7.2%), concomitant EGFR mutations, and ALK expression were found.

**Conclusions:** A previously published study conducted in Latin American patients with lung cancer showed a similar frequency of EGFR mutation in women’s tumors, and in the age of the patients (64 years old). The same study showed a 26.4% rate for EGFR mutations (Arrieta et al., JTO 2015). Moreover, in our research, we found a slightly higher frequency of 30.05%. All of our cases were evaluated before EGFR mutational analyses and ALK expression with TTF-1 to confirm pulmonary adenocarcinoma. The frequency of ALK expression by IHC was 13.7%, higher than those reported by previous investigations (6.8%) (Arrieta et al., 2018). We report a higher-than-expected IHC EGFR mutation frequency and ALK expression, which may be explained by the broad ethnicity of the region, patients’ baseline characteristics, underlying genetic differences, environmental factors, sample selection, and detection (Midha et al., AJCR 2015).

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**ST74. Tissue Requirements of a Novel 27-Gene Immuno-Oncology Algorithm Measuring Tumor Microenvironment to Predict Response to Immunotherapies**

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**Introduction:** Immunotherapies have revolutionized cancer treatment, however relatively few patients respond. A 27-gene immuno-oncology algorithm has been described with improved predictive response relative to PD-L1 IHC and TMB in both NSCLC and TNBC. A real-time qBDA polymerase chain reaction technology that combines blocker displacement amplification (BDA) with unique molecular identifiers (UMIs) to achieve multiplexed enrichment of DNA variants by >1,000-fold. Results: We show here that our qBDA pan-cancer panel amplifies more than 300 hotspot regions from 61 oncogenes covering >8,000 characterized cancer mutations with high sensitivity and reproducibility. Furthermore, we have validated our panel on clinical samples and healthy control cfDNA. Conclusions: By designing primers with competing thermodynamics, variant alleles originally down to ≤0.1% variant allele frequency (VAF) are preferentially amplified and detected with minimal sequencing depth. The incorporation of UMIs to mark each distinct molecule allows qBDA to quantify the absolute number of variant molecules in a sample.

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**ST75. Pan-Cancer Liquid Biopsy Assay for Mutation Profiling in 61 Genes by Low-Depth Sequencing**

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**Introduction:** Liquid biopsies represent a minimally invasive approach to disease monitoring and therapy selection. Next-generation sequencing of cell free DNA (cfDNA) isolated from plasma allows for the characterization of genomic alterations associated with diseases, in particular, cancer. Yet, the low proportional abundance of circulating tumor DNA within cfDNA presents a challenge for accurately and economically quantifying mutations. Methods: Here, we develop quantitative multiplexed blocker displacement amplification (qBDA) polymerase chain reaction technology that combines blocker displacement amplification (BDA) with unique molecular identifiers (UMIs) to achieve multiplexed enrichment of DNA variants by >1,000-fold. Results: We show here that our qBDA pan-cancer panel amplifies more than 300 hotspot regions from 61 oncogenes covering >8,000 characterized cancer mutations with high sensitivity and reproducibility. Furthermore, we have validated our panel on clinical samples and healthy control cfDNA. Conclusions: By designing primers with competing thermodynamics, variant alleles originally down to ≤0.1% variant allele frequency (VAF) are preferentially amplified and detected with minimal sequencing depth. The incorporation of UMIs to mark each distinct molecule allows qBDA to quantify the absolute number of variant molecules in a sample.

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**ST76. WITHDRAWN**

**ST77. Spatially Resolved Gene Expression Profiles in Human Glioblastoma**


**Introduction:** Glioblastoma is a common type of malignant brain tumor with a median survival time of 12 to 14 months. The histological features of these tumors are often used for evaluation by pathologists. Sequencing of RNA from these diseased tissues can provide gene expression measurements from biomarkers, helping support a pathologist's interpretation of the samples. Our objective is to determine the minimum amount of tissue and tumor content required to generate a reproducible gene expression profile with minimal sequencing depth. The incorporation of UMIs to mark each unique molecule allows qBDA to quantify the absolute number of variant molecules in a sample.

**Results:** We utilized the 10x Genomics Visium Spatial Gene Expression Solution to examine intact normal cortical and glioblastoma samples and elucidate the gene expression profiles in the context of tissue anatomy. Methods: Serial sections of fresh-frozen healthy human cortical and glioblastoma solid tumor samples were placed onto Visium Spatial Gene Expression slides. The slides consisted of arrays of ~5,000 spots with uniquely barcoded capture probes. Subsequent tissue permeabilization allowed for the capture of native mRNA. Sequencing of captured molecules was performed on an Illumina NovaSeq at a depth of 50,000 reads per spot. Using the Space Ranger v1.1 analysis pipeline, the RNA-seq data were merged with H&E stained tissue images to align reads, perform clustering, and gene expression analysis. Additional analyses and data visualization were performed on the Loupe Browser v4.1 desktop software. Results: Spatial gene expression data were generated from 4 serial sections each of both healthy cortical tissue and glioblastoma solid tumor. Data clustering by gene expression profile provided insight into differential expression between tumor and healthy tissue. Clustering from the glioblastoma samples demonstrated a loss of laminar organization, which was present in the healthy samples. Differential gene expression analysis confirmed the upregulation of glioblastoma markers in diseased tissue. More importantly, the spatial gene expression profiles within the tumor revealed regional variability in tumor and immune cell marker expression. Conclusions: Our results suggest that spatial gene expression profiling provides a powerful technique that can complement traditional histological practices, as well as, single cell and bulk RNA-seq. This higher resolution...
microenvironment is critical to the development of diagnostic biomarkers. TNBC is an aggressive, complex disease with a poor prognosis due to resistance by loss of HER2, estrogen receptors, and progesterone receptors. TNBC is an intermediate 21 Gene Recurrence Score.

**Conclusions:** The PI3K pathway may be involved in recurrence and metastasis of early breast cancer with low to intermediate RS. Further research is warranted to better understand the role of this pathway in the progression of breast cancer and possible preventive therapies.

**ST70. Spatially Resolved Molecular Interrogation of Triple Negative Breast Cancer**

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**Introduction:** Triple negative breast cancer (TNBC) accounts for 10% to 20% of all diagnosed breast cancer cases in the US and is characterized by loss of HER2, estrogen receptors, and progesterone receptors. TNBC is an aggressive, complex disease with a poor prognosis due to resistance to traditional therapies. Investigation of the underlying biology and tumor microenvironment is critical to the development of diagnostic biomarkers and to guide the search for effective therapies. **Methods:** Spatial transcriptomics technology can complement pathological examination by combining the benefits of histological stains with the throughput and deep biological insight of RNA-seq. We investigated serial sections of TNBC, using the 10x Genomics Viusm Spatial Gene Expression Solution, to spatially resolve its expressed microenvironment. Viusm incorporates ~5,000 molecularly barcoded, spatially encoded capture probes in spots over which a tissue section is placed, imaged, and permalelized. Next, native mRNA is captured. Imaging and RNA sequencing data are processed together, resulting in whole transcriptome gene expression mapped to the tissue image. **Results:** We captured spatial patterns of gene expression and mapped the information back to H&E-stained images with cellular annotations. Serial sections were then subject to fluorescence immunohistochemical staining for immune infiltrate paired with spatial gene expression capture. Next, we combined these data with 3’ single-cell RNA-seq from the same tumor, generating cell-type expression profiles that were used to automatically annotate cell types across the cancer sections. This allowed for an understanding of the tumor microenvironment that could not be captured using image-based techniques alone or using subgroups of spatially and biologically distinct immune, stem, and cancer progenitor cells. Finally, we digitally annotated tumor and normal tissue regions using expressed genetic mutations alone. Annotated tumor regions (digital and manual) expressed more deleterious mutations than normal regions and we were able to automatically cluster regions of tumor versus normal cells without any prior histopathological information. We also found intratumor gradients of mutational burden in both known oncogenes as well as non-cancer associated loci. **Conclusions:** Taken together, we demonstrate that spatial gene expression profiling can provide a powerful complement to traditional histopathology, enabling both targeted panels and whole-transcriptome discovery of gene expression. This spatially resolved molecular information provides an unprecedented view into the tumor microenvironment and a powerful new tool for discovery of new biomarkers and therapeutic targets.

**ST79. Quantitative Assessment of Functional Activity of Multiple Signaling Pathways in Recurrent Breast Cancer with Low to Intermediate 21 Gene Recurrence Score**

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**Introduction:** The molecular characterization of breast cancer has been shown to correlate with prognosis and predict risk of recurrence. The 21-gene recurrence score (RS) is one of the most widely used commercially available tests to predict risk of recurrence in breast cancer based on a molecular approach. Even when classified as low-intermediate risk by RS score (≤10), 6.8% to 14.3% and 4% to 7.2% of patients will experience distant or locoregional recurrences, respectively. In this study, we investigated the functional activity of key signal pathways in recurrent breast cancers with low to intermediate RS score to identify molecular features that may predict recurrence. **Methods:** This is a case-control study of patients with recurrent breast carcinoma with low to intermediate RS score and a control cohort of patients with non-recurrent breast cancer controlled for age, RS score, and follow-up time. Cases were diagnosed between October 2012 and December 2017 and tested with the 21-gene RS assay. The cases and controls were retrospectively identified from NYU Langone Health Pathology Database. We collected clinicopathologic, treatment, and outcome data. The tissues were microdissected, and RNA was extracted. mRNA expression of genes involved in ER, AR, PI3K, MAPK signaling pathways was measured by RT-qPCR and translated into quantitative pathway activity scores to characterize the signaling activity of these tumors using the OncoSignal (PhilipsMolecular Pathway Dx) assay. Statistical analysis was performed using student t test and Fisher exact test. **Results:** A total of 18 cases with locoregional recurrence or distant metastatic disease and available formalin-fixed, paraffin-embedded tissue were included. An additional 15 non-recurrent controls matched for age, RS score, and follow-up were retrieved. There were no statistical differences between the groups’ characteristics, including age, race, pathologic characteristics (histologic type, grade, staging, lymphovascular invasion, resection margins, lymph node positivity, ER/PR percentage, Ki-67 proliferation index), Oncotype score, follow-up time, and treatment parameters (type of surgery, percentage receiving hormonal treatment, chemotherapy, and radiotherapy). The PI3K pathway showed significantly higher activity score in cases with locoregional recurrence or distant metastatic disease compared to controls (35.2 ± 8.65 versus 23.8 ± 10.03; p = 0.0014). There was no difference in ER, AR, and MAPK pathways between the groups. **Conclusions:** The PI3K pathway may be involved in recurrence and metastasis of early breast cancer with low to intermediate RS. Further research is warranted to better understand the role of this pathway in the progression of breast cancer and possible preventive therapies.

**ST80. Prospective Study Using Virtual Enrollment to Assess an RNA-FIT Assay for Noninvasive Detection of Colorectal Cancer, Advanced Adenomas, and Other Precancerous Adenomas**

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**Introduction:** Non-invasive screening for colorectal neoplasms has focused on detection of early stage colorectal cancer (CRC). However, reduction in CRC morbidity and mortality requires cancer prevention through adenoma detection. A noninvasive diagnostic that can accurately detect advanced (AAs) and other precancerous adenomas (OPAs) requires a sensitive biomarker panel that can withstand a robust study design. **Methods:** A multi-factor assay (RNA-FIT) was developed that evaluates 8 stool-derived RNA biomarkers, a fecal immunochemical test (FIT), and patient demographics (smoking status). Using virtual enrollment and a centralized IRB, 1,305 average-risk, asymptomatic patients were identified. Collection kits were shipped directly to each patient’s residence and patients returned stool samples for testing, with sample transit time of up to 4 days. Stool samples were received from all 48 continental states and subsequent screening colonoscopies were performed at more than 600 different endoscopy sites. A pragmatic study design was employed to facilitate reproducible results. This included: collecting stool prospectively; using a patient population that matched the intended use; using numerous/geographically diverse testing sites; using varied reagent lots and test systems; mitigating features employed in the RNA-FIT assay; and assessing the model performance by setting thresholds within the folds of the internal cross-validation. **Results:** In the study, the average age of participants was 55 years old (range = 44 to 80) and 63% were female (37% male). A total of 13% of participants were African American, 2% were Asian-American, 6% were Hispanic, and 75% were Caucasian (6% other). The cohort was derived from hundreds of different cities, many of which were in rural areas. A total of 21% of the study population was low income (<$29,999 per year) and 24% were on public insurance. When evaluating the RNA-FIT assay performance, internal cross validation of the training set (n = 939) attained a 100% sensitivity for CRC (n = 3), 59% sensitivity for AAs (n = 66), 22% sensitivity for OPAs (n = 279), 80% specificity for hyperplastic polyps (n = 155) and 86% specificity for no findings on a colonoscopy (n = 436). Assessment of a 366-patient hold out test is ongoing. **Conclusions:** Using virtual enrollment, the
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samples. We compared the performance of these Compromised FFPE damage characteristics typically found in FFPE-preserved tissue biopsy. These materials were developed to mimic DNA manufacturing process for reference materials that produces formalin-fixed, paraffin-embedded (FFPE) tissue using Qiagen RNA prep. Fusion gene extraction from formalin-fixed, paraffin-embedded (FFPE) tissue using Qiagen RNA prep. Fusion gene as internal control. In next step, fusion genes were identified using a custom designed Archer FusionPlex panel (94 target genes) and the Illumina NextSeq 550. Results: The first case had a history of HPC in parietal lobe of brain. Almost 10 years later, she was diagnosed with malignant SFT of liver. The second case had multiple lesions in brain and bone/soft tissue in 16 years’ time frame with a diagnosis of SFT and clear cell meningioma in brain. In all lesions tested, 2 lesions from patient 1 and 3 lesions from patient 2 at various time point, we identified the same breakpoint (NAB2ex6-STAT6ex16). The archer fusion panel identified NAB2 (e6) → STAT6 (e16) fusion with genomic location of breakpoint being chr12:5478381 and chr12:5479323. Conclusions: Our study demonstrated multiple SFT/HPCs in the different location and different time share the same fusion breakpoint, using both RT-PCR and Archer fusion panel. To our knowledge, this is the first report testing tumors from the same patient in different locations, i.e., CNS and outside CNS, and is a novel molecular application to address a specific clinical question to guide clinical therapy.

ST82. Development and Performance of Formalin Compromised FFPE Reference Materials

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Introduction: Genomic profiling of cancer patients typically analyzes tissue biopsy that is formalin-fixed, paraffin-embedded (FFPE), a process that introduces damage of the nucleic acid. FFPE reference materials that closely mimic the damage profile of cancer patient samples are difficult to design, manufacture, and/or source. We have developed an FFPE manufacturing process for reference materials that produces formalin-compromised DNA as surrogates to cancer patient samples: Seraseq Compromised FFPE Tumor DNA (mutation-positive) Seraseq Compromised FFPE WT. These materials were developed to mimic DNA damage characteristics typically found in FFPE-preserved tissue biopsy samples. We compared the performance of these Compromised FFPE DNA reference materials to that of FFPE reference materials with manufactured with minimal fixation damage. Methods: Biosynthetic DNA containing 31 cancer variants were introduced into the GM24385 reference cell line (Coriell) to simulate SNVs, indels, and structural rearrangements. The RNA-FIT assay presented here provides a robust method to noninvasively detect CRC, AAs, and OPAs with high sensitivity and specificity.

ST83. CNV Detection from a Multi-Cancer NGS Panel: A Single-Tube, Multiplex-PCR Based NGS with 309 Tiled Amplicons

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Introduction: SLImamp technology allows multiplex-PCR of tiled amplicons in a single tube, which enables targeting of large exons for next-generation sequencing (NGS) analysis with a streamlined process. Copy number variants (CNVs) are DNA segments that are present with variable copy number of copies compared to a normal genome. CNVs have a high prevalence in the pathogenesis of cancer and their characterization is important to acquire a more comprehensive picture of the mutations present in a patient sample. Pillar developed a proprietary CNV algorithm and combined it with SLImamp to develop an integrated multi-cancer plus CNV detection NGS panel which identifies CNVs in the ERBB2, EGFR, MET, and MYC genes. Methods: To assess the ability of the ONCO/Reveal Multi-Cancer with CNV Panel to detect ERBB2 CNV compared to DISH (dual in situ hybridization), libraries were created from 44 well-characterized formalin-fixed, paraffin-embedded (FFPE) breast cancer samples plus 3 Genome in a Bottle (GiAB) samples. Twenty-three tumor samples had known ERBB2 amplification, 21 were known non-amplified tumors, and 3 GiAB samples were used as negative controls. Libraries were sequenced on an Illumina MiSeq and data were analyzed by the Pillar Variant Analysis Toolkit (PiVAT). The previous DISH results were not known to Pillar. Results were provided to an independent collaborator who unblinded the study and compared the NGS results to DISH. Results: Overall the assay performed well with a mapping rate of 99.4%, on-target rate of 99.1%, and coverage uniformity >0.2x mean coverage of 93.5%. The assay and software detected 100% of ERBB2 amplification negative samples as confirmed by DISH. For amplification positive samples, the NGS assay detected 100% of positive samples. The software correctly identified 91% of amplification positive samples. The 2 samples that were not called by PiVAT had normalized gene counts of 1.2 and 1.4, and also had the lowest DISH scores. All samples with a normalized gene count >1.5 were correctly called by PiVAT. The NGS assay was able to accurately call CNV independent of tumor cellularity. Conclusions: The ONCO/Reveal Multi-Cancer with CNV Panel is a robust assay for the detection of CNVs, SNVs, and indels of interest across multiple solid tumor cancer types. The workflow is streamlined,
with same day loading of finished libraries when starting from as little as 5ng of isolated input DNA. The assay and software demonstrate detection of low CNV with the recommended cutoff set at 1.5. With further optimization of the calling algorithm, accurate calling of even lower CNVs may be possible. The detection and identification of CNVs along with other mutations provides a more comprehensive overview of the mutations present in a sample, supporting better clinical management of patients.

ST64. Rapid Isolation of High-Quality Ultra-High Molecular Weight Genomic DNA from Blood, Bone Marrow Aspirates, and Frozen Human Tumors
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Introduction: Genetic alteration is hallmark of human cancer and plays a key role in both tumor initiation and progression. Short read next-generation sequencing has been instrumental in the identification of single nucleotide variants (SNVs) and small insertions and deletions (indels) in coding and non-coding regions in hematopoietic cancers and solid tumors, but larger structural changes are often missed. Array CGH can detect copy number variation (CNV), but balanced translocations and inversions will be missed in the absence of a pre-designed fluorescence in situ hybridization (FISH) assay. Optical mapping with Bionano genome imaging is a powerful tool for comprehensive detection of structural variations (SVs) in cancer genomes. A crucial step in this workflow is isolating intact ultra-high molecular weight (UHWM) genomic DNA (gDNA). For routine study of leukemic samples, we developed Bionano Prep SP kits for rapid, solution-based isolation of high-quality gDNA from blood and BMAs. We also developed a rare variant bioinformatic pipeline that detects SVs at 5% allele fraction. Together, these can potentially replace a suite of “classical” cytogenetic tests for leukemia analysis (karyotyping, FISH, CNV microarrays) with a single Bionano genome imaging analysis. Methods: For solid tumors, we developed a novel protocol to isolate UHWM gDNA from small amounts (9 to 12 mg) of fresh frozen tissues. Homogenized tissues are processed with the Bionano Prep SP Tissue & Tumor Kit, where lysis is coupled with purification steps to bind, wash and elute UHWM gDNA. With this protocol, a batch of 8 samples can be processed in ~6h. Eluted UHWM gDNA (3 to 19 µg DNA/10 mg) is compatible with the Direct Label and Stain protocol. We then tested this protocol on 10 different fresh frozen human tumors obtained from BioIVT (bladder, brain, colon, kidney, liver, lung, ovary, prostate and thyroid). Results: For each tumor, at least 2 different pieces were processed in 2 different preparations, with results well above the minimal concentration required for DLS labeling (35 ng/µl) and the average final DNA yields for each tumor ranged from ~6 to 16 µg/10 mg input tissue. The single molecule quality metrics for all of the samples easily passed specification. The collected data for each sample were subjected to the Bionano rare variant pipeline (RVP) against the hg38 human reference assembly and structural variations detected and annotated. Conclusions: This novel UHWM DNA isolation protocol facilitates structural variation analysis of a variety of solid tumor types on the Bionano Saphyr system including large indels, inversions, inter- and intra-chromosomal translocations, and CNVs.

ST65. Novel Amplicon-Based NGS Library Preparation Protocols Compared and Evaluated across Two Sequencing Technologies
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Introduction: SLiMamp technology allows multiplex-PCR of tailed amplicons in a single tube, which enables targeting of large exons for next-generation sequencing (NGS) analysis with a streamlined process. This technology has been previously demonstrated to be a sensitive and robust NGS library prep method for Illumina’s MiSeq, MiSeqDX, NextSeq, and NextSeq CN500 platforms. However, to benefit the largest number of patients and researchers, SLiMamp library prep was made compatible for multiple sequencing platforms that are common worldwide including the MGISEQ portfolio. Methods: Five different ONCO/Reveal assays for MG1 were evaluated (multi-cancer, multi-cancer with CNV, multi-cancer cfDNA, lung fusions, and lung and colon cancer) on the MGISEQ-2000. Libraries were prepared and sequenced by 2 different laboratories (Zhengu Shanghai and MGI R&D Application Center). Additionally, 14 CRC samples were tested and compared on both the MGISEQ-2000 and NextSeq CN500 platforms. Automated library preparation by the MGISP-100 instrument was also evaluated. Results: Across the 5 assays, mapping (range: 97.1% to 99.5%) and on-target rates (range: 93.6% to 99.3%) and coverage uniformity at 0.2x of the mean (92.2% to 95.1%) demonstrated high performance and were generally >95%. Variant calls from 14 CRC samples for the ONCO/Reveal Multi-Cancer Panel sequenced on the MGISEQ-2000 and NextSeq CN500 demonstrated an R² value of 97%. Variants were concordantly called across a wide range of frequencies from 2% VAF to 65% VAF. The lung fusions and lung fusions were detected in all expected fusions from a positive reference control sample and did not yield any false positives from known negative formalin-fixed, paraffin-embedded (FFPE) samples. Automation on the MGISP-100 reduced total assay time from 8 hours to 5 hours with only 30 minutes of hands-on-time. Library yields and results from automated library prep were similar to those produced from manual library prep performed by experienced scientists. Conclusions: All 5 ONCO/Reveal assays demonstrated high performance when sequenced on the MGISEQ-2000 as well as Illumina platform. Automated library preparation utilizing the MGISP-100 reduced hands-on-time while providing similar sensitive and robust performance. Concordance was demonstrated across platforms, specifically the NextSeq CN500 and MGISEQ-2000. ONCO/Reveal assays are sensitive and robust NGS library prep solutions, which are available across multiple sequencing platforms that are commonly used by researchers across the globe.

ST66. Chromosomal Microarray Analysis of Benign Mesenchymal Tumors with RB1 Deletion
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Introduction: Spindle cell lipomas/pleomorphic lipomas, mammary-type myofibroblastomas, and cellular angiofibromas are distinct neoplasms that share clinical and imaging features with breast RB1 tumors and that share histologic overlap at low levels. This study compared a series of spindle cell lipomas/pleomorphic lipomas, mammary-type myofibroblastomas, and cellular angiofibromas with breast RB1 tumors, as well as other spindle cell neoplasms.

Methods:
- 136 cases were included in the study, consisting of 17 breast RB1 tumors, 10 spindle cell lipomas/pleomorphic lipomas, 11 mammary-type myofibroblastomas, and 10 cellular angiofibromas.
- Standard histopathology, immunohistochemistry, and genetic analysis were performed.
- Standard karyotype and fluorescence in situ hybridization (FISH) were performed.
- Molecular studies were performed using a panel of 25 genes, including RB1, p53, and other genes associated with spindle cell neoplasms.

Results:
- For breast RB1 tumors, the most common genetic alteration was RB1 deletion (93.7% of cases).
- For spindle cell lipomas/pleomorphic lipomas, the most common genetic alteration was MYB amplification (68.4% of cases).
- For mammary-type myofibroblastomas, the most common genetic alteration was MYB amplification (81.8% of cases).
- For cellular angiofibromas, the most common genetic alteration was MYB amplification (90.0% of cases).

Conclusions:
- The genetic alterations found in spindle cell lipomas/pleomorphic lipomas, mammary-type myofibroblastomas, and cellular angiofibromas are distinct from those found in breast RB1 tumors.
- The genetic alterations found in spindle cell lipomas/pleomorphic lipomas, mammary-type myofibroblastomas, and cellular angiofibromas are distinct from each other.
- The genetic alterations found in spindle cell lipomas/pleomorphic lipomas, mammary-type myofibroblastomas, and cellular angiofibromas are similar to each other.
- The genetic alterations found in spindle cell lipomas/pleomorphic lipomas, mammary-type myofibroblastomas, and cellular angiofibromas are similar to breast RB1 tumors.

S84
ST87. A Novel Nanoparticle-Based Approach to Improve Extraction of Circulating Tumor DNA (ctDNA)  
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Introduction: Liquid biopsies, especially those that use plasma ctDNA, are emerging as a powerful complement, and, in some cases, alternative to solid tumor biopsies for the molecular characterization of cancer. Non-small cell lung cancer (NSCLC) has proven particularly amenable to liquid biopsies due to the availability of an FDA-approved tyrosine kinase inhibitor for patients whose tumors have EGFR exon 19 deletions or exon 21 L858R mutations, as well as the need to monitor disease progression and treatment response. However, collection of ctDNA is challenged by the low abundance of ctDNA in blood, the potential for contamination by genomic DNA, as well as other factors. These challenges can lead to false negative results.

Methods: We compared the recovery of ctDNA using a commercial column-based approach (Roche) to a novel magnetic hydrogel particle (Ceres Nanosciences) method. We created contrived liquid biopsy specimens by spiking fragmented EGFR wild-type and mutant DNA sequences into pooled donor plasma at varying concentrations. Recovered DNA was quantitated and tested for EGFR mutations using a semi-quantitative multiplex PCR assay (COBAS 4800). The performance of these 2 ctDNA extraction methods were further compared using plasma derived from NSCLC patients with known mutational profiles.

Results: In the contrived specimens, the column-based approach recovered 48% to 63% of spiked DNA, whereas the magnetic hydrogel particles recovered 79% to 84% of spiked DNA. Importantly, the hydrogel particle-based approach prevented genomic DNA contamination for up to 24 hours post-collection. Extracted ctDNA from both approaches demonstrated comparable performance in the detection of EGFR mutations, with EGFR exon19del, L858R, and T790M mutation detection possible at plasma DNA concentrations of 0.5, 4, and 20 ng/ml, respectively. Patient data demonstrated concordance between ctDNA and tissue analysis in most cases, regardless of ctDNA extraction method. ctDNA analysis revealed an EGFR L858R mutation not detected by solid tumor testing. Conclusions: The magnetic hydrogel particle-based ctDNA extraction technique was highly efficient in recovering ctDNA from plasma. Moreover, this extraction technique required less starting material (1 ml plasma) than column-based approaches with similar performance in downstream applications. These results demonstrate the potential benefit of a magnetic hydrogel particle-based ctDNA extraction technique in liquid biopsy testing of NSCLC patients. On-going work is focused on integrating this extraction technique into a multi-gene liquid biopsy assay.

Technical Topics

TT01. Dimensionality Reduction for Noise Filtering of Big Data Sets  
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Introduction: Fundamental to effective bioinformatic pipeline development is the reduction of noise in favor of true signal. In most instances, this process involves a heuristic workflow, validated with orthogonal experimentation. For large exploratory datasets, however, confirmatory orthogonal testing may not be possible, and “best guesses” may be the only viable option. The use of dimensionality reduction techniques in big-data analyses is commonplace; however, the use of these techniques for big-data noise-filtering is less well described. Described herein is the use of dimensionality reduction-based statistical modelling on next-generation sequencing (NGS)-quality metrics to effect noise reduction. A principal component analysis (PCA)-based dimensionality reduction approach was applied, along with a simple over-fitting decision-tree algorithm, to filter raw reference-aligned and variant-called NGS data. This approach was tested on a large cohort of acute myeloid leukemia NGS data (Wellcome Sanger Institute, EGAS00001000275). Variant annotation with variant effect predictor (VEP) was performed. Comparison of filtered and unfiltered variant calls served to generate accuracy metrics. Results: Without the PCA-filter, from among 1,897 assessable specimens, 218,450 variants were identified, including 32,186 (15%) with VEP clinical significance assertions (CSAs). In contrast, with filtering applied, only 63,168 variants were called, including 13,051 with VEP CSAs (21%; Z = 35.73, p < 0.00001). Of those variants with VEP CSAs, only 5.1% were flagged as likely pathogenic/likely pathogenic without filter, as compared to 10.5% with filtering (Z = 20.73, p < 0.00001). Of those variants not passing the filter, only 1.4% were subsequently noted to have a VEP CSA of likely pathogenic/likely pathogenic. Also, of those variants not passing the filter and without a VEP CSA, only 1.5% demonstrated copy number greater than the median and with a population allele frequency <2%. From within the last subset, 0.2% of variants had a SIFT or PolyPhen score of either deleterious, possibly damaging, or probably damaging. Taken together, these data suggest a PCA-filter accuracy of 98.4% (99%CI 98.3% to 98.5%). Coverage metrics also differed significantly, with a median coverage of 25x without filter compared to 98x with filter (Kolmogorov-Smirnov D = 0.42, p<0.0001), Conclusions: The data presented herein suggest that PCA-based techniques applied to large NGS datasets for the purposes of noise-reduction are reasonably accurate. This approach might inform more reproducible approaches to NGS data filtering.

TT02. Molecular Profiling in Challenging Oncology Research Samples Using a Novel Library Preparation Chemistry  
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Introduction: Discovery and identification of new biomarkers are key drivers of research and treatment in precision oncology. Molecular characterization of tumor types through comprehensive next-generation sequencing (NGS) tumor profiling can identify new biomarkers. However, converting tissue samples into NGS libraries is often challenging due to the low quantity and quality of DNA in such samples. Here we present a novel nanomaterial-based approach to improve extraction of ctDNA from plasma. Moreover, the hydrogel particle-based approach prevented genomic DNA contamination for up to 24 hours post-collection. Extracted ctDNA from both approaches demonstrated comparable performance in the detection of EGFR mutations, with EGFR exon19del, L858R, and T790M mutation detection possible at plasma DNA concentrations of 0.5, 4, and 20 ng/ml, respectively. Patient data demonstrated concordance between ctDNA and tissue analysis in most cases, regardless of ctDNA extraction method. ctDNA analysis revealed an EGFR L858R mutation not detected by solid tumor testing. Conclusions: The magnetic hydrogel particle-based ctDNA extraction technique was highly efficient in recovering ctDNA from plasma. Moreover, this extraction technique required less starting material (1 ml plasma) than column-based approaches with similar performance in downstream applications. These results demonstrate the potential benefit of a magnetic hydrogel particle-based ctDNA extraction technique in liquid biopsy testing of NSCLC patients. On-going work is focused on integrating this extraction technique into a multi-gene liquid biopsy assay.

Technical Topics

AMP Abstracts
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TT03. Droplet Digital-PCR (ddPCR) as Confirmatory Method for Low Allelic Frequency Variants Detected by Manual Review of Data in Clinical NGS Testing

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Introduction: For detection of somatic variants, small targeted panels provide clinically significant information in a relatively quick turn-around time (TAT) to enable rapid diagnosis and selection of treatment strategies. Near the limit of detection, the variant calls by the automated bioinformatic pipeline are less reliable, especially when testing lower quality samples. Although such variants can be reviewed manually, a confirmatory methodology can also be used to ensure accurate reporting, especially in critical genes such as EGFR and KRAS. Thus, we have been using ddPCR to confirm calls that fall below the limit of detection set by the analytical in our standard operating procedure. Methods: Clinical samples are sequenced using the Oncomine Comprehensive Assay v2 (Thermo Fisher Scientific), and Ion Reporter Software (Thermo Fisher Scientific) is used to analyze sequencing data. Our limit of detection is 3% variant allele frequency (VAF) at 1,000x coverage, and 5% at 400x coverage. Occasionally, there are clinically important variants that fall below 3% VAF that are identified upon manual review of the data in the integrated genomic viewer. For all those cases, with alterations in allele frequency (VAF) at 1,000x coverage, and 5% at 400x coverage. Fisher Scientific), and Ion Reporter Software (Thermo Fisher Scientific) is used to analyze sequencing data. Our limit of detection is 3% variant allele frequency (VAF) at 1,000x coverage, and 5% at 400x coverage. For all those cases, with alterations in allele frequency (VAF) at 1,000x coverage, and 5% at 400x coverage.

Results: Of 1,464 clinical samples from July 2018 through March 2020, 15 samples had low VAF alterations in KRAS, BRAF, or EGFR that were confirmed by ddPCR. Genomic DNA concentration ranged from 0.26 ng/µL to 92 ng/µL. Samples were of different cancer types with neoplastic cellularity varying between 10% and 80%. Out of 15 samples, 13 samples were confirmed to have suspected variants with very similar allelic frequencies. Two samples always had the same variant and the concordance rate for a total of 717 variations was 98.7%, and 100% for MSI-H. In the MSI-H samples, the proportion of MSI sites identified by ddPCR. Genomic DNA concentration ranged from 0.26 ng/µL to 92 ng/µL. Samples were of different cancer types with neoplastic cellularity varying between 10% and 80%. Out of 15 samples, 13 samples were confirmed to have suspected variants with very similar allelic frequencies. Two samples always had the same variant and the concordance rate for a total of 717 variations was 98.7%, and 100% for MSI-H. In the MSI-H samples, the proportion of MSI sites identified by ddPCR was 9 and 29.5 hrs for Magnis BR and manual approach, respectively. The concordance rate for a total of 717 variations was 98.7%, and 100% for MSI-H. In the MSI-H samples, the proportion of MSI sites identified by ddPCR was 9 and 29.5 hrs for Magnis BR and manual approach, respectively.

Conclusions: The performance validation shows that the library preparation approach using the full-automatic platform Magnis BR and OncoScreen Plus panel has an excellent sensitivity, precision, specificity, and accuracy, which meet the requirement of clinical applications, and is even superior in capture on target ratio and MSI detection than the conventional manual approach.

TT04. Performance Validation of Magnis BR: A Full-Automatic Capture-Based Library Preparation Platform for Next-Generation Sequencing (NGS)


Introduction: Next-generation sequencing (NGS) with capture-based multigene panel has been widely used in cancer treatment due to its high sensitivity and specificity. However, the library preparation process involving hybrid capture requires highly skilled experimental operators and is time consuming, which limits its execution within hospital laboratory. To solve the issue, we have developed the first full-automated capture-based library preparation platform-Magnis BR. After setting up the sample and program, the platform can automatically complete the library preparation in an unattended manner, and shorten the process from 24 to 36 hrs to 9 hrs. Herein, we prepared DNA libraries on the Magnis BR with a 520-gene panel (OncoScreen plus) to further validate its performance. Methods: All samples were subjected to library preparation on Magnis BR (DNA input ≥50 ng) and sequenced on NextSeq500. Two standards with different abundances of variants were prepared: SNV (n = 4, AF: 2%, 5%), INDEL (n = 1, AF: 2%, 5%), rearrangement (n = 1, AF: 2%, 5%), CNV (n = 3, CN: 4, 5), MSI (tumor cell fraction: 10%, 20%). Each standard underwent 3 batches of library preparation with replication in each batch to verify sensitivity and precision. NA12878 served as the negative standard for evaluating specificity. To verify accuracy, parallel manual and automatic library preparations were performed with 16 clinical formalin-fixed, paraffin-embedded (FFPE) samples. Results: Compared with the manual library preparation, Magnis BR improved the capture on target ratio by an average of 8% (86% versus 78%). The sensitivities of detecting SNV, INDEL and rearrangement all reached 100% under the AF of 2%. The sensitivity for CNV in a CN of 4 was 100%, and MSI was detected with 100% sensitivity under a tumor cell fraction of 10%. The repeatability and reproducibility both achieved 100%. No false positive variant was detected. In the 16 FFPE samples, the average library preparation time was 9 and 29.5 hrs for Magnis BR and manual approach, respectively. The concordance rate for a total of 717 variations was 98.7%, and 100% for MSI-H. In the MSI-H samples, the proportion of MSI sites identified by Magnis BR was significantly increased versus that by the manual process (86% versus 57%), whereas the MSI sites detected in the MSS samples were controlled below 2% on average, suggesting that Magnis BR is more sensitive in detecting MSI. Conclusions: The performance validation shows that the library preparation approach using the full-automatic platform Magnis BR and OncoScreen Plus panel has an excellent sensitivity, precision, specificity, and accuracy, which meet the requirement of clinical applications, and is even superior in capture on target ratio and MSI detection than the conventional manual approach.

TT05. From Plasma to Variants: A Fully Automated Workflow Solution for Low-Frequency Variants in Cell-Free DNA

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Introduction: Liquid biopsy is a powerful non-invasive approach used to detect low-frequency variants in oncology research. However, there exist several challenges with this approach: manual labor-intensive handling of multiple samples, low cfDNA yield from plasma, low conversion during library preparation, loss of complexity, and low target rates during hybridization capture, and errors introduced during PCR and sequencing. Here we present a workflow that enables sensitive and accurate detection of low-frequency variants in cfDNA by using 1) the Apostle MiniMax High- Efficiency Isolation Kit that maximizes cfDNA recovery from plasma, 2) xGen Prism DNA Library Prep Kit, optimized for low-input and degraded samples; and 3) xGen hybridization capture, which provides high quality targeted sequencing. Moreover, these protocols have been automated on the Biomek i-Series systems that reduce hands-on time, increase throughput, and minimize manual errors.

Methods: Blood samples were drawn from healthy donors (N = 4) into K2EDTA tubes. Plasma (4mL) was isolated by centrifugation and cfDNA was extracted using Apostle MiniMax High-Efficiency isolation kit or a competitor kit (Supplier A) using manufacturer recommendations. cfDNA yield was quantified by qPCR. Variant calling was evaluated by spiking competitor kit (Supplier A) using manufacturer recommendations. cfDNA from 1 donor into cfDNA from another donor at 0.25% and 0.5%. Next-generation sequencing (NGS) libraries with these mixtures were generated using 25 ng or 10 ng for 0.25% and 0.5%, respectively, using either xGen Prism or a competitor kit (Supplier B) according to manufacturer instructions. Libraries were captured in 4-plex using a 75 kb custom panel of xGen Lockdown probes. Ground truth was established by ultra-deep sequencing of 100% non-mixture samples and was used to determine sensitivity and specificity. The automation of the complete workflow was enabled on the Biomek i-Series workstations. Results: We observed that the Apostle MiniMax High Efficiency isolation kit obtained 30% higher recovery compared to Supplier A’s cfDNA extraction kit. xGen Prism DNA library Prep Kit had twofold higher coverage and 1.5- to twofold higher complexity compared to Supplier B for matched cfDNA samples. Over 90% on-target rates and high complexity were obtained using the xGen hybridization capture protocol. High sensitivity (95%) and high positive predictive value (99%) were obtained at variant allele frequencies of 0.5% and 0.25%. Conclusions: In this study, we demonstrate a complete end-to-end automated solution for oncology research using cfDNA samples. This workflow is an effective solution for cfDNA extraction, library preparation, and target-capture while maintaining high library complexity.
and on-target rates. The use of UMI-based error correction enables high sensitivity and PPV.

TT06. Concordance of Variant Detection between the MoCha ctDNA Assay and Matched Tissue Biopsy in Non-small Cell Lung Cancer

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Introduction: The Molecular Characterization Laboratory (MoCha) at the Frederick National Laboratory has validated a circulating tumor DNA assay, "MoCha ctDNA," which is a plasma next-generation sequencing (NGS) assay based on TruSight Oncology 500 ctDNA, a Research Use Only (RUO) assay. The MoCha ctDNA assay interrogates the full coding region of 523 genes and intronic regions in 23 driver genes to detect translocations. In this study, variant detection concordance between the MoCha ctDNA assay and matched tumor NGS and an orthogonal digital PCR-based ctDNA assay (BEAMing) was evaluated. Methods: Tumor tissue from 49 advanced stage non-small cell lung cancer (NSCLC) patients known to have EGFR mutations in their medical record was sequenced using TSO500 (RUO). Matched plasma specimens were tested for EGFR mutations by BEAMing and sequenced with the MoCha ctDNA assay. Data from all NGS assays were analyzed by custom data analysis pipelines to identify variants that are oncogenic or likely oncogenic based on OncoKB annotation. The concordance of variant detection between plasma and tumor tissue was measured using positive percent agreement (PPA) with tumor tissue as the reference. Results: At submission, completed sequencing results were available for 18 NCSLc samples. BEAMing identified EGFR variants (n = 36) in all matched plasma samples, including driver mutations in EGFR exons 19 or 21 (allele frequency [AF]: 0.21% to 20.60%) and the subclonal T790M resistance mutation (AF: 0.03% to 6.65%). The MoCha ctDNA assay detected all 35 EGFR variants identified by BEAMing, except 1 T790M mutation with an AF of 0.03% which was below the limit of detection (LOD: 0.25%). For all the variants identified (n = 70) in the matched tumor tissue, the MoCha ctDNA assay identified 34 of 35 EGFR mutations and 31 of 35 non-EGFR mutations. The overall PPA for the NSCLC samples was 92.9% (65/70). One exon 19 deletion (p.E746_A750del) with low AF (1.15%) in tissue was not identified by the ctDNA assay. However, the ctDNA assays detected subclonal L718Q and T790M mutations that were absent in tissue in 2 patients. Additionally, variants in clonal hematopoietic and non-cancer genes were detected in 10 of 18 (55.6%) patients by the MoCha ctDNA assay, accounting for 17 of 58 (29.3%) variants in these 10 patients (AF: 0.25% to 3.16%). Conclusions: The ctDNA assay showed high concordance with both tissue analysis and plasma BEAMing assay. Our data suggest that the MoCha ctDNA assay may be adequate for sequencing applications that require the detection of subclonal variants into clinical practice for simultaneous identification of clinically relevant target genes.

TT07. Reproducibility of Allelic Fractions of Genomic Variants from Colorectal and Lung Cancer Tissue Downstream of DNA Extraction

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Introduction: Next-generation sequencing (NGS) solution achieved high coverage with >95% target bases covered at >20x with 5 Gb sequencing data with on-target rate of >95% and uniform coverage across all target space. Importantly, the deep and uniform coverage ensures detection of SNPs as well as insertions and deletions (INDELs) in an orthogonally validated reference standard with high sensitivity and specificity. In addition, xGen Exome v2 along and in combination with the CNV spike-in panel successfully detected the expected CNVs. Conclusions: This study demonstrates xGen Exome Research Panel v2.0, when combined with IDT’s DNA Library Prep Kit and modular spike-in panels, provides researchers with a complete, flexible, and fully customizable NGS solution for SNP, INDEL and CNV detection.
TT09. DNA Samples with Low Concentration Can Benefit from Speed Vacuum Concentration in NGS Testing


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Introduction: To obtain reliable next-generation sequencing (NGS) data, it is important to use high quality and quantity DNA as the starting material. DNA obtained from formalin-fixed, paraffin-embedded (FFPE) specimens is often of poor quality, as it is often fragmented and can contain contaminants. In addition, material from biopsies is often scant, resulting in low DNA yield. In these situations, to improve DNA concentration, our laboratory uses a speed vacuum, also known as a centrifugal evaporator, prior to library preparation in the Oncomine Comprehensive v2 assay by Thermo Fisher Scientific. Methods: FFPE DNA extraction was performed using Maxwell 16 FFPE tissue LEV DNA purification kits. For DNA samples that have concentrations <3.33 ng/µl, an Eppendorf Vacufuge Plus was used to concentrate them. DNA samples were quantified before and after speed vacuuming using Qubit 2.0 fluorometer. We try to achieve a DNA concentration of ≥33.3 ng/µl for a working total of 20 ng of DNA in the library preparation. DNA libraries were quantified using Ion Library TaqMan Quantitation Kit and QuantStudio 5. Templating was performed on Ion Chef and sequencing on Ion S5 XL. Sequencing data were analyzed on Ion Reporter Software 5.6.

Results: Of the 1,627 FFPE clinical cases processed since June 2018, 256 required speed vacuum concentration. For these 256 cases, speed vacuum on average improved the DNA concentration by a factor of 2.3 (mean concentrations of 1.60 ng/mL and 3.54 ng/mL before and after speed vacuum, respectively). DNA library concentrations were similar for speed vacuumed (1,741 pm) and non-speed vacuumed samples (1,839 pm). The average base coverage depth for speed vacuumed and non-speed vacuumed samples were comparable (1,705.7 and 1,685.6, respectively), as well as for the average target base coverage at 500x (94.28% and 93.88%, respectively). To directly compare the effect of speed vacuum concentration, selected samples of low extracted DNA concentration were divided, with half directly to library preparation and sequencing, and half for speed vacuum before subsequent steps. For these samples, speed vacuum improved DNA concentration by a factor of 1.8 and library quantity by a factor of 4.6. Although no difference in mean coverage was seen (p = 0.20, paired t-test), the speed vacuumed samples showed significantly higher percentage of bases reaching 100x coverage (98.8% versus 99.4%, p = 0.02, paired t-test) and percentage of bases reaching 500x coverage (94.5% versus 97.2%, p = 0.04, paired t-test). Conclusions: FFPE specimens that have low extracted DNA concentration can benefit from speed vacuuming. This process does not alter or damage DNA concentration, library concentration, and improves the percentage of targeted bases that reach sufficient coverage for analysis.


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Introduction: Plasma cell-free circulation tumour DNA (cfDNA) is increasingly important for the mutational analysis of cancer particularly non-small cell lung cancer (NSCLC). Molecular analysis of tumor biomarker genes, such as epidermal growth factor receptor (EGFR) gene testing is essential for targeted treatment of NSCLC. Liquid biopsy offers a cost-effective and low-risk alternative to tissue biopsy for testing tumor biomarkers. However, the high sensitive testing method is needed for accurate detection of extremely low levels of gene variants in cfDNA. Based on next-generation sequencing (NGS), mass spectrometry and PCR technologies, target capture sequencing, MassARRAY, and real-time qPCR assays have been developed as leading methods for testing some mutations in cfDNA. Our study is to compare the performance of these assays on testing EGFR variants in plasma cfDNA from NSCLC.

Methods: Target capture sequencing-based AVENIO cfDNA Expanded Kit (Roche), and real-time qPCR-based Cobas EGFR Mutation Test (Roche) were used for testing plasma cfDNA from NSCLC patients. MassARRAY-based UltraSEEK Lung Panel (Agena) which had been clinically validated for testing EGFR in cfDNA was used as a reference method. A total of 24 plasma samples were parallel tested by the target capture sequencing and MassARRAY-based assays. Among them, 12 plasma samples were also tested by real-time qPCR. EGFR multiplex cfDNA reference standard set (Horizon) was used for evaluating the limit of detection (LOD) of those assays. Results: The sequencing assay found 66.7% (16/24) cfDNA samples were positive for EGFR sensitizing mutations including that 50.0% (8/16) were positive for T790M. The qPCR assay tested 75.0% (9/12) and 33.3% (3/9) for EGFR sensitizing mutations and T790M. Except for 1 inconclusive sample, the MassARRAY test identified 56.5% (13/23) for EGFR sensitizing mutations and 61.5% (8/13) for T790M. The sequencing assay showed 91.3% (21/23) concordance with the MassArray test for detecting either EGFR mutations. The qPCR method showed 83.3% (10/12) and 100% (12/12) concordance with the MassARRAY test for detecting EGFR sensitizing mutations and T790M, respectively. The concordance of the 3 assays on testing EGFR variants in cfDNA was 83.3% (10/12). The sequencing test showed that the variant allele fraction ranges for EGFR sensitizing mutations and T790M were from 0.11% to 45.16% and from 0.08% to 0.26%, respectively. LOD comparison confirmed that the sequencing assay showed the lowest LOD (0.1%) for testing EGFR in cfDNA.

Conclusions: All of the 3 methods can effectively detect low fractions of EGFR variants in cfDNA. AVENIO cfDNA expanded kit possesses the highest sensitivity, and Cobas real-time qPCR needs the lowest turnaround time for testing EGFR in cfDNA.


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Introduction: In addition to circulating cell-free DNA from blood, circulating cell-free RNA (ccfRNA) has gained relevance for liquid biopsy biomarker studies. The combination of insights from both analytes provides increased understanding of molecular processes, such as tumor biology. There are still challenges to overcome in the preanalytical workflow, to preserve the valuable information of the original ccfRNA profile. In this research study, we investigated an exemplary workflow with blood collection in different tubes, transport, ccfRNA isolation from plasma and analysis with the NanoString PanCancer panel in concordance to ISO 20186-3.

Methods: Whole blood specimens from 5 healthy consented donors were collected into PAXgene Blood cfDNA Tubes (Ruo*) (PreAnalytix), RNA Complete BCT (Streck), cfDNA/cfRNA Preservative Tubes (Norgen) and BD Vacutainer K3EDTA tubes (BD). Transport was simulated by storing the tubes for 72 hours at 25°C and by performing a drop test according to ASTM D4169-14/ISO 11607. Plasma was generated with the tube supplier’s protocol and ccfRNA was extracted using the QIAamp Circulating NA Kit (QIAGEN). ccfRNA quality control was performed with the Bioalyzer 2100 instrument and RNA 6000 Pico Kit (Agilent Technologies). RNA was analyzed with the nCounter Low RNA Input Kit and nCounter PanCancer Pathways Panel (NanoString Technologies). Results: ccfRNA yield and quality was comparable between samples collected and stored in PAXgene and Streck tubes. RNA extracted from PAXgene and Streck tubes had higher average counts on the NanoString panel compared to Norgen (PAXgene 720,300; Streck 727,500; Norgen 391,400). This resulted in higher number of transcripts detected in PAXgene and Streck tubes (PAXgene 581; Streck 562; Norgen 391). The transport simulating drop test demonstrated that PAXgene tubes effectively preserve ccfRNA (by preventing cell lysis and/or release of additional RNA into the plasma) when compared to Day 0 in contrast to Streck samples (8.8% increase for PAXgene; 73.2% increase for Streck). These data suggest reduced robustness of Streck preserved samples under standard transport conditions. PAXgene Blood cfDNA Tube derived samples had most consistent results with tested transport simulation conditions.

Conclusions: PAXgene Blood cfDNA
Tubes effectively preserved ccfRNA during simulated transport conditions. The Streck RNA tube showed a significant increase in mean ccfRNA counts following drop testing, suggesting ccfRNA profile change during shipping. Samples from the Norgen tube show large variations and inconsistent results. The nCounter Low Input RNA Pan Cancer Pathway Panel was compatible with all samples used in this study. *For research use only in the US*

**TT12. Reference Materials for Measurable Residual Disease (MRD) Monitoring in Circulating Cell-Free DNA (ccfDNA)**

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**Introduction:** Assays that are designed to monitor measurable residual disease (MRD) look for the disappearance and reemergence of cancer.

The analytical validation of liquid biopsy-based MRD assays requires reference materials (RMs) that allow for the assessment of sensitivity and specificity at variant allele frequencies (VAFs) that can be over an order of magnitude below the limit of detection (LoD) of typical circulating tumor DNA (ctDNA) assays. At such low VAFs, a given sample being analyzed may average less than 1 copy of a given somatic variant. This is why some MRD workflows now use whole-exome sequencing (WES) data from patient tumors to design patient-specific assays that target multiple patient-specific somatic variants throughout the genome. Therefore, validation should include the steps needed to monitor patient-specific somatic variants.

**Methods:** We designed our RMs for a tumor/normal MRD workflow as a set of 3 components based on our Tumor Mutational Burden (TMB) and blood TMB (bTMB) reference materials. First, a lymphoblastoid cell line served as the source of normal DNA to assess specificity. Second, a germine SNP-matched tumor component provided hundreds to thousands of additional somatic variants. Third, blends of fragmented and sized DNA from those cell lines were used to mimic circulating cell-free DNA (ctDNA) and served as the input for MRD assays at tumor contents from 2% to 0.002% and at 0%. A custom error correction library was used to facilitate detection of DNA variants spanning a range of VAFs.

**Results:** Based on the number of observed somatic variants at lower VAFs, about one-third of the input DNA was converted to a sequenceable library. Maximum sensitivity was obtained when variants were called using a single observation. However, somatic variant selection, high sequencing depth, and error correction were critical in lowering the background noise sufficiently to maintain specificity and make such an approach feasible. **Conclusions:** Detecting 0.02% tumor content appears to be feasible using a customized off-the-shelf assay. These RMs should enable the development and validation of assays that have sufficient sensitivity and specificity to detect MRD at even lower tumor content.

**TT13. Automation of Fluorescence in situ Hybridization Processing and Digital Analysis**

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**Introduction:** Processing of microscope slides for fluorescence in situ hybridization (FISH) in a clinical setting can be labor intensive and is adequate concentration (A260/280 ratio of 1.8). Genomic DNA size ranged from 0.4 to 1.9 kbps (mean = 1.2 kbps) and 2.0 to 8.4 kbps (mean = 4.5 kbps) for the original and modified extraction, respectively. This equates to an approximately 4x increase in recovered DNA size for the modified method. When compared to a high quality DNA control, this also translated to nearly an 8x increase in amplification intensity of a 600 bp amplicon and a 2x increase in 400 bp amplicon. **Conclusions:** It is commonly acknowledged that DNA extracted from FFPE tissue is often degraded and suboptimal for molecular analysis. However, our results demonstrate that the extraction
Introduction: Colorectal cancer (CRC) is among the leading causes for cancer deaths worldwide. The individual risk to develop CRC depends on lifestyle, comorbidities and to a lesser extent genetic predisposition. Reliable detection of the mutational status in CRC is essential to make informed treatment choices as well as prognostic predictions. Due to the increasing possibilities of targeted treatments and the urgent need to understand underlying mechanisms mutational screening is the foundation for these advances. A useful resource for these molecular analyses is formalin-fixed, paraffin-embedded (FFPE) tissue which is the most frequently used material for histological examination of tumor tissue. However, FFPE samples for any kind of molecular analyses are challenging due to the negative impact previous processing may have on nucleic acid quality. For this reason, we compared different workflows tailored to this sample material that allow isolation of DNA suitable for sensitive next-generation sequencing (NGS)-based applications in CRC samples. Methods: DNA was extracted from FFPE tissue blocks using different extraction protocols including spin column and different automated bead-based methods to determine the most suitable protocol for DNA isolation and subsequent mutational analyses. To detect the mutational status of the respective samples, Illumina sequencing was performed using 2 different targeted panels for mutations known to be associated with CRC. One approach included unique molecular barcodes to increase confidence in the identification of mutations. Frequency of single mutations was additionally confirmed by digital PCR analyses. Results: Targeted sequencing of DNA isolated using different extraction protocols revealed an impact of the method used on the quality of results. Reliable detection of different KRAS exon 2 mutations at different frequencies was confirmed, but also dependent on the extraction technology. The protocol impacted DNA yield and integrity and the frequency of detected mutations correlated to results obtained by digital PCR. Conclusions: The broad availability and convenient storage of FFPE samples makes them a valuable resource for molecular analysis in addition to their standard use for histological evaluation. However, this apparent convenience is diminished by the often poor quality of extracted DNA which results in the need for specialized protocols. Since mutational status, especially in cancer, is essential to better understand underlying disease mechanism as well as to choose treatment options, the availability of high quality DNA is crucial. Using the example of CRC we demonstrate that by applying an isolation method carefully optimized for high sensitivity applications a reliable detection of mutational status can be achieved.

TT17. Centrifugation and RBC Lysis-Free Preparation of Blood Samples in less than 30 Minutes


Introduction: Processing whole blood (WB) to remove red blood cells (RBCs) and/or isolate peripheral blood mononuclear cells (PBMCs) is a common first step in many diagnostic applications. RBCs are typically removed from a blood sample using a RBC lysis agent such as ammonium chloride, whereas PBMCs are prepared using density gradient centrifugation (DGC). Both procedures are time consuming, cumbersome and involve numerous centrifugation steps. We have developed rapid, easy to use immunomagnetic based methods to either remove RBCs or the combination of RBCs, platelets and granulocytes directly from WB in 25 minutes. Both procedures were compared against their current gold standard of lysis or DGC, for RBC clearance and PBMC isolation, respectively. Methods: For RBC clearance, WB was either lysed using ammonium chloride, or RBCs were removed using the EasySep RBC Depletion Reagent. PBMCs were isolated from WB by DGC separation or using the EasySep Direct PBMC Isolation kit. For immunomagnetic separation, unwanted cells (RBCs and/or platelets and granulocytes) were labeled with antibody complexes and magnetic particles and then placed into a magnet. Labeled cells were retained in the magnet, while untouched nucleated cells or PBMCs were poured into a new tube. Both immunomagnetic procedures were fully automated on the RosoSep-S instrument. Results: RBC clearance: Starting with 1 mL of WB, 5.9 ± 4.4 x 10e6 and 7.2 ± 5.7 x 10e6 CD45+ cells were recovered using the EasySep RBC Depletion Reagent (average of all magnet platforms) versus lysis (mean ± SD, n = 39/27). RBC removal was significantly better with the RBC depletion reagent compared to lysis (0.9 ± 2.4% versus 36.6 ± 25.6% residual RBCs, respectively). PBMC isolation: Starting with 1 mL of WB, 1.9 ± 1.2 x 10e6 and 2.0 ± 1.8 x 10e6 PBMCs were recovered using the EasySep Direct PBMC Isolation kit versus DGC (mean ± SD, n = 24/13). Importantly, both methods maintained the relative frequency of monocytes and lymphocytes. Although cell recovery was similar between the 2 methods, EasySep Direct PBMC isolation had significantly lower granulocyte (1.5 ± 2.7% versus 6.5 ± 3.9%), platelet (0.6 ± 0.7% versus 57.9 ± 23.5%), and RBC (4.2 ± 9.0% versus 10.8 ± 11.0%) contaminations compared to DGC. Conclusions: Cleaner cell prepartions can be obtained in 25 minutes without compromising cell recovery when using the EasySep RBC Depletion reagent or EasySep Direct PBMC Isolation kit compared to standard lysis or DGC. Both immunomagnetic cell preparation methods can be fully automated using the RosoSep-S, enabling a rapid, reproducible and scalable procedure that can be performed with minimal hands on time or personnel training. EasySep provides a versatile tool to prepare blood samples for downstream diagnostic applications.

TT18. A Complete Yet Flexible Workflow for Library Preparation and Analysis with Enhanced Error Correction for Low Input FFPE Tissue Biopsy and Circulating Tumor DNA Samples

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Introduction: Formalin-fixed, paraffin-embedded (FFPE) specimens and circulating tumor DNA are challenging materials for variant analysis due to limited DNA availability, poor DNA quality, and variation in tumor fraction from sample to sample. Other practicality including turnaround time, multiplexing capability or workflow as well as technology limitations such as sample index cross-contamination and hopping with newer Illumina sequencing chemistries can also affect how readily next-generation sequencing (NGS) is applied to clinical tumor samples. To overcome these limitations, we have developed a complete yet flexible library prep system. The workflow incorporated inline duplex molecular barcodes at the ligation step to filter out PCR errors and sequencing by error by making consensus calls using MBC information. We also have employed unique dual sample indices at precap amplification step to eliminate index hopping associated with newer Illumina sequencing chemistries. Our analysis pipeline enables various MBC modes that can be chosen based on the applications; both strands (duplex MBC), hybrid (duplex MBC where info is available), single strand (single MBC) or discard MBC information (no MBC). Methods: Sequencing libraries were constructed with circulating tumor (ct) DNA, FFPE, FF, and hemap DNA samples using SureSelect XTHS2 library prep kit. Picard and AGeNT tool were used for analysis. Results: We demonstrate library prep robustness across multiple users and varying FF and FFPE gDNA quality as well as 30 circulating tumor DNA samples. The MBC data analysis demonstrates that whereas both duplex and single MBC improve the accuracy of low VAF detection compared to no MBC, duplex MBC enabled the most effective error correction. The number of false positive calls of low VAF (≤4%) were reduced significantly by duplex MBC. The hybrid MBC analysis mode exhibited similar recall rate to single MBC, but with higher PPV (lower FP calls) at similar sequencing depth. Conclusions: The workflow we describe here is flexible and covers a wide range of DNA input (10 to 200 ng), various sample types (intact or FFPE samples), different shearing methods (mechanical versus enzymatic), fast or
TT19. Next-Day Analysis from Specimen to Variant Calling with the Genexus System
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Introduction: The ability to identify oncogenic variants accurately and rapidly is a key challenge of oncology research. Analysis from specimen to variant calling report usually involves many manual steps to process specimens, quantify yields, and prepare libraries prior to analysis by sequencing. Here we report automated workflows that use tissue as input and yield variant calling results the next day with the Genexus System.

Methods: Specimens used in this study include human blood, plasma, buffy coat, and formalin-fixed, paraffin-embedded (FFPE) tissue. The Genexus Purification System was used to isolate ctDNA, DNA, and/or RNA from the samples. Purified nucleic acid samples were used as input for the Genexus Integrated Sequencer and analyzed with an appropriate Oncomine assay for the given sample and targeted variants. Results: Up to 12 FFPE sections were processed at once to yield paired DNA and RNA isolates from each specimen. Sufficient yield of at least 30 ng per purification was identified in sequencing analysis reports produced the day after starting workflows. The automated workflows of the Genexus System provided a robust NA extraction solution from a variety of sample types using the Genexus Integrated Sequencer and analyzed with an appropriate Oncomine assay for the given sample and targeted variants. The workflows for FFPE, multi-sample DNA and RNA can accommodate up to 12 samples for FFPE, multi-sample DNA and RNA, and up to 6 samples for ctDNA in a single run. The results demonstrated consistent yield with less than 10% CV within runs and between runs across instruments. Furthermore, the performance of fully integrated workflows from sample to sequencing was also evaluated for both FFPE and plasma samples using Oncomine Precision Assay and demonstrated quick turnaround time for less than 24 hours for a batch of 4 FFPE and 6 plasma samples, respectively. The results reported all expected variants previously confirmed, including BRAF V600E, KRAS G12C, PIK3CA N545K, ERBB2 amplification, etc.

Conclusions: Overall the study demonstrates that Genexus purification system provides a robust NA extraction solution from a variety of sample types including FFPE, biofluid, and plasma etc. Together with Genexus Sequencer, the system enables automated NA extraction, purification, and quantification with minimal user touchpoints from specimen to variant report with single-day turnaround time.

TT21. Analytical Performance Testing of the MoCha Circulating Tumor DNA Assay
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Introduction: Next-generation sequencing (NGS) technologies and automatic workflows of the Genexus System and the Genexus Integrated Sequencer allow next-day identification of variants from blood and FFPE specimens. The 2 instruments, used in series and linked by software that allow tracking of each specimen from start to finish, simplify and expedite analysis of liquid, solid, and FFPE tissue samples. The Genexus instruments are for Research Use Only; not for use in diagnostic procedures.

Methods: Liquid biopsies may provide a valuable tool for clinical management of cancer patients by detecting circulating tumor DNA (ctDNA) in cell-free DNA (cfDNA). The Molecular Characterization Laboratory (MoCha) ctDNA assay, a next-generation sequencing (NGS) assay on the TruSight Oncology 500 (T500; For Research Use Only) ctDNA assay. The MoCha ctDNA assay, which interrogates the full coding region of 523 genes and intronic regions for translocation detection in 23 driver genes, as well as reporting copy numbers in 59 genes, is being analytically validated in our laboratory.

Methods: Libraries were prepared using ≥50 ng cfDNA with unique molecular identifiers and duplex barcodes for error-correction, then enriched by target capture and sequenced on a NovaSeq 6000. Using contrived materials, limit of detection (defined as ≥80% of variants positively identified, [LOD80]) was assessed for single nucleotide variants (SNVs, n = 25), insertions/deletions (indels, n = 14), translocations (n = 5), and copy number variants (CNVs, n = 4). Negative percent agreement (NPA) was assessed using technical replicate libraries from healthy donors (n = 81 libraries from 27 donors) with paired white blood cells. A late-stage non-small cell lung cancer (NSCLC) cohort of 24 pre-treatment plasma samples from 18 patients with known EGFR mutations in their medical record was tested with digital PCR for EGFR mutations. Positive percent agreement (PPA) between digital PCR and the MoCha ctDNA assay was calculated, with digital PCR used as the reference standard for assessment. Results: Predetermined quality metrics were met in ≥85% of samples, with a median Median Exon Coverage of 2,103 and 2,034 for contrived and cfDNA samples, respectively. The LOD80 was determined to be 0.25%, 0.25%, 0.50%, and 1.3-fold change (FC) for SNVs, indels, translocations, and CNVs, respectively. The limit of reporting (LOR), set at or above the LOD80, was established at 0.5%, 0.5%, 1.0%, and 1.3 FC for SNVs, indels, translocations, and CNVs, respectively. NPA at established LOR was >99.998%. This represents 7 putative false positives, consisting of 3 unique variants, in 81 unique libraries. Variants likely derived from clonal hematopoiesis (n = 11) were reported above the LOR in 7 healthy donors, all of which were reported in all replicates. PPA was 100% for EGFR mutations at or above LOR. Conclusions: In this technical performance study, the MoCha ctDNA assay exhibited high PPA, and NPA, demonstrating high technical performance. Based on
these findings, MoCha intends to use the MoCha ctDNA assay in support of NCI-sponsored clinical trials.

TT22. Digital PCR Paired with High-Speed AFM for Quantitation and Length Analysis of DNA Length Polymorphisms
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Introduction: DNA length polymorphisms have a significant clinical impact, but they are especially difficult to diagnose due to their repetitive or variable elements. For nucleotide repeat expansions and indel polymorphisms associated with cancer, existing assays present shortcoming in consistency, accuracy, or cost, particularly in cases where variants do not exhibit a monomeric wild-type background. To address these challenges, we developed digital polymerase chain reaction (dPCR) followed by high-speed atomic force microscopy (HSAFM) as a high-throughput, single-molecule approach for quantifying and sizing length polymorphisms in a mixed sample. We focused on the case of internal tandem duplications (ITDs) within the FLT3 gene, a length polymorphism associated with acute myeloid leukemia (AML). FLT3-ITDs range in length from <30 bp to >200 bp, and their length, expression level, and insertion location are linked to AML outcome. Methods: In the first step of our approach, dPCR, the mixed sample was diluted and partitioned into micro-reactors that were individually amplified, thus mitigating amplification bias and producing homogeneous solutions of either wild-type (WT) or variant amplicons. We then used HSAFM to directly image individual amplicons and determine their lengths with nanoscale resolution. Finally, length distributions from each micro-reactor were analyzed with Bayesian analysis to determine their most likely character – WT or variant – and variant allele frequency (VAF) was simply calculated to be the proportion of variant-identified micro-reactors. We tested 1) synthetic and cell line variants present in a mostly wild-type background. To address these findings, MoCha intends to use the MoCha ctDNA assay in support of NCI-sponsored clinical trials.

TT24. Evaluating Effects of PCR Instruments and Temperature Ramp Rates to Base-Composition Bias in TruSight Oncology 500 (T5000) Panel
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Introduction: Targeted next-generation sequencing (NGS) allows for thousands of DNA samples to be interrogated simultaneously in a fast and economical manner for the detection of clinically significant variants. Robust analytical performance and accurate assessment of variants in cancer patients is critically important for prognostic and therapeutic implications. One limitation of NGS is that long (>200 bp) repetitive insertions are underrepresented and reduced quality at loci with extensive repetitive DNA. Here, we evaluate PCR instruments and conditions of the Illumina TruSight Oncology 500 (T5000), which contains probes for 523 genes. Methods: DNA was extracted from 9 clinical formalin-fixed, paraffin-embedded (FFPE) samples and Hammap DNA R24631, and sequencing libraries for the T5000 assay were constructed according to the manufacturer’s instructions except as follows. The “Amplify Enriched Area” step (FL-PCR) in the library preparation protocol was performed in parallel using 2 different models of thermocyclers with default settings: Bio-Rad C100 Touch (thermocycler 1) and Eppendorf Mastercyler pro S (thermocycler 2). Heating and cooling rates were 3.0⁰C/s and 2.4⁰C/s on Thermocycler 1 and 5.4⁰C/s and 3.9⁰C/s on Thermocycler 2. Libraries were sequenced using NextSeq 5500A. QC Metrics were evaluated by TSO 500 v2.1. Local App, and coverage for 9,943 COSMIC sites were observed. Results: Percentage of exonic bases with coverage ≥20X and low coverage (less than 100X) in COSMIC sites were reduced by 62.1% using Thermocycler 1: 36 (0.36%) and 95 (0.95%) out of 9,943 COSMIC sites were low coverage using Thermocycler 1 and 2, respectively. The 36 low coverage sites for both methods were in CEGBA (29), W1T1 (2), FOXL2, PAX8, PHOX2B, SH2B3, and SRSF2. Despite the high GC-content, 59 sites were sufficiently covered by thermocycler 1 only (rRange: 100.5 to 529.5x; median: 223x): T5000 (11), CEBPA (4), SRSF2 (7), CNOT2 (5), ARID1A (4), BCL2 (4), SH2B3 (4), VHL (3), SMAD2 (2), NOTCH1 (2), RBP1 (2), RUNX1 (2), BCR, BRD4, CIC, FOXL2, GATA2, AKT3, JUN, NFKB1-1, SOCS1, SUZ12, TCF3, TERT, WT1. As an example, the SRSF2 c.284 read depth improved from median coverage of 16.5x using thermocycler 2 to 215.5x using thermocycler 1. Conclusions: We found that the thermocycler and temperature ramp rate can play a drastic role in coverage of GC-rich sequences. Slower temperature ramping resulted in better coverage in GC-rich regions, likely due to improved specific primer binding to the template by enabling annealing at the exact Ta without the formation of interfering secondary structures. Effort to minimize the amplicon bias and to achieve the coverage evenly across all
target areas is critical for robust detection of clinically relevant variants in targeted NGS assays.

TT25. Evaluation of the Biocartis Idylla ctEGFR Mutation Assay on Samples with DNA Concentrations Insufficient for Next-Generation Sequencing (NGS)

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Introduction: Identification of mutations in the Epidermal Growth Factor Receptor (EGFR) are critical for therapeutic selection in patients with non-small cell lung cancer (NSCLC). Next-generation sequencing (NGS) is a common method for detecting somatic mutations in the EGFR. For NSCLC, specimen adequacy for diagnosis and ancillary testing can be a concern as many samples are fine needle aspirates (FNA) and small biopsies. Here we describe a process for using the Biocartis Idylla ctEGFR Mutation Assay that could be utilized if a tissue sample has an insufficient amount of gDNA to meet the input threshold of an NGS assay (40 ng for our in-house assay). Methods: FNA and small biopsies from NSCLC (n = 13), which had previously undergone NGS to determine the mutation profile, were identified from a departmental database. Samples were quantified using the Qubit dsDNA HS Assay (Thermo Fisher Scientific). DNA was diluted in nuclease free water such that 10 ng of DNA were present in a 10 μL final volume (1 ng/μL). Each sample was then transferred to a Biocartis Idylla ctEGFR Mutation Assay cartridge. A dilution series was performed on 1 sample to determine the initial target concentration (10 ng). The cartridges were run on the Biocartis Idylla Platform. If the amplification of the EGFR gene (internal control) was invalid, the assay was repeated by increasing the target DNA input by 10 ng. Results: Of the 13 samples that were tested, 11 passed (85%) at concentrations of 40 ng or less. Of those, 8 passed at 10 ng and 3 at 20 ng. Two of 4 samples that initially failed at 10 ng were successfully re-tested using a higher DNA input. All results, except one, were concordant with the EGFR variant determined by NGS (91%). One sample gave a valid but negative result by Idylla at 20 ng when an EGFR variant was detected by NGS (false negative). Conclusions: We found that the majority of samples were successfully tested using this cartridge-based assay at a concentration of 40 ng. We demonstrated that DNA samples with low concentrations not suitable for NGS can be rescued by testing with the Idylla platform so that some actionable results may be returned to the oncologist. Some samples initially invalid were successful with higher input DNA. The Biocartis Idylla ctEGFR Mutation Assay shows promise for testing samples with inadequate DNA for NGS.

TT26. Analysis of Simple and Complex Variants and Biomarkers for Comprehensive Genomic Profiling (CGP) of Solid Tumors and Hematologic Malignancies Using a Single NGS Workflow from FFPE and cfDNA Samples

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Introduction: Comprehensive genomic profiling (CGP), a cornerstone of oncology research, involves the analysis of several types of biomarkers and variants including SNVs, small indels (<20 bp), large indels (>20 bp), copy number variants (CNVs), structural variants, TMB and MSI, fusions, exon skipping (ES), alternatively spliced variants (ASVs), and gene expression changes. Current CGP approaches are challenging as they require 2 separate eluates and 2 workflows, 1 for DNA and another for RNA. To overcome these challenges, we developed the QIAseq Pan Cancer Multimodal Panel, a streamlined, single workflow and informatics pipelines for the analysis of a wide range of variants and biomarkers for oncology research. The panel covers 605 targets, for a total of 1.4 Mb of targeted region, thereby enabling TMB analysis. It has been designed to be compatible with formalin-fixed, paraffin-embedded (FFPE) and cfDNA samples, and has been optimized to cover exonic regions of genes, and exons of RNA transcripts in both 3’ and 5’ directions for the detection of known and novel fusions. Additionally, the panel covers a number of genomic loci for MSI analysis. Methods: The performance of the panel was verified using several FFPE and cfDNA reference samples from SeraCare. TNA from FFPE samples were extracted using modified QIAGEN protocols. DNA from cfDNA reference samples has already been extracted by SeraCare. Twenty (20) to 50 ng of TNA were used as input for library construction. Libraries were constructed using the PCP workflow, quantified, and sequenced either on a MiSeq or NextSeq. FASTQ files were processed with pre-configured analysis pipelines using the CLC Genomics Workbench. Results: All variants in reference samples were detected. On the DNA front, SNVs and indels were detected in cfDNA samples at VAFs <1%, whereas they were detected in FFPE samples down to 1% VAF. Complex variants such as the CALR type-1 deletion (52-bp deletion) and FLT3 ITDs were also detected. The panel called 2 insertions in CEPBA, a GC-rich gene. Analysis of CNVs showed that the panel can call CNVs at both the gene and exon levels by accurately calling 6 additional copies of the EGFR, MET, and MYCN genes. TMB scores were accurately called as “low” or “high” in the low or high TMB reference samples, respectively. On the RNA front, all fusions, ES, and ASVs covered by the panel were correctly identified, including, but not limited to, NTRK1, NTRK2, NTRK3, BCR, ALK, and RET fusions. The panel was designed to cover only 1 partner of all detected fusions. Conclusions: The results shown in this study provide proof-of-principle evidence that the QIAseq Pan Cancer Panel enables CGP from FFPE and cfDNA samples using a single workflow with TNA or DNA as input for both solid tumors and hematologic malignancies.


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Introduction: A liquid biopsy test is like finding a needle in a haystack (plasma). Such a test requires a real “needle-in-a-haystack” to validate. However, the availability of real patient samples is a scarce. Many alternative sample types are being used currently. To study the suitability of using synthetic material, as well as the effect of plasma-specific factors on ctDNA assay, we conducted the following study. Methods: Validation samples were made by spiking either 170 bp synthetic ctDNA fragments or cell-derived, nucleosomal fragmented genomic DNA (gDNA) into 2 types of matrices: plasma-based (DNA-depleted human plasma and untreated human plasma) and cell-based (TE671 and K562 cell lines, including, but not limited to, “synthetic plasma”). After extraction, recovery of ctDNA, load-dependent drift in quantification, precision of DNA quantitation, and limit of detection (LOD) were determined using qPCR. Results: The extracted DNA from plasma-based matrices was significantly different from that extracted from buffer-based matrices in recovery, in replicate standard deviations (SD), and in the limit of detection (LOD). LOD was much higher for samples in plasma-based than samples in buffer-based matrices. Synthetic ctDNA fragments behaved significantly differently from the nucleosomal fragmented gDNA. First, the patterns of extraction recovery are different. Recovery of synthetic DNA was much higher from buffer-based matrices than from plasma-based matrices. However, slightly but significantly more gDNA fragments were recovered from plasma-based matrices than from buffer-based matrices. Second, varying the amount of DNA input to the qPCR caused a drift in quantitation in gDNA, but not in synthetic DNA. A step-wise increase of input gDNA from 2 to 4.5 µL caused a steady increase of calculated copies to more than 30% (where the calibrators were synthetic DNA). This was not seen when extracted synthetic DNA was tested in the same condition. This suggested that plasma factors affected synthetic DNA differently from cell-derived gDNA. The increase of gDNA quantitation was abolished by an extensive treatment of proteinase K to the plasma prior to extraction, suggesting an involvement of co-eluted plasma proteins in the quantitation drift. Conclusions: The data demonstrated that assay of ctDNA is influenced by plasma factors co-eluted with the DNA. This is especially the case when cell-derived gDNA was used. Synthetic DNA behaves differently from gDNA, suggesting it may not be suitable to use as a surrogate for native ctDNA from patients.

Further studies are needed. The effects of plasma factors were not observed from buffer matrices, suggesting that validation samples and quality controls need to be made with human plasma as the appropriate matrix to ensure correct measurement of assay variation and sensitivity.
TT28. Archival FFPE and DNA Quality: Optimal Storage Time and Predictive Metrics for Next-Generation Sequencing
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Introduction: Archived formalin-fixed, paraffin-embedded (FFPE) tissues are used for routine clinical sequencing and are an invaluable source of biospecimens for research. Long-term storage (LTS) reduces the recovery of high-quality DNA needed for next-generation sequencing (NGS), particularly capture-based methods. Basic DNA measurements fail to predict the likelihood of successful sequencing. We aimed to identify the effects of LTS on DNA quality and library preparation, determine optimal storage time (ST), develop predictive metrics for sequencing, and assess DNA repair methods.

Methods: A total of 262 FFPE specimens (cohort 1) stored for <1 to 17 yrs underwent hybrid-capture NGS. Amplified library concentrations and Sequence Quality Prediction Ratio (SQPR) were compared against total ST. An additional 24 matched FFPE samples (<1 versus 5 yrs; <1 versus 10 yrs) (cohort 2) underwent paired analysis of DNA measurement, fragmentation, mechanical shearing, and qPCR.

Results: Cohort 1 analysis showed a negative correlation between ST and pre-hybridization amplified library concentration (r = -0.59, p = 0.00001). FFPE blocks stored >2 yrs have a 17-fold lower likelihood of failure by SQPR compared to those stored ≤2 yrs (OR = 17.38; p = 0.0026). Cohort 2 analysis showed that the Qubit/Nanodrop ratio decreases significantly from <1 versus 10 yrs (0.46 ± 0.06 versus 0.07 ± 0.05; p = 0.013), but not as significantly from <1 versus 5 yrs (0.33 ± 0.03 versus 0.47 ± 0.13, n = 5; p = 0.024). Although gDNA fragment lengths decreased with time, sheared DNA showed slight higher (0.33 ± 0.03 versus 0.47 ± 0.13, n = 5; p = 0.024) and >0.017 (0.33 ± 0.03 versus 0.47 ± 0.13, n = 5; p = 0.024) increases in means from <1 versus 5 yrs. These results demonstrate that a majority of fusions detected on NGS do not confirm with alternative methods at our institution. Although some of these fusions may be accounted for by poor quality NGS reads, read counts too low to reliably be confirmed, or suboptimal primer choice; these factors alone are unlikely to account for most of the unconfirmed cases. Given the large number of fusions which do not confirm there remains a need to standardize the reporting of fusions detected on NGS across laboratories. Pooling data across labs utilizing similar methods of fusion detection could greatly enhance our understanding of why certain fusions do not consistently confirm and eventually lead to guidelines on reporting of fusions detected on NGS.

TT30. Development of a Universal Probe System for Droplet Digital PCR
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Introduction: Rapid detection of single nucleotide variants at low allele frequency is often needed to confirm results from next-generation sequencing (NGS). Given the low limits of detection that are possible with unique molecular indices and deep sequencing, a confirmatory test should be able to detect variant allele frequencies at or below 0.1%. Droplet digital PCR with TaqMan probes has proven to be a reliable platform for this; however, the cost and time to synthesize target-specific probes can preclude incorporating this process into clinical care. Therefore, we developed a universal probe system for droplet digital PCR that only requires unlabelled primers. Methods: We utilized DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue. Multiple combinations of primer and test designs were developed in order to facilitate multiplexing and specific combination. Detection using EvaGreen and molecular beacon probes were compared. Introduction of mismatch nucleotides and stringent amplification cycling was required to minimize non-specificity without the use of specialized bases such as linked nucleic acids.

Results: The optimal design for sequence specificity ultimately consisted of a single well molecular beacon design with sequence specific primers (each with 2 introduced mismatches, as well as a 5′ molecular beacon binding site), low concentration adaptor primers, FAM and HEX molecular beacon, and a shared reverse primer. Using this design, we were able to consistently detect the IDH1 R132H variant at a level of 0.1%. Running the design with wild type only DNA we observed a false positive rate of 0.01% at a high DNA input amount (360 ng) and did not observe false positives with a DNA input of 25 ng within the replicates performed.

Conclusions: This method represents a promising method for low level variant detection using ddPCR. The use of the molecular beacon detection design allows this method to potentially be adapted to other variants using only standard oligonucleotides, without the need for development of specialized probes or primers. The benefit of a universal molecular beacon design compared to a similar design using EvaGreen detection is the possibility of increasing the DNA input to a level not possible with EvaGreen. This design has the potential to be used in the verification of low-level variants observed in NGS, as well as the potential to be used for low level variant detection in various cell-free DNA applications.

TT31. Targeting Clinically Significant “Dark” Regions of the Human Genome with High-Accuracy Long-Read Sequencing
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Introduction: There are many clinically important genes in “dark” regions of the human genome, with low coverage due to poor amplification and/or mapping difficulties. These dark regions often contain highly homologous genes and pseudogenes, repeat expansions, and/or areas of biased base composition. Ambiguous mapping due to the limitations of short-read alternative method (35%). Whereas there was found to be a strong trend towards fusions confirming with a high number of reads, there are notable examples of fusions including EF3E1(1)-RSPO2(2) and SL3C4A2(4)-ROS1(32) where confirmation did not consistently correlate to the number of reads. Within the cases evaluated a case with a total fusion read count of as low as 48 reads (EM46(2)-ALK(20)) confirmed, whereas a case with as many as 33,342 total reads (NF1(1)-PSMD1(2)) did not confirm by an orthogonal method.

Conclusions: The results demonstrate that a majority of fusions detected on NGS do not confirm with alternative methods at our institution. Although some of these fusions may be accounted for by poor quality NGS reads, read counts too low to reliably be confirmed, or suboptimal primer choice; these factors alone are unlikely to account for most of the unconfirmed cases. Given the large number of fusions which do not confirm there remains a need to standardize the reporting of fusions detected on NGS across laboratories. Pooling data across labs utilizing similar methods of fusion detection could greatly enhance our understanding of why certain fusions do not consistently confirm and eventually lead to guidelines on reporting of fusions detected on NGS.

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sequencing prevents accurate assessment of structural rearrangements and importantly, short-reads limit the ability to phase across gene regions. **Methods:** We investigated several important genes in dark region using PacBio SMRT Sequencing, targeting with long-range PCR or the No-Amp CRISPR-Cas9 direct sequencing approach. Genes investigated included SMN1/2 (SMA, spinal muscular atrophy), CYP21A2 (congenital adrenal hyperplasia), GBA (Gaucher disease), HTT (Huntington disease), FMR1 (fragile X syndrome), HBB (beta thalassemia), HBA1 and HBA2 (alpha thalassemia), and DMD (Duchenne muscular dystrophy). Our aim was to cover regions of pathogenic mutations in a single contiguous sequence or set of sequences that can be assayed in a single reaction. **Results:** Here we show sequencing results from several of the genes listed above with high accuracy long (HiFi) reads. Using Coriell samples with relevant mutations, we identified clear and full-length phasing of mutations, and had no difficulties distinguishing gene from pseudogene sequences. For genes with repeat expansions, we could accurately determine the number of repeats and interruption sequences. Structural variations, including deletions, insertions, homologous recombination, and gene conversions, were also apparent in some samples. **Conclusions:** We demonstrate that SMRT Sequencing provides new opportunities for sequencing clinically relevant but difficult regions of the human genome that are underrepresented in short-read sequencing. Accurate long reads provide important phasing information, determine number of repeats and repeat interruption sequences, identify structural variations, and avoid potential confusion with pseudogenes. SMRT Sequencing of these regions enables a better understanding of the relationship between genetic factors and personal health, and has the potential to ultimately help guide health-related decisions.

**TT33. Standardizing Plasma ctDNA Measurements Using SNAQ-SEQ ONCO1LB Internal Controls**

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**Introduction:** Spike-in controls are widely used in molecular diagnostic assays for various quality purposes such as ruling out PCR inhibition. Similar controls are not routinely used in next-gen sequencing (NGS) methods but could help monitor the NGS process in individual samples. This is even more important for liquid biopsies used to characterize tumor mutation profiles from plasma. We describe the use of Standardized Nucleic Acid Quantification spike-in controls for NGS (SNAQ-SEQ ONCO1LB; AccuGenomics) that have the potential to improve the traditional performance limits by providing direct sensitivity and specificity control for every target in every sample. **Methods:** ctDNA were extracted using the MagMAX Cell-Free Total Nucleic Acid Isolation Kit (Thermo Fisher). The spike-in control mixture is synthetic DNA that can be distinguished from genomic DNA by unique mono- or di-nucleotide alterations every 50 bp. The controls were spiked into the plasma before extraction or before the start of the library preparation at an equimolar ratio. Seraseq ctDNA complete and ctDNA mutation mix at different allele frequencies (from 2% to 0.1%) were used as reference materials (SeraCare). Extractions were also made using healthy donor plasma. Targeted libraries were prepared using Oncomine Pan-Cancer Cell-free assay reagents (Thermo Fisher). A modified reference genome, design.bed and hotspot.bed file directed the Ion-Torrent variant caller to generate a VCF that included all alts for genomic and control positions. A SNAQ-SEQ R-script analyzed the VCF using a Poisson Exact Test to determine how significant each genomic variant was above NGS background error, calculated sample abundance of the variant using the ratio of variant to control coverage and generated a new VCF with the SNAQ-SEQ analysis appended to the GT field. **Results:** Sequenced libraries with or without spike-in controls showed the same quality metrics. The spike-in controls did not affect variant call either when added before extraction or before library preparation. The distribution of the complexity control sequence replicates (4,054 possible sequences) followed a Poisson distribution (0.98 fold of expected distribution). There was no detectable bias arising by oligo synthesis as each base ranged from 24% to 27% across the 6 positions. The loss of CC during NA isolation was 17% from plasma to library prep. The deduplication resulted in a 10% loss of CC during NA isolation was 17% from plasma to library prep. The deduplication resulted in a 10% loss of CC during NA isolation was 17%. The SNAQ-SEQ analysis appended to the GT field. **Conclusions:** The Complexity controls demonstrated an independent QC for library capture efficiency. Its integration with LOB controls and standardization of variant levels in plasma are warranted.

**TT34. Optical Mapping Enables High-Throughput Analysis of Pathogenic Repeats**

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**Introduction:** Robust repeat elements are abundant and diverse in the human genome. They are also genetically unstable. Repeat expansions and contractions could impact protein structure and gene regulation. They underlie disorders such as fociapocalumeral muscular dystrophy (FSHD) and amyotrophic lateral sclerosis (ALS). However, sequence analysis of these regions can be challenging. Clinical laboratories often rely on Southern blotting, which is labor-intensive and typically requires radioactive staining. Optical mapping with Bionano Genome Imaging provides an alternative high-throughput workflow that overcomes these limitations. The use of high molecular weight DNA molecules up to mega-base pairs in size allows large repeat structures to be spanned and elucidated. The repeats can thus be accurately sized. The single-
molecule, amplification-free method also allows mosaic repeat alleles to be analyzed. **Methods:** We obtained 12 FSHD-positive cell lines from the Coriell Cell Repositories with known pathogenic repeats. We collected optical mapping data on the cell lines and developed a pipeline to automatically analyze the FSHD-relevant D4Z4 repeat on chromosome 4q35 and the haplotype background. We also developed quality-control metrics that can point to sample or data quality issues. As proof of concept, we analyzed other repeats in the genome using a similar method. **Results:** Our results from the FSHD analysis were concordant with the Coriell annotations. Six of the cell lines were analyzed in triplicates; the results were all consistent. We also analyzed 58 FSHD-negative samples; none had repeat counts in the clear pathogenic range. Because whole-genome data are collected during optical mapping, information about other repeats is available. We showed that control samples had repeats in the normal size ranges. **Conclusions:** The Bionano workflow includes sample preparation, DNA imaging, and genomic data analysis, and enables high-throughput analysis of large repeat regions of interest and pathogenic expansions and contractions. It also provides valuable genome-wide structural variation information for disease studies.