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O11. Multitumor Profiling of Lymphocyte Activation Gene 3 (*LAG*-3) and Association with Immune Cell Phenotypes

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Introduction: *LAG-3* negatively regulates T-cell activation, is expressed on exhausted T cells, and may promote regulatory T-cell activity. By limiting antitumor T-cell activation, *LAG-3* may contribute to immunotherapy nonresponsiveness, as observed in patients with melanoma who progressed during prior anti-PD-(L)1 therapy (Ascierto *et al. Ann Oncol*. 2017;28(Suppl_5): abstract LBA18). Here we describe first results from comprehensive multitumor profiling using quantitative immunohistochemistry (IHC) to characterize expression of LAG-3 and its ligand, MHCII, in the context of inflammation markers, as well as a bioinformatic investigation of *LAG-3* using The Cancer Genome Atlas (TCGA).
Methods: Urothelial, gastric, non-small cell lung cancer, renal cell carcinoma (RCC), squamous cell carcinoma of the head and neck, and melanoma tumor specimens (N=245) were stained by IHC for LAG-3, CD8, FOXP3, CD68, CD163, PD-L1, and MHCII. The proportion of total nucleated cells in the tumor microenvironment expressing a given marker was determined using image analysis, and unsupervised clustering was used to identify subgroups within tumor types. A 160-gene T-cell-inflamed signature was applied to TCGA RNA-sequencing data to assess correlations between LAG-3 and IFNγ-induced gene expression.
Result: Unsupervised clustering of IHC results revealed inflammation-high, -moderate, and -low subgroups, and LAG-3 expression generally correlated with the level of inflammation: CD8 (r=0.65); CD68, CD163, and FOXP3 (r=0.49-0.53). MHCII tumor-cell expression was observed in inflammation-high and -low tumors and did not correlate with PD-L1 positivity, whereas LAG-3 was significantly higher in tumors with MHCII expression ≥1% vs < 1% (*P*=0.001). In 6 individual tumors with heterogeneous MHCII tumor-cell expression, LAG-3 was higher in MHCII^{hi} (>70%) vs MHCII^{lo} (< 10%) regions (*P* range=0.001-0.070). TCGA analysis was consistent with IHC analyses, demonstrating a strong correlation of LAG-3 mRNA expression with CD8, PD-1, and CTLA-4 (r=0.81; r=0.87; r=0.69), moderate correlation with PD-L1 and MHCII (r=0.47; r=0.58), and correlation of LAG-3, CD8, and PD-1 mRNA expression with T-cell-inflamed gene signatures across tumor types. Exploratory analyses of clinical trials in RCC and melanoma showed increased mean LAG-3 mRNA expression after nivolumab (anti-PD-1) treatment.
Conclusion: *LAG-3* expression correlates with tumor inflammation and is enriched in tumors with MHCII^{hi} tumor cells. Preliminary data suggest that preferential localization of LAG-3-expressing leukocytes to MHCII^{hi} tumor regions potentially serves as a mechanism for *LAG-3* checkpoint pathway activation. These findings, and the observation that nivolumab may induce LAG-3 expression, underscore the importance of studies to define predictive biomarker profiles for relatlimab (anti-LAG-3) therapy in PD-1-naive and -progressed patients.

These results were previously presented as a poster presentation at SITC 2017. Mustimbo Roberts is listed as an author for presenting purposes only and did not contribute to the study.

O12. QIAseq Digital Sequencing and Biomedical Genomics Workbench: Application to Liquid Biopsy in Bladder Cancer

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Introduction: QIAGEN combines a powerful chemistry based on unique molecular indexes (UMIs) and a UMI-aware bioinformatics workflow in the Biomedical Genomics Workbench. The use of DNA panels is a cost-

effective approach to reach the high coverage needed for research using liquid biopsy. In such scenarios, the capability to distinguish between sequencing or amplification errors and real findings is crucial, in order to detect clinically relevant mutations at low allele-fraction levels. UMIs address this challenge, if combined with bioinformatics capable of exploiting their added value. Liquid biopsy is emerging as a new and non-invasive approach to characterize cancer mutation profiles. However, some tumors like urothelial cancer are particularly challenging, due to their location and biology. In this work we explore the performance of our workflow and its insights on bladder cancer, comparing data from both tissue and plasma.
Methods: Different mixtures of NA12878 and NA24385 (2% and 10%) as well as commercially available Horizon Discovery samples HD780 and HD700 were used to measure performance. DNA extracted from tumor biopsy as well as plasma from 8 individuals with bladder cancer was sequenced, using QIAseq UMI chemistry.
All analyses were performed with CLC Biomedical Genomics Workbench.
Results: This work illustrates the features of QIAseq Panel Analysis Plugin and how its workflow leverages UMIs, to reach impressive performances in detecting low allele-fraction variants. The connection with other QIAGEN solutions such as Ingenuity Variant Analysis (IVA) and QCI Interpret allows to further explore the results for their biological and pathological relevance. The QIAseq Panel Analysis workflow shows competitive performance even at allele fractions as low as 1%, detecting SNVs with a sensitivity of 91.5% and a precision of 96.7%. We compared the results from tissue and plasma libraries from individuals with bladder cancer, and show that the recovery of variants (i.e. variants identified in the tissue also called in the plasma) ranges above 70% for SNVs. A very good achievement, in the biological context of urothelial cancer. We further explore the results from a pathological perspective, and show the success in capturing biologically relevant mutations, classified by their pathogenicity and actionability according to existing ACMG/AMP and AMP/CAP guidelines.
Conclusion: QIAseq Targeted Panel Analysis uniquely combines state-of-the-art molecular biology with accurate and computationally efficient variant detection, and can be successfully used in liquid biopsy research.

O13. Comprehensive Molecular Characterization of Metastatic Cancer Using Whole-Genome Sequencing: A Dutch National Initiative

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Introduction: Using whole-genome sequencing (WGS) it is now possible to perform comprehensive molecular characterization of (small) tumors biopsies. As a national initiative, the Hartwig Medical Foundation (HMF) provides high-quality WGS for two large Dutch clinical studies: the Center for Personalized Cancer Treatment (CPCT-02) study and the DRUP (The Drug Rediscovery Protocol) trial, in which more than 45 hospitals participate. Sequencing results are used for building of a large database of metastatic cancers, and to generate an extensive patient report.
Methods: Fresh-frozen tumor biopsies are collected and confirmed by central pathology to contain sufficient tumor cells (≥30%). At least 50 ng of DNA is required using an Illumina X10 setup in a dedicated ISO accredited laboratory. For all patients, tumor sample(s) as well as a blood sample are analyzed by WGS to identify all somatic aberrations. An in-house bioinformatic pipeline has been established and all scripts are available at GitHub. Median turnaround time from biopsy to sequencing report is 25 days, and ~40 patients are processed every week.
Results: Currently, more than 2000 tumor biopsies have been successfully sequenced from patients with advanced metastatic disease who are either receiving (last line) regular therapy or for whom all regular options have been exhausted.
With an average sequencing depth of 100x, WGS enables sensitive identification of genome-wide somatic variants, indels and copy number variants. Gene fusions and structural variations (SV) (e.g. BRCA inactivation by SV instead of a mutation) are detected, which cannot be identified reliably using gene panels or exome sequencing. Assessment of the tumor cell purity is performed, which is used to determine local ploidy of each variant (e.g. AAAB), loss-of-heterozygosity (LOH) and an indication of sub-clonal events. All data is deposited in the HMF database and investigators can request access to answer specific research questions. The HMF patient report provides an overview of all aberrations in 124 cancer related genes. In addition, higher-level molecular features such as mutational load and microsatellite (in)stability are provided. To support interpretation of the observed variants, the report provides evidence items from the Clinical Interpretation of Variants in Cancer (CIViC) knowledgebase and clinical trials for which the patients is potentially eligible. At this time more than 150 patients have received an off-label treatment in the DRUP trial and preliminary analysis indicates a response of about 40%.
Conclusion: WGS provides a complete view of all aberrations of a tumor including the mutational load and microsatellite status. Moreover, information on LOH, tumor purity and variant ploidy, gene fusions and other SV types are provided, all using a single biopsy. Together, this data facilitates new biomarker discovery, and the best possible treatment decisions or clinical trial inclusion.

O14. Exploratory Analysis of Janus Kinase 1 (*JAK1*) Loss-of-Function (LoF) Mutations in Patients with DNA Mismatch Repair-Deficient/Microsatellite Instability-High (dMMR/MSI-H) Metastatic Colorectal Cancer (mCRC) Treated with Nivolumab + Ipilimumab in CheckMate-142

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Introduction: *JAK1* LoF mutations were reported as a mechanism of resistance to anti-PD-1 therapy in patients with metastatic melanoma (n = 2) or mCRC (n = 1) (Zaretsky JM *et al. N Engl J Med*. 2016;375:819-829; Shin D *et al. Cancer Discov*. 2017;7:188-201). Here, we report an exploratory analysis of CheckMate-142 (NCT02060188) investigating whether JAK1 LoF mutations were present in patients with dMMR/MSI-H mCRC who achieved clinical benefit with nivolumab + ipilimumab treatment.
Methods: Patients received nivolumab 3 mg/kg + ipilimumab 1 mg/kg Q3W × 4 doses and then nivolumab 3 mg/kg Q2W. Whole-exome sequencing was performed on pretreatment biopsies preserved in RNAlater and germline DNA from peripheral blood mononuclear cells. Single nucleotide variants and insertions/deletions were called by 2 variant callers in paired normal and tumor samples, and the union of mutations was included in the downstream analysis as previously described (Carbone DP *et al. N Engl J Med*. 2017;376:2415-2426). Best overall response was assessed by investigators (RECIST v1.1).
Results: *JAK1* LoF mutations were identified in 4 of 40 patients evaluated in this analysis. Of these 4 patients, 1 had an ongoing partial response of 9.7 months at data cutoff, and 3 had stable disease (Table); 2 patients with stable disease were still on treatment (12 and 17 months) at data cutoff, and 1 discontinued treatment after 2.9 months due to an unrelated adverse event and had not progressed after 10 months off treatment.
Conclusion: Clinical benefit was achieved with nivolumab + ipilimumab in patients with dMMR/MSI-H mCRC who harbored a JAK1 LoF mutation. Additional analyses are needed to assess the effect of concurrent JAK1 loss of heterozygosity and other genetic alterations in patients with dMMR/MSI-H mCRC.

	JAK1 LoF mutant n = 4	JAK1 wild type n = 36
Best overall response, n		
Complete response	0	1
Partial response	1[a]	18
Stable disease	3[b]	11
Progressive disease	0	4
Not evaluable	0	2

fs, frameshift. [a]JAK1 mutation: I975*. [b]JAK1 mutations: D899Gfs*10, K860Nfs*16, and K142Rfs*26.

[Best overall response in patients with dMMR/MSI-H mCRC identified as having wild-type JAK1 *or a* JAK1 *LoF mutation]*

These results were previously presented as a poster presentation at AACR 2018. Zachary Boyd is listed as an author for presenting purposes only and did not contribute to the study.

O15. Development and Characterization of Multigene Molecular Cytopathology Cell Slides as a Potential Fine Needle Aspirate (FNA)-Mimetic Specimen for Proficiency Testing

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Introduction: In the era of personalized medicine, with an increasing need for molecular testing, cytologic specimens comprise a crucial component in providing prognostic & predictive information for clinical management of lung cancer patients. Fine needle aspirate (FNA) of the lung is the most common sampling method which provides good quality nucleic acid for downstream molecular testing. However, due to the lack of reference standards mimicking FNA specimens, there is a lack of validation of different assays & standardization of the specimen across laboratories. Cytopathology reference material can be employed for assay development, quality assurance & proficiency panels to validate assay performance & understand cross-site and inter-operator variabilities across different laboratories. Cell-line based reference standards are ideal, as they represent a biologically-relevant, reproducible & renewable source of control materials. Using CRISPR/Cas9 technology, we engineered a reference bank of cell lines containing ~300 oncogenic point mutations, insertions & deletions, spread across 40 genes including *EGFR, KRAS, BRAF, PIK3CA, NRAS, HRAS & TP53*. Here, we show the utility of these cell-lines for development of a multigene molecular cytopathology panel, with the goal of mimicking low allelic frequency, in clinical FNA samples.
Methods: CRISPR/Cas9 was used to engineer the *EGFR* E746-A750del, *EGFR* T790M & *KRAS* G13D mutations into three separate RKO cell lines. These cell lines were mixed to make four separate cytopathology

slides, containing all three mutations at mutant allele fractions of 0%, 1%, 5%, and 10%. Mixed cells were ethanol-fixed & deposited onto slides by CytoSpin centrifugation at ~ 200,000 cells/slide. Deposition process was optimized to allow Kwik-Diff staining, with minimal cell-detachment & enable subsequent DNA extraction & analysis. Inter- & intra- slide cell distribution was assessed by digital images. Genomic DNA extraction efficiency was assessed & allelic frequencies were validated by digital PCR (dPCR) & Next-generation Sequencing (NGS).
Results: We obtained homogeneous distribution of the cells on glass slide using the CytoSpin. The coverage area is ~10% using 200,000 cells +/- 40,000 cells. Total extractable genomic DNA is ~ 300 ng/slide. Allelic frequencies were highly reproducible across a set of three slides tested with dPCR & NGS.
Conclusion: CRISPR/Cas9-engineered cell lines provide a renewable, biorelevant source of molecular reference standards. These engineered lines can be reproducibly incorporated into cell-based cytopathology slides & provide a valuable alternative to clinical samples, particularly for difficult to source oncogenic variants. Deposition of ~200,000 cells/slide mimics a clinical FNA specimen & enables the use of precisely manufactured multigene slides for cross-site proficiency panel studies and/or for the training of clinical lab personnel without sacrificing precious clinical specimens.

O16. External Quality Assessment Schemes for Gene Mutation Testing in Oncology: Comparison of Performance between FFPE-Tissue and ctDNA in Plasma

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Introduction: The European Society of Pathology (ESP) has an extensive database on laboratory performance for variant testing in cancer samples. This database is the result of several subsequent rounds of External Quality Assessment (EQA) schemes assessing the standard of testing in Formalin-Fixed Paraffin-Embedded (FFPE) samples from lung and colon cancer patients. In addition to these lung and colon schemes on tissue, a pilot EQA scheme was organized in 2017 to assess the standard of testing in circulating cell-free DNA (cfDNA) in plasma samples. The aim of this study is to determine whether laboratories can detect clinically relevant variants in plasma samples with the same success rate as in tissue samples.
Methods: The starting point of this pilot study are the 32 laboratories that participated in the first joint cfDNA pilot scheme in 2017 organized in collaboration with AIOM, EMQN, ESP EQA and UK NEQAS, under the umbrella organization IQN Path. Registrations of the schemes were analyzed to determine how many of these 32 laboratories have participated in lung or colon EQA schemes on tissue organized by ESP. In addition, the variants that were used in the 2017 cfDNA pilot EQA scheme formed the basis of this comparison. The genotyping score in plasma was compared to the genotyping score in tissue retrieved from the lung and colon EQA schemes database.
Results: A total of 18 laboratories were selected that have previously participated in at least one other lung or colon EQA scheme organized by ESP (between 2015-2017) as well as in the 2017 cfDNA pilot EQA scheme. The samples used in the cfDNA pilot EQA scheme contained five different variants in three separate genes:
· *KRAS* c.35G>C p.(Gly12Ala)
· *NRAS* c.182A>G (Gln61Arg)
· *EGFR* c.2235_2249del p.(Glu746_Ala750del)
· *EGFR* c.2573T>G p.(Leu858Arg) and *EGFR* c.2369C>T p.(Thr790Met)
The ability of the laboratories to detect these variants in plasma samples will be compared to the ability to detect the same variants in tissue sample used in the other ESP EQA schemes. This resulted in an analysis of 14 samples across six different schemes from 18 laboratories. The detailed analysis of the genotyping score of the laboratories and the detection method will be presented.
Conclusions: Our analysis will give insight into the performance of laboratories in detecting the same variants in both FFPE-tissue and cfDNA from plasma. These conclusions will provide feedback on the overall performance of laboratories in oncological diagnostics and will further improve biomarker testing in clinical setting.

The joint cfDNA pilot scheme was organized in collaboration with AIOM (N Normanno, Italy), EMQN (S Patton, UK), ESP EQA (see authors) and UK NEQAS for Molecular Genetics (Z Deans, UK), under the umbrella organization IQN Path (J Hall, Luxemburg). This comparison study is performed by the ESP EQA team.

O17. Use of Highly Multiplexed Reference Materials to Facilitate Validation of a Clinical NGS Tumor Fusion RNA Assay

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Introduction: Next Generation Sequencing assays for detection of tumor RNA fusions must undergo rigorous validation before clinical implementation. Validations include assessment of the assay´s accuracy, precision, reproducibility and limits of detection or reportable range. Obtaining samples with all needed variants is difficult and time consuming. When found, they are often in limited quantity such that repeated testing for precision and reproducibility studies is not possible. Sample heterogeneity or lack of characterization further complicates interpretation of results. This study demonstrates how highly characterized, uniformly

manufactured reference materials can facilitate clinical NGS assay validation.
Methods: An RNA panel representing decreasing levels of 16 different RNA fusions and exon skipping events compared to the total cellular RNA was generated. GM24385 cell line was engineered to contain the fusion RNAs then formalin fixed, and the total RNA was extracted using the Maxwell RSC FFPE RNA kit. The fusion RNA was serially diluted into total RNA from similarly processed non-engineered GM24385 cells to create a panel with decreasing levels of each fusion. The panel was characterized by fusion-specific, TaqMan digital PCR assays to obtain a "truth set" on which to compare NGS results. Each panel member was tested in triplicate on three separate days as part of a validation study.
Result: Precision and reproducibility were assessed quantitatively through analysis of the NGS unique reads. The intra-run precision varied among fusion targets with many targets having %CV of less than 15%, while a few targets had significantly higher variability between replicates. The linearity of the assay was good, with R-squared value greater than 0.95. Lower limits of detection for each fusion target were estimated using the digital PCR data and were different for different fusion targets; for example, detection of EML4-ALK fusion was five-fold more sensitive than detection of CD74-ROS1 fusion.
Conclusion: Manufactured reference materials can supplement validation studies and their uniformity and digital PCR characterization allows greater insight into assay performance such as limits of detection than use of remnant specimens alone. The sensitivity of the NGS panel used was not the same for different fusion targets and, and underscores the need for highly multiplexed reference materials and for labs to test all clinically important fusions during validation.

O18. Assessment of Circulating Tumor DNA in Patients with Metastatic Colorectal Cancer Treated with Cetuximab Monotherapy

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Introduction: Currently, third line systemic treatment for patients with RAS wild-type metastatic colorectal cancer (mCRC) includes anti-EGFR therapy with monoclonal antibodies such as cetuximab. Here, we examined whether circulating tumor DNA (ctDNA) can be used to get insight into mechanisms underlying primary and acquired resistance in mCRC patients receiving cetuximab.
Methods: 34 patients with RAS wild-type mCRC (exon 2-4) received biweekly cetuximab monotherapy (500mg/m^2) as third line therapy. Four milliliter plasma was obtained at baseline and progression according to RECIST 1.1. Cell free DNA (cfDNA) was isolated using the QiaSymphony Circulating DNA kit. Next, 20 ng cfDNA was used for a targeted next generation sequencing (NGS) approach with unique molecule identifiers for detection of somatic mutations in ctDNA. All cfDNA samples underwent IonPGM sequencing for a CRC-specific 14 gene panel covering 242 hotspots, which was analyzed using a standard variant calling pipeline. *KRAS* and *BRAF* mutational status was assessed as part of clinical routine work-up in either primary or metastatic tumor tissue. Outcome was defined as clinical benefit (CB; progressive disease > 8 weeks) versus no clinical benefit (NCB; progressive disease ≤ 8 weeks).
Results: 33 baseline and 26 progression cfDNA samples were of sufficient next generation sequencing (NGS) quality (median molecular coverage ≥ 1000). In 5/12 (42%) baseline cfDNA samples of patients with NCB, we detected somatic mutations in *KRAS* (n=3) and *BRAF* (n=2). Two of these *KRAS* mutations were not detected in tissue tested at routine diagnostic work-up. *BRAF* mutations found in cfDNA were also present in tissue, one *BRAF* mutation in tissue was not detected in cfDNA. One patient with CB had a polyclonal KRAS mutation in cfDNA at baseline, which was absent in tissue. In patients with initial CB an enrichment of mutations in genes associated with resistance (*KRAS, NRAS* and *BRAF*) was found in 12/17 (70%) at progression. Moreover, in 8/17 (47%) patients *EGFR* mutations in codons coding for the epitope binding site of cetuximab emerged at progression. At progression, in 9/17 patients (53%) multiple mutations in the same gene occurred suggesting the presence of multiple subclones.
Conclusions: Mutations in genes associated with cetuximab resistance were already present at baseline in a subset of mCRC patients with NCB on cetuximab therapy and can be used to identify patients who could refrain from anti-EGFR antibodies. In patients with CB, mutations associated with acquired resistance were enriched at progression in the majority of patients. Further studies are necessary to reveal whether the emergence of these mutations precedes clinical progression and if so, can guide treatments.
Keywords: circulating tumor DNA, colorectal cancer, EGFR inhibition

P01. For Research Use Only? Regulatory and Quality Frameworks in Clinical NGS

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Introduction: The rapid evolution in next generation sequencing (NGS) technologies and data analytics has transformed research in genetics and biomedicine, but the transition of these methods from a research or translational research setting into clinical practice is challenging. When techniques move from the lab into the hospital, questions regarding quality assurance, assay validity, clinical utility, the handling of sensitive data, and risk management need to be addressed.
Methods: A systematic review of directives and regulations, quality management frameworks, lab

certification schemes, and best-practices and recommendations published by professional societies was conducted. Specific recommendations were noted and categorized into several broad topics, and examined for recurring themes.
Results: Over 400 specific recommendations were compiled from a selection of 30 primary sources. Documents could be broadly classified as high-level regulations, such as the EU IVDR, guidelines specific to a medical setting, such as ISO 15189, and technical documents providing guidance specifically on NGS-related topics, such as interpreting and reporting variants, validating bioinformatics systems, or ensuring data quality. While scope and topics were varied, where best practices did overlap recommendations were highly consistent, and several cross-topic themes emerged.
Conclusion: Quality assurance and compliance with regulatory standards are essential for ensuring standard-of-care and patient safety during the wider adoption of clinical sequencing. By gathering, analyzing through the lens of clinical NGS, and disseminating best practices and guidelines, we hope to instigate wider discussion on how to implement quality control and assurance systems within clinical sequencing labs.

P02. Methylation Profiling for Improved Tissue Diagnosis of CNS Tumors: The Utrecht Experience on >150 Cases

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Introduction: Colleagues from Heidelberg have now convincingly demonstrated that detailed methylation profiling (MP) of routinely processed (FFPE) CNS tumor tissue can be used for improved diagnosis. From November 2016 through January 2018 we have performed MP on >150 tumors. Here we report on the added diagnostic value of MP in this series.
Methods: The Infinium Methylation EPIC beadchip (detecting methylation events of 850.000 CpG sites) was used for MP of (mostly) FFPE CNS tumor tissues, and the patterns were subsequently classified according to the Heidelberg classifier. Simultaneously, MP provides information on chromosomal copy number variations (CNVs) and *MGMT* promoter methylation. Our results are correlated with the clinical and pathological context.
Results: Out of the 167 cases, 86 (51%) concerned CNS tumors in adult patients, 81 (49%) in kids. In the PMC, the policy is to perform MP on every new pediatric CNS tumor. In a subset of cases, MP was explicitly requested for detection of (clinically relevant) molecular subgroups. In adult patients, requests for MP frequently concerned the need to assess a combination of molecular features and/or an unclear histological diagnosis. In 41% of the runs, 8 diagnostic samples were analysed, which is optimal for a cost efficient workflow. The mean number of diagnostic samples per run was 6.2. The quality score of the methylation class prediction was >0.9 in 77% of the cases, indicating that the diagnosis (´methylation class´) suggested by MP was to be taken very seriously. In 11% the result was not interpretable. *MGMT* status was methylated in 25%, not methylated in 57%, and inconclusive in 18% of the cases. In a substantial subset of patients, MP provided very valuable information on CNVs (e.g. 1p/19q co-deletion, combined chr. 7 gain & chr. 10 loss, *EGFR* or *MYC*/*NMYC* amplification).
Conclusion: MP is of great help for improving and refining the diagnosis of difficult to classify CNS tumors, both in adult and in pediatric patients. Additionally, MP is a (relatively) cost-effective diagnostic tool in cases where particular molecular information at different levels is required for diagnosis and/or treatment.

P03. Tumor Intrinsic Properties Associate with Differential Effects on CD8+ Tumor-Infiltrating Lymphocyte Density and Immune Gene Expression in Non-Small Cell Lung Cancer (NSCLC) Samples

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Introduction: Anti-tumor immune response is controlled by a complex interaction between the immune system, tumor cells, and associated stroma. Expression of major histocompatibility complex class I (MHC I) and MHC class II (MHC II) antigens may be dysregulated in cancer, leading to alterations in the tumor antigen presentation profile. Stage I-III resected NSCLC tumor samples were profiled to study the relationship between MHC I, MHC II, and programmed death ligand 1 (PD-L1) tumor cell (TC) expression with tumor-associated inflammation. Here we report the potential impact of respective profiles on tumor immune response.
Methods: Fifty three (53) adenocarcinoma (NSCLC-AD) and 51 squamous cell carcinoma (NSCLC-SQ) stage I-III resected formalin-fixed, paraffin-embedded tumor specimens from commercial sources were stained by immunohistochemistry for MHC I (HLA-A,B,C), MHC II (HLA-DP, DQ,DR), and PD-L1 and TC expression was assessed by manual pathologist review. CD8+ cell density was quantified using Definiens image analysis algorithms for the intraepithelial tumor region. RNA extracted from the samples was analyzed by RNAseq, with data available for 48 NSCLC-AD and 46 NSCLC-SQ specimens. A 25-gene IFN-gamma gene signature (IFNG score) was used to study the association of T-cell inflammation with other biomarkers.
Results: Of all NSCLC-AD and NSCLC-SQ specimens, 85% showed either complete (< 20% TC) or partial (20-

80% TC) loss of TC MHC I, while TC MHC II was increased (>1%; MHCII^{hi}) in 60% of NSCLC-AD and 18% of NSCLC-SQ. Quantitative analysis of CD8 in the tumor microenvironment (TME) revealed a significantly reduced intraepithelial density of CD8+ tumor-infiltrating lymphocytes (TILs) in NSCLC-AD (*P*=0.009) and NSCLC-SQ (*P*=0.01) with complete MHC I loss. In contrast, intraepithelial CD8+ TILs were significantly increased in MHCII^{hi} tumors for both NSCLC-AD (*P*=0.004) and NSCLC-SQ (*P*=0.006). Tumors displaying both MHCII^{hi} and retained MHC I demonstrated the highest TIL density. Gene expression associated with IFN-gamma response was increased in tumors with retained MHC I (*P*=0.0036) and MHCII^{hi} samples (*P*< 0.001). In NSCLC-AD and NSCLC-SQ, 38% and 59% had PD-L1 expression ≥1%, respectively. PD-L1 expression correlated with CD8 density and IFNG score in NSCLC-AD, but not in NSCLC-SQ.
Conclusion: Understanding the role of the antigen presentation machinery in immune activation and evasion in the TME is important to predict responses to immunotherapy. These data suggest that the expression of MHC I, MHC II, and PD-L1 by tumor cells may play a role in guiding the localization of CD8+ T cells in the TME with differential effects dependent on histologic subtype. The lack of correlation between inflammation and PD-L1 expression in stage I-III NSCLC-SQ suggests an alternate PD-L1 induction mechanism in a subset of those tumors.

These results were previously presented as a poster presentation at AACR 2018. Christine Ward is listed as an author for presenting purposes only and did not contribute to the study.

P04. Comprehensive Routine Diagnostic Screening to Identify Predictive Mutations and Copy Number Gains in FFPE Tumor Material

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Introduction: Detection of clinically relevant molecular aberrations to guide therapeutic decisions is gaining importance. Panel Next Generation Sequencing (NGS) is becoming a standard diagnostic approach for simultaneous detection of multiple molecular aberrations, using a limited amount of formalin-fixed, paraffin-embedded (FFPE) tissue.
Methods: In collaboration with pathology laboratories of two other academic centres, and input from medical and pulmonary oncologists, we designed a novel 29-gene panel for Predictive Analysis in Therapy (PATH), aimed to target all clinically relevant coding sequences for molecular testing of lung cancer, colorectal cancer, melanoma and gastro-intestinal stromal tumors. For this we expanded our experience with single molecule Molecular Inversion Probes (smMIPs), that include unique molecule identifiers, which allows reliable identification of clinically actionable sequence variants with 1% analytical sensitivity and specification of the sensitivity of sequencing on a case by case basis by determining the actual number of analyzed gDNA molecules. Moreover, we validated the concomitant analysis of amplifications of 13 genes.
Results: According to our quality guidelines, we formulated the diagnostic requirements prior to implementation and validation of the panel. In a validation series of 58 cases, our panel fulfilled all predefined criteria and we identified a total of 60 mutations in 37 cases of which 35% provide potentially actionable therapeutic targets. In addition, we provide a framework for analysis and clinical reporting of copy number changes using NGS analysis, including analytical specifications of sensitivity. A series of positive and negative controls provided cut-offs for copy number variation calling and in a validation series of 58 clinical samples we identified a total of 9 clinically relevant gene amplifications.
Conclusion: Reliable and sensitive detection of both mutations and gene amplifications in lung cancer, colorectal cancer, melanoma and gastro-intestinal stromal tumors can be achieved in routine diagnostics using an integrated NGS approach.

P05. Comparison of Comprehensive Solid Tumor DNA/RNA Panels for the Detection of Small Nucleotide Variants, Copy Number Variants and Fusions

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Introduction: Simultaneous sequencing of DNA and RNA (cDNA) targets from solid tumors can provide a comprehensive genetic profile including small nucleotide variants, copy number changes and fusion products. Commercial panels are now available for use in the clinical diagnostic laboratory to ascertain somatic variants from paraffin embedded solid tumors, but consistency across panels has not been assessed.
Methods: We investigated the equivalency of two panels, Oncomine Comprehensive Assay v3 (OCAv3, Life Technologies) and TruSight Tumor 170 (TST170, Illumina) using 53 solid tumor samples including lung, astrocytomas, gliomas, cell lines and an artificial FFPE control DNA. OCAv3 libraries were sequenced with an Ion Torrent S5XL and analyzed using Ion Reporter v5.2. TST170 libraries were sequenced with a NextSeq500 and analyzed using Basespace v1.0.0 and variants filtered in Catagenia. Only regions covered by both platforms were included in the analysis.
Results: Filtering out common variants in ExAC population database (>1%), allele frequencies less than

5% and regions with a read depth of less than 100, 224 SNV/Indel variants were detected, 209 of which (93.3%) were concordant between OCAv3 and TST170. The remaining 15 variants were discordant novel variants not in oncology hotspot regions. Of the discordant variants, 5 were found by OCAv3 only. One of these variants had a read depth of 131, close to the filter threshold of 100x, the remaining 4 were small insertions and deletions. Ten variants were found by TST170 alone, four of which were recurrent with low allele frequencies (near 10%) suggesting technical artifacts. The remaining 6 were small insertions and deletions. A total of 86 concordant copy number variants were found in both platforms. Filtering out low quality CNVs (fold change parameter in Basespace ≥ 1.95 OR ≤0.60 and CNVs with gain ≥ 3.5 or loss ≤1 in Ion Reporter) removed all platform-specific CNV calls. Known fusions observed by FISH analysis included 17 fusions (ROS and ALK) that were detected in both platforms. In an additional 6 samples suspected of having BRAF fusions based on pathological morphology and disease progression, 3 BRAF fusions were found by both OCAv3 and TST170. Other fusions found by both panels included 3 glioma samples with EGFR deletions from exons 2-7 and also one lung sample with Met exon 14 skipping.

Conclusions: Comparison of 53 tumor samples tested on both OCAv3-Ion Torrent S5XL and TST170-Illumina platforms demonstrate a concordance of >93% for SNVs and small indels, with a small comparable number of platform-specific unique indels identified by each platform. In addition, copy number variants and fusions detect by both the OCAv3 and TST170 are highly concordant. Overall, these results demonstrate that users can expect high concordance across panels but optimizing filtering to remove low quality variant calls is critical for good performance.

P06. Structural Variation Detection by Proximity Ligation from FFPE Tumor Tissue

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Introduction: The clinical management and therapy of many solid tumor malignancies is dependent on the utilization of genomic tools for comprehensive detection of medically actionable or diagnostically relevant genetic variation. However, a principal challenge in tumor sequencing is the use of archival formalin-fixed paraffin-embedded (FFPE) tissue which produces highly fragmented low-molecular weight nucleic acid. This sub-optimal input specimen, coupled with the short read nature of DNA and RNA next-generation sequencing protocols, proves challenging to generate long-range (Mbp+) genomic sequence data that are able to accurately and robustly provide information on underlying large-scale structural variation and phasing from these specimens.

Methods: As a proof-of-principle we have developed a Hi-C chromosome conformation capture methodology for FFPE tissue called “Fix-C” which yields phased read pairs spanning distances up to full chromosomes and enables unambiguous structural variation detection and variant phasing in archival specimens. We have applied our method to 15 clinical lung adenocarcinoma and sarcoma specimens spanning a broad range of tumor purity for structural variation fusion detection and somatic mutation phasing.

Result: Our method achieves a 90% concordance rate with concurrent FISH testing, which is currently used as the clinical gold standard. Additionally, this global structural detection approach enables unbiased whole-genome perspective for structural variation detection and identifies novel structural events in our cohort missed by FISH and traditional RNA and DNA sequencing on these specimens.

Conclusion: Here we present a novel sequencing approach that leverages the genome scale structural resolution of Hi-C with the throughput of traditional Illumina sequencing methods. We demonstrate the ability to identify known and novel structural events in tumor tissue. This advance is a paradigm shift in FFPE nucleic acid analysis and will enable high-detailed resolution of global structural variation underling solid tumor malignancies while simultaneously allowing unambiguous somatic variant phasing.

P07. Robust Measurement of Signal Transduction Pathway Activity in Cancer Using RNA Sequencing on Cells and FFPE Tissue

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Introduction: With the introduction of high throughput genomics technologies, more and more molecular information of cancers has become available, but the translation into pathophysiological characteristics to improve treatment selection is still a large challenge. We have developed a method to quantitatively and reliably measure the activity of signal transduction pathways in cancer cells, based on the interpretation of expression levels of transcription factor target genes via a Bayesian computational model. We have previously shown that this works well on expression levels measured with RT-qPCR or microarrays *(*Verhaegh *et al*., Cancer Res 2014 Jun 1;74(11):2936-45*)*, showing expected driving signaling pathways in various cell culture model systems and PDx mice and clinically relevant findings in multiple cancer types. Using RNA sequencing (RNAseq) to reliably measure expression levels has so far proven more difficult, because of the large variation in protocols and results, especially for formalin-fixed, parafin-embedded (FFPE) tissue.

Methods: We have evaluated several RNAseq protocols for quality of expression quantification and have chosen two for further validation and testing in breast cancer cell

lines and colon cancer samples. Models for estrogen receptor (ER) and Wnt pathway activity were calibrated for RNAseq to measure pathway activity in these samples.
Results: We will share results showing that signalling pathway activity scores based on validated RNAseq protocols are very robust. We show a dose-dependent inhibition of the ER pathway after Fulvestrant treatment of breast cancer cells. Secondly, we show strong activation of the Wnt pathway in colon adenoma samples as compared to normal colon samples, with similar quality of results for FFPE and fresh frozen samples.
Conclusions: Upon proper selection and validation of protocols, signaling pathway activity prediction based on expression levels of transcription factor target genes measured with RNAseq shows good results. Specifically, although RNAseq on FFPE samples has been reported to be very challenging, we obtained results on FFPE samples of similar good quality as for fresh samples.

P08. Number of Fusions Detected in FFPE Cancer Tissue Depends on RNA Isolation Method
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Introduction: RNA derived from formalin fixed, paraffin embedded (FFPE) tissues are invaluable for gene expression analysis of a myriad of disease states. However, the process of fixing the tissue and embedding it in paraffin severely affects the functionality of the RNA in downstream assays. These challenges are compounded by the labile nature of RNA and the ubiquitous presence of RNase that makes RNA difficult to work with throughout the sample preparation process. The advent of robust next-generation sequencing (NGS) technologies has enabled low quality and low input RNA for RNAseq workflows. Nevertheless, the reliability and accuracy of RNAseq data is still dependent on the extent of template RNA quality and amount. In this study, the effect of RNA quality on RNAseq fusion call sensitivity as a function of RNA extraction. Interrogating the FFPE derived RNA quality is a critical and yet overlooked step towards achieving optimal NGS results.
Methods: RNA was isolated from FFPE curls by both FormaPure (Beckman Coulter) and a column-based purification method. Matched samples were sequenced using both TruSeq Stranded Total RNA (Illumina) and TruSight Tumor 170 (Illumina). Data was analyzed using both TopHat and STAR-Fusion.
Results: RNA isolated using FormaPure Total was significantly more intact, as shown by higher DV200 values. The quality control metrics for RNA libraries do not show any difference between the two sample preparations. However, more fusion calls are detected in samples with higher DV200 values; sensitivity cannot be rescued in the lower quality sample by addition of more starting material. Higher numbers of fusion calls with higher quality input are seen for both kits tested as well as for both methods of data analysis.
Conclusions: Fusion detection in cancer tissues is dependent on RNA quality, as shown by the DV200 value. RNA from FFPE tissues is often degraded further during RNA isolation, and minimizing this degradation allows for detection of more fusion events.

P09. Incorporation of Gene Expression Profiling for Cell-of-Origin Determination (Lymph2Cx Testing) Using Formalin-Fixed Paraffin-Embedded Tissue Sections in Routine Workflow for the Work-Up of Diffuse Large B-Cell Lymphoma Cases
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Introduction: Diffuse large B-cell lymphomas (DLBCL) represent a clinically heterogeneous group that is classified together based on similarities in morphology and immunophenotype. However, it was discovered that gene expression profiling (GEP) could classify DLBCL into distinct molecular subgroups based on cell-of-origin (COO), including germinal center B-cell type (GCB), activated B-cell type (ABC), and unclassified (UNC) type. COO assignment of DLBCL has important biological and prognostic significance, as well as potential therapeutic implications, with the ongoing development of selective agents for treatment of specific DLBCL subtypes. Here, we describe the development of a clinical GEP assay (Lymph2Cx testing) to perform COO assignment in the routine work-up of DLBCL using FFPE tissue sections and summarize the results of the first 90 clinical DLBCL cases analyzed by the laboratory.
Methods: Lymph2Cx COO analysis was performed on 90 clinical DLBCL cases. RNA was extracted from macrodissected FFPE tissue sections and hybridized to the 20 fluorescently-labeled gene probes in the Lymph2Cx panel, including 8 genes overexpressed in ABC, 7 genes overexpressed in GCB, and 5 housekeeping genes. Probe/RNA complexes were purified on a NanoString nCounter Prep Station and transferred to a NanoString nCounter Digital Analyzer for quantification. The counts were processed using the National Cancer Institute's Lymphoma/Leukemia Molecular Profiling Project Lymph2Cx DLBCL COO Classifier (patented algorithm) for subtyping. Minimum laboratory time required to perform the Lymph2Cx assay is approximately 28 hours/2.4 lab days.
Results: We have successfully analyzed 90 DLBCL cases from 63 male and 27 female patients using the Lymph2Cx assay and detected 42 GCB, 34 ABC, and 14 UNC cases. 77 of the 90 total cases were analyzed by immunohistochemistry using the Hans algorithm, and included 48 GCB and 29 non-GCB cases, with an overall concordance rate of 79%. 89 of the 90 DLBCL cases were also analyzed by FISH and detected 7 cases of high-grade B-cell lymphoma, with MYC and BCL2 and/or BCL6 rearrangements, including 4 cases with genetic double-hit type rearrangements and 3 triple-hit cases. Lymph2Cx analysis of these 7 cases detected 5 GCB type and 2 ABC type lymphomas. The 90 DLBCL cases subjected to Lymph2Cx analysis included 34 nodal and 56 extranodal lymphomas. Most of the 14 total UNC

cases were comprised of extranodal lymphomas, suggesting that UNC lymphomas may arise more frequently in extranodal sites. In addition, ABC cases occurred 2 times or more frequently at extranodal sites versus nodal locations in both male and female patients.
Conclusions: we have demonstrated that the Lymph2Cx COO assay can be performed relatively rapidly in the clinical laboratory and can be incorporated into the routine workflow for the workup of DLBCL cases. The assay is highly robust and reproducible and can provide valuable biological, prognostic, and potential therapeutic information for DLBCL patients.

P10. Improved Methods for Next Generation Sequencing Library Cleanup and Size Selection

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Introduction: Next Generation Sequencing (NGS) libraries require high quality nucleic acid inputs of varying quantity, concentration, and size depending on the library preparation methods and sequencing platforms used. Most methods use a magnetic bead-based chemistry throughout the overall protocol, with usage falling into two basic categories of function: 1) Sample clean up to remove unwanted components (adaptors, primers, dNTPs, enzymes, etc.), and 2) Size selection to remove nucleic acid fragments or library molecules that are outside an optimal size range for downstream sequencing. Although commercially available beads are used extensively in NGS library prep methods, there are several performance issues that can be improved upon, including significant DNA loss, poor reproducibility, difficulty in pipetting due to viscosity of chemistry, and retention of undesired high molecular weight DNA.
Methods: Comparisons between commercially available size-selection beads and a newly developed chemistry were performed to assess library yield, reproducibility, size-selection precision, and final sequencing results. Whole genome sequencing was performed using the NEBNext Ultra II library prep kit on a range of DNA inputs from 1ng - 1µg. Size selection steps were executed in parallel with both size-selection chemistries, followed by Illumina sequencing. In addition, measurements were conducted to determine surface adhesion and bead response time between chemistries as these parameters can significantly affect reproducibility.
Result: DNA yields increased 20% or more per sample cleanup step cycle with the newly developed chemistry, and substantial reduction of undesired HMW DNA from libraries was achieved. Viscosity and bead response time are both reduced at least 5-fold, resulting in greater reproducibility and 2 to 4-fold reduction in library yield standard deviation. Sequencing results demonstrating duplication rates, coverage uniformity and reproducibility will be presented.
Conclusion: Improved DNA recovery from NGS library size-selection can enable working with smaller starting sample inputs and reductions in PCR cycling, resulting in fewer duplicate reads, greater coverage uniformity, and higher confidence in mutation calling. Reduced variability and better removal of HMW fragments in sample preparation can minimize costly sequencing failures and wasted reads. All of these enhancements can serve to improve any sequencing experiment, but are especially important when sequencing limited, heterogeneous samples such as those derived from formalin-fixed paraffin-embedded (FFPE) or circulating cell-free DNA.

P11. *TP53* Mutations in Circulating Cell-Free DNA as Longitudinal Biomarker for High-Grade Serous Ovarian Cancer

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Introduction: High-grade serous ovarian carcinomas are characterized by *TP53* mutations. The goal of this study was to establish a workflow for detection of circulating tumor DNA (ctDNA) by *TP53* mutations in cell-free DNA (cfDNA) isolated from archived serum samples and to define whether ctDNA is suitable as biomarker for clinical disease monitoring.
Methods: We investigated TP53 for expression in tissue and for mutations in tissue and serum (< 1mL) in two distinct subsets (n=10 each) of advanced stage (FIGO III-IV) ovarian cancer patients with high-grade serous histology. All patients underwent debulking surgery and received platin-based chemotherapy. Subset I compared patients with ≤2cm versus >2cm residual disease and subset II patients with progression-free survival (PFS) ≤6 months versus PFS after 5 years. Tumor tissue of both subsets were first evaluated for mutations by next-generation sequencing (NGS) using Ion AmpliSeq panels and for protein expression by immunohistochemistry. In subset I, cfDNA from sera collected at diagnosis, after chemotherapy and at the time of disease progression were analyzed by AmpliSeq *TP53* community panel and customized digital PCR assays (dPCR). In subset II, cfDNA from sera collected at diagnosis were analyzed for 10 genes including *TP53* using NGS Oncomine cfDNA assays with molecular barcoding.
Results: *TP53* missense mutations and protein expression were observed in all analyzed tissue specimens, except for three patients. Half of the patients in each subset of residual disease had ctDNA in sera at diagnosis. Thus, in serum cfDNA at diagnosis, *TP53* mutations were demonstrated in serum cfDNA at diagnosis in 4/8 cases by dPCR and only in 2/8 cases by AmpliSeq NGS from subset I, and in 6/9 cases by the Oncomine NGS from subset II. In one patient of subset II a *KRAS* mutation was detected in cfDNA but not the concurrent *TP53* mutation. Evaluation of cfDNA from longitudinal sera by dPCR detected no mutations in sera taken while on therapy, whereas at disease progression *TP53* mutations were observed in 5/8 patients. In two patients ctDNA levels measured by dPCR were compared with serum CA125 levels over time and

showed in one patient an increase of ctDNA occurring a few months before increase in CA125 levels and radiologic progression. Finally, ctDNA in sera at diagnosis was observed in all cases with PFS ≤6 months, but only in 2/5 cases with no or residual disease after 5 years.
Conclusion: By using *TP53* mutations as biomarker for advanced stage high grade serous ovarian cancer, we were able to detect ctDNA in 25% to 67% of sera taken at time of diagnosis, depending on methodology. *TP53* mutations were undetectable in cfDNA while on chemotherapy but re-appeared at disease progression, showing that ctDNA shows promise as biomarker for clinical disease monitoring in high grade serous ovarian cancer.

P12. Validation of the GeneReader NGS System and Its Workflow for BRCA 1/2 Sequencing Test
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Introduction: Next generation sequencing (NGS) is able to simultaneously interrogate many genomic loci efficiently. However, multiple barriers still exist for its broader adoption in the clinical laboratory, such as fragmented workflow and complex bioinformatics analysis and interpretation. GeneReader NGS system (QIAGEN, Hilden, Germany) includes all sample processing steps starting from nucleic acid extraction to an integrated bioinformatics solution that enables direct access to real-time updates from literature, clinical knowledge, and evidence. We evaluated the performance and utility of GeneReader NGS Platform for BRCA 1/2 sequencing test.
Methods: We used previously validated samples from 100 patients who were diagnosed breast or ovarian cancer and tested Sanger based BRCA 1/2 sequencing test. Libraries were prepared using the QIAGEN Library Kit, which amplifies 253 amplicons covering 37.5 kb. Emulsion PCR and bead enrichment steps were carried out using the GeneReader QIAcube and the Clonal Amp Q Kit. The pooled libraries were sequenced using the GeneReader platform. All the procedures were performed according to the manufacturer's protocols. QCI Analyze software performed read alignment, quality control, variant calling, and clinical report generation. The final BRCA 1/2 results generated by GeneReader were compared to data generated by Sanger sequencing. For the reproducibility of variant calling, we performed the process in triplicate using 20 samples and analyzed the results according to variant type. We also reviewed the turnaround time (TAT) of total process and its hands on time.
Results: The percentage of reads in on-target regions was about 90%. Greater than 99.9% of the target regions showed read depths >100x. Variants generated from the GeneReader achieved 86.7% (13/15) and 98.9% (722/730) concordance with those generated from Sanger sequencing in insertion/deletions (indels) and single nucleotide variations (SNVs), relatively. The annotation of the GeneReader system showed over 99.9% (744/745) concordance with databases in the description of variants such as Clinvar. All of 272 SNVs and 7 indel variants showed 100% calling reproducibility. Additionally, the GeneReader provided a truly integrated workflow and the most process were consisted of hands off time (3250 min, 87% of total TAT).
Conclusions: The GeneReader NGS system for BRCA 1/2 testing showed good performance and provides short hands on time. With a full end-to-end solution with integrated sample preparation to bioinformatics interpretation, the GeneReader NGS System can be useful and practical for clinical laboratories.

P13. MYD88 Mutation Detection by Ultra-Sensitive Droplet Digital PCR Reveals High Potential of Aqueous Humor to Detect Vitreoretinal Lymphoma
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Introduction: Diagnostic work-up of patients suspected with vitreoretinal lymphoma (VRL) is primarily based upon vitreous fluid (VF) analysis, including the recently emerging *MYD88* mutation analysis. Aqueous humor (AH) paracentesis is a relatively less invasive and safer procedure than taking VF specimens. Therefore, AH-based *MYD88* mutation analysis would provide an additional 'liquid biopsy' tool to diagnose and monitor VRL patients. Our objective was to investigate whether MYD88 p.(L265P) detection by highly sensitive *droplet digital PCR* (ddPCR) is feasible in AH of VRL patients.
Methods: This is a retrospective cohort study of 96 AH and VF samples from 63 patients (23 cases with VRL and 40 uveitis cases) at the University Medical Center Utrecht, the Netherlands. Ocular fluids were randomized and blinded before MYD88 p.(L265P) analysis, which was performed using an in-house validated *droplet digital PCR* (ddPCR) platform. We assayed for the presence of MYD88 p.(L265P) mutation detected by ddPCR in AH and VF.
Results: MYD88 p.(L265P) was detected in the majority of VRL patients (17/23[74%]) and in none of the uveitis patients. It was detectable in both VF and AH. In paired VF and AH samples (n=17), the mutation was detected in 89% of AH samples of MYD88 p.(L265P)-positive VF samples. After treatment, the mutation was no longer detectable in any of the ocular fluids. *MYD88* ddPCR showed a sensitivity of 75% (95%CI: 43-93) in VF and 67% (95%CI: 35-89) in AH, and 100% specificity in both (100%; 95%CI: 46-100). The performance of MYD88 p.(L265P) in AH compared to routine laboratory tests was proportional to the performance of MYD88 p.(L265P) in VF.

Conclusion: The high concordance between AH and VF suggests that use of the easily accessible AH is nearly as informative as VF to identify key somatic mutations in VRL. This approach may provide an additional minimally invasive tool for accurate diagnosis, detection of recurrence and monitoring of treatment.

P14. Accurate Determination of DNA Concentration for Next Generation Sequencing Applications Is Harder Than You Think

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Introduction: Next Generation Sequencing (NGS) requires the input of DNA of sufficient quantity and quality to ensure a high-quality sequencing output. This is even more critical when performing whole exome sequencing or whole genome sequencing (WGS). To ensure samples submitted into England's 100,000 Genomes Project (both for the cancer and rare disease programme) are of appropriate quantity to enable successful WGS, NHS England with UK NEQAS for Molecular Genetics have developed an external quality assessment (EQA) scheme for the concentration measurement of double-stranded DNA. All laboratories providing DNA samples for inclusion into the project were required to participate in this EQA scheme.
Methods: Since December 2014, laboratories have participated in three practical EQAs. A combination of DNA samples derived from formalin-fixed paraffin-embedded (FFPE) tissue, whole peripheral blood and DNA reference samples were provided. Laboratories were instructed to measure the concentration of DNA using their usual protocol and return the data to UK NEQAS for analysis. A consensus median and scoring criteria were established. The known concentration of commercially available reference samples was reported alongside the consensus median. An overall combined score for DNA concentration measurement was generated and laboratories bench-marked against each other.
Results: Results demonstrated the use of a broad range of DNA concentration methodologies and a high variation of measurement between methods and also laboratories performing the same assay was observed. Spectrophotometric methods of DNA quantification were less accurate than fluorometric methods for DNA derived from all samples types, especially DNA extracted from FFPE tissue. Furthermore, spectrophotometric methods consistently detected DNA where none was present. Continued participation in EQA runs showed a trend in variation regardless of sample type and sample quality.
Conclusions: Laboratories often are not aware of the limitations of methods applied to determine DNA sample concentrations. Participation in DNA quality measurement EQAs highlighted that not all concentration determination methodologies used for routine molecular genetics applications are suitable for samples destined for WGS. Application of quality metrics, inter-laboratory bench-marking and continued participation allowed laboratories to identify areas of concern promptly and address them efficiently and effectively.

P15. Using the Genereader NGS System to Identify Pathogenic Mutations in Challenging Homopolymer Regions of *BRCA1* and *BRCA2*

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Introduction: Breast cancer is the most frequent cancer in women with nearly 1 in 8 developing invasive disease in their lifetime. In 2018 it is estimated that >260,000 new cases will be diagnosed in the US. The key tumor suppressor genes *BRCA1* and *BRCA2* have essential roles in DNA repair and maintenance of chromosomal stability. Inherited or acquired mutations in these genes allow cells to grow and divide unregulated, leading to the formation of tumors. Targeted next generation sequencing (NGS) has proven to be invaluable for identification of mutations and has the future potential to dramatically improve detection and enable earlier intervention. However, unlike some genes that possess well characterised hotspots, mutations throughout the sequence, especially insertion and deletion (InDel) events, in *BRCA1/2* can result in oncogenic transformation. The detection of medium to long insertions is challenging, especially in the homopolymer-rich regions present in BRCA1/2.
The GeneRead QIAact BRCA Advanced Panel in combination with the QIAGEN GeneReader NGS System provides a solution to test for single nucleotide variants (SNV) and InDels in the entire coding region, plus flanking intronic regions, of *BRCA1/2.*
Methods: The GeneRead QIAact BRCA Advanced Panel has been designed to detect mutations throughout *BRCA1/2*, with a key feature of the panel being the addition of a unique molecular index (UMIs) to tag individual molecules prior to target enrichment by PCR. This allows duplicate reads originating from the same molecule to be identified and used for correction of PCR and sequencing artefacts. To demonstrate accuracy, reference FFPE samples from Horizon (HD810) and FFPE clinical samples were used. Following target enrichment, libraries were sequenced on the GeneReader and mutations analysed using QIAGEN Clinical Insight (QCI) Analyze for GeneReader.
Results: Reference samples confirmed the ability of the panel to detect a range of pathogenic DNA mutations in both *BRCA1/2*, even in challenging homopolymer regions. Interestingly, the assay was also able to detect two small pathogenic deletions in a homopolymer region of *BRCA2* that were previously missed by other methods. All expected mutations (5% allele frequency threshold appropriate for formalin-fixed paraffin-embedded (FFPE) DNA) were repeatedly identified both within and between runs. Results confirmed UMI coverage of the

entire coding region of the two genes and testing of clinical samples consistently detected the expected DNA mutations that had been previously reported.
Conclusions: The data presented here illustrate the reliability of the GeneRead QIAact BRCA Advanced Panel on the GeneReader NGS System. This study demonstrates the ability of the new GeneRead QIAact BRCA Advanced Panel to detect even challenging insertions and deletions in key tumor suppressor genes, which are often overlooked by other NGS methods. This allows a laboratory to efficiently and effectively detect pathogenic breast cancer mutations with high precision.

P16. Evaluation of Customizable Circulating Tumor DNA (ctDNA) Reference Materials with Multiple Assays

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Introduction: The utility of circulating cell-free DNA (ccfDNA) has led to the development of an ever-increasing number of ctDNA assays that also make use of fundamentally different analytic methods. However, the limited quantities of ccfDNA in plasma make it difficult to assess the accuracy and performance of such assays because there is often insufficient material to perform a comprehensive analysis. Additionally, there have been reports of discordance between different ctDNA assays with patient samples at lower variant allele frequencies (VAF), but the limited amount of material is a bottleneck to investigating the causes of discordance. To overcome this, we developed customizable reference materials that are similar to native ctDNA in both size and adapter ligation efficiency.
Methods: Reference materials were designed by adding synthetic variant-containing DNA to genomic DNA derived from the GM24385 lymphoblastoid cell line at defined frequencies and converting the mixtures into ccfDNA-like molecules. DNA fragment size was evaluated by Bioanalyzer DNA 1000 assay and next generation sequencing (NGS). These reference materials were analyzed by digital PCR as well as by several NGS assays that utilized a variety of different approaches including amplicon, anchored multiplex PCR and hybrid/capture.
Result: The reference materials were found to have a size distribution that is similar to that of ccfDNA and to be broadly compatible with different NGS assays with input amounts of 20-50 ng that represent typical input amounts for ctDNA NGS assays. Observed variant frequencies were generally similar between assays, and variants around 0.125% were routinely detectable above noise with most assays.
Conclusion: We have developed customizable ccfDNA-like materials for analysis by ctDNA assays. This should enable the development, improvement and validation of assays that analyze ctDNA because sufficient material can be generated in order to assess sensitivity and other performance characteristics. Additionally, this should also allow for concordance studies that attempt to analyze the same material with multiple assays and for multi-lab studies typically performed in CDx applications.

P17. Microsatellite Instability Analysis and NGS with Fragmented Sample Types

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Introduction: A significant hurdle to using fragmented DNA for genomic studies is obtaining a sample of sufficient quantity and quality for rigorous downstream applications like next generation sequencing (NGS). Having effective tools to isolate, characterize, and analyze fragmented DNA containing samples, such as circulating cell free DNA (ccfDNA) and formalin-fixed paraffin-embedded (FFPE) tissues, can prevent downstream failures, ultimately saving hours of work and precious samples. Here we present optimized methods for use with even highly fragmented DNA samples. Using this toolset, we demonstrate successful NGS and microsatellite instability (MSI) workflows using matched FFPE tissues and plasma samples.
Methods: Plasma and FFPE tissue samples were obtained from three individuals with colorectal adenocarcinoma. DNA was isolated with Promega's Maxwell RSC Instrument using the Maxwell RSC FFPE DNA Kit for FFPE tissues and the Maxwell RSC Circulating DNA Kit with the large volume custom protocol for plasma. DNA was then quantified with the ProNex DNA QC Assay. Following quantitation, MSI analysis and NGS library preparation using the TruSeq Custom Amplicon Low Input Kit from Illumina was performed. NGS libraries were checked for size and quantity and then sequenced on the MiSeq System (Illumina).
Results: Full MSI profiles were obtained from DNA obtained from both ccfDNA and FFPE samples from each individual. Following successful determination of MSI-status, NGS libraries were produced from each sample. Sequencing of these libraries produced mean amplicon read depth greater than 3000x and mean coverage uniformity greater than 90%. In addition to excellent sequencing quality metrics, variants in mismatch repair genes identified in FFPE samples were also detected in matched plasma samples.
Conclusions: Proper molecular tools and assays are essential to success in exacting downstream applications like NGS and multiplex PCR. This work introduces streamlined methods for DNA isolation, library preparation, and multiplex microsatellite instability analysis from fragmented sample types and demonstrates their effective use with matched FFPE and ccfDNA samples.

P18. Development of Well Characterized Breast, Lung, and Brain Cancer Copy Number Variation Reference Materials

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Introduction: Copy number variation (CNV), including gene amplification and deletion, can be a key driver of oncogenesis. Pathogenic CNVs are often associated with unfavorable prognosis and drug resistance, therefore detection of these types of genetic changes will be important for personalized treatment. Many tumor profiling workflows can detect CNVs in addition to somatic mutations, but well-characterized reference materials for CNVs are not widely available, which makes it difficult to assess accuracy and sensitivity. CNV content of cell lines can be variable and may change with passages, the genomic background may not be diploid, and germline and somatic variants may not be well characterized. Remnant patient samples often lack characterization and the volumes needed for such characterization and larger validation studies. Therefore, we developed and evaluated Seraseq CNV reference materials to serve as accuracy, precision, and limit-of-detection (LOD) controls for tumor profiling assay development and validation. By providing a consistent source of purified DNA with stable CNV content, these reference materials allow for repeatable assessment of clinical diagnostic assays targeting CNVs.
Methods: Seraseq CNV reference materials were prepared by mixing genomic DNA with additional copies of target genes from the well-characterized cell line GM24385. Copies of *ERBB2*, *FGFR3*, and *MYC* were combined with genomic DNA to simulate CNVs found in breast cancer, while sequences for *EGFR*, *MET*, and *MYCN* were used to mimic lung and brain cancer CNVs. Three levels of amplification were targeted: +3, +6, and +12 additional copies. The copy number of each gene was precisely quantified using digital PCR and confirmed on several NGS-based assays internally and externally.
Result: Testing revealed CNVs of each target gene were close to the expected copy number. Good correlation was observed between digital PCR data and the data from NGS-based assays for all of the genes covered by these assays. The internal and external NGS data show a broad assay compatibility.
Conclusion: While CNVs observed clinically can be upwards of 50 additional copies and more are easily detectable, LOD controls such as those described here are imperative for development, validation and harmonization of assays that are used to assess CNV. Collectively, the data demonstrate the utility of biosynthetic Seraseq CNV reference materials, which serve as superior alternatives to DNA from cell lines or patient-derived material.

P19. Detection of Microsatellite Instability in Circulating Cell-Free DNA from Colorectal Cancer Patients

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Introduction: The success of checkpoint inhibitors targeting programmed cell death protein-1, programmed cell death ligand-1, and cytotoxic T lymphocyte antigen-4 in the treatment of patients with colorectal cancers displaying microsatellite instability underscores the need for novel technologies to evaluate the progression of such instability as a means of monitoring therapeutic response. This is complicated by the fact that serial tumor specimens are usually not available while on therapy. We hypothesized that liquid biopsies could provide an important source of cancer-derived DNA readily obtainable by minimally invasive means in patients undergoing treatment.
Methods: Liquid biopsy samples were from 13 patients harboring stage IV colorectal cancers, including 10 with microsatellite instability previously documented in their primary tumor specimens and 3 in whom the primary lesions lacked such instability. The liquid biopsies were collected at various time points relative to treatment and tested for microsatellite instability by evaluating the electrophoretic mobility of 5 different mononucleotide repeats amplified by PCR using primers purchased from Promega. We also documented cancer-driving mutations in liquid biopsy and solid tumor specimens from 5 of the 13 patients processed on the Vela Dx Sentosa NGS platform using the Vela Dx colorectal cancer panel. Absence of contamination of circulating cell-free DNA with genomic DNA in the liquid biopsies was verified using an Agilent TapeStation Bioanalyzer.
Results: Both the electrophoretic profiles of microsatellite biomarkers and the mutational profiles in liquid biopsies were identical to those in the matched solid tumor samples from each patient examined except one. Gradual loss of microsatellite instability in serial liquid biopsies from patients with high microsatellite instability in their respective primary tumors matched their clinical response to immunotherapy and coincided with disappearance of driver mutations in the liquid biopsies. The only patient with discordant results between the liquid biopsy and the solid tumor had only minimal instability in the tumor in each of the 5 biomarkers suggesting that only a small fraction of the cancer cells displayed instability in the primary tumor sample. This patient, who did not respond to immunotherapy, showed no detectable instability in the liquid biopsies despite the fact that a KRAS G12D mutation present in the tumor was also detected in the liquid biopsy.
Conclusions: The prospect of using liquid biopsies to evaluate microsatellite instability status in patients with cancers associated with such instability might be useful

not only in monitoring patients undergoing therapy, but also in the follow up of patients with Lynch syndrome with no clinical evidence of cancer. Microsatellite instability can be detected in liquid biopsies of patients with colorectal cancer.

P20. Patients Treated for Gastrointestinal Neoplasms Show Higher Abundance of Lactobacilli in Their Stool Compared to Non-Treated Patients through Application of 16S rRNA Gene Sequencing

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Introduction: The gut microbiome is a vital component of the gut environment regulating and modulating both health and disease. Disturbances in the microbiota can result in development of several types of gastrointestinal tract (GIT) malignancies either by the microbial metabolites or the pro-carcinogenic activities. Stool specimens represent a non-invasive method for studying the gut microbiome. 16S rRNA gene sequencing from stool samples has demonstrated enrichment or deletion of certain bacterial types in the various types of GIT neoplasms such as *Fusobacterium species* and *Bacteroides fragilis* in colorectal cancer (CRC). Moreover, *Helicobacter pylori* infection has been linked with increased incidence of gastric carcinoma. We investigated the diversity and the abundance of gut microbiota in different gut neoplasms with special focus on comparing the bacterial profile in relation to location of the tumor in the GIT and between treated and non-treated cancer patients.
Methods: The study was carried out on stool samples from 86 GIT neoplasia patients (65 non-treated and 21 treated) in addition to 13 healthy controls. All patients and controls were of Finnish origin. Samples from the non-treated 65 patients were categorized according to the tumor location into five categories; stomach, pancreas, small intestine, colon, and rectum. However, the samples from the 21 treated patients were categorized as a separate group (Treated) regardless of tumor type/location. Next generation based 16S rRNA gene sequencing was performed on all stool samples.
Results: Comparison of the gut microbiota with the controls revealed lower relative abundance of Lactobacillaceae in patients with colon neoplasms. Similarly, Bifidobacteriaceae at both the family and genus taxa levels revealed significantly lower relative abundance in patients with stomach and rectal neoplasms. Comparison of the treated cancer group with the non-treated cancer groups revealed elevated abundance of *Lactobacillus* at the three taxonomic levels (family, genera and species) in stool samples from the treated cancer patients.
Conclusion: This study demonstrates that the gut microbiota in stools differ according to the GIT tumor location and to cancer treatment status as well. Since *Lactobacillus* are considered part of the healthy gut microbiota, their higher levels in treated cancer patients in comparison to non-treated cancer patients could be indicative of effective therapy. This could also represent recovery of the essential activity of the normal bowel bacteria, thus restoring gut homeostasis.

P21. Whole Exome Sequencing and Data Analysis of FFPE Tumor Samples to Find Clinically Relevant Alterations for Pediatric Cancer Treatment

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Introduction: Pediatric cancers carry relatively few mutations in genes that code for "druggable" targets and the spectrum of the genetic alterations underlying these malignancies differs from the adult tumors, causing inefficient application of targeted gene panels in the market. Whole exome sequencing (WES) is an efficient and cost-effective method to identify complex genetic changes in the coding region of all genes in the genome. For integrating WES into personalized therapy decision protocol, it is necessary to consider limitation of the technology by contamination of normal cells, uncovered tumor heterogeneity, low amounts of tissue and DNA damages common in formalin-fixed paraffin-embedded (FFPE) specimens. Variant filtering and interpretation of large datasets produced by WES also poses a serious challenge for molecular diagnostic laboratories. Complex workflow on FFPE specimens from sample process and sequencing to data analysis is necessary for identifying function-impacting and clinically relevant variants in FFPE samples.
Methods: Capture-based paired-end sequencing was performed with 100x average coverage on 14 FFPE samples derived from pediatric cancer patients. All samples were validated and tumor ratio was determined by the pathologist before DNA extraction. Data from vcf files were filtered by using a combination of Ingenuity web tool and software of IGV with manual steps. A filtering step to select clinically relevant genes of 893 was integrated to our workflow. Hits were evaluated by our curated evidence database and in-house developed algorithm.
Results: We have successfully sequenced the samples (100%) with an average coverage of 155x from input DNA of 500-2000 ng. The average on-target/off-target ratio estimating the enrichment efficiency was 83%. Variants with an average of 140 638 were listed in the vcf files. We have selected variants with an average of 586 in 265 genes by using specific filter settings in Ingenuity tool to exclude low quality, common, likely benign and benign and non-cancer driver variants by predictions and

databases, and to keep variants in genes selected in our virtual gene panel. Finally, we listed variants that were exonic and non-synonymous and considered as true positive by manual steps using IGV to visualize mapped reads. We have identified all the variants in 213 genes as 24% of our custom panel were affected. 81% of these genes were not included by the Cancer Gene Census (Cosmic). In 6 cases (42%) positive association with off-label therapy was found.
Conclusions: We have developed a complex workflow of WES analysis of FFPE samples to improve our data for more effective clinical analysis and interpretation for pediatric cancer. The whole process from sample delivery to molecular pathology report takes less than 20 working days and can be well-integrated to the clinical practice. Our results demonstrate that 42% of cases tested in our laboratory had clinically relevant findings that can be integrated into therapy strategies.

P22. Accurate *FLT3*-ITDs and *CEBPA* Variant Detection in Acute Myeloid Leukemia by Anchored Multiplex PCR and Next Generation Sequencing
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Introduction: Acute Myeloid Leukemia (AML) is clinically and biologically heterogeneous, requiring the detection of mutations across multiple genes for characterization. Internal tandem duplications (ITDs) in *FLT3*, detected in about 25% of AML cases, cause aberrant cell growth leading to tumorigenesis and are associated with poor prognosis. *CEBPA* has an important role in myeloid differentiation, and mutations in the gene are the most common mutations detected in cytogenetically normal AML. Consequently, the WHO recommends characterization of *CEBPA* mutations for hematopoietic and lymphoid tumor classification. While *FLT3*-ITDs and *CEBPA* mutations represent important markers in *AML*, they are difficult to detect by NGS due to the highly variable nature of ITDs, the high GC content of *CEBPA*, and the difficulty in mapping repeated sequences to a wild-type reference.
Methods: We developed Archer VariantPlex myeloid assays based on Anchored Multiplex PCR (AMP) to detect important mutations in myeloid malignancies, including *FLT3*-ITDs and *CEBPA* variants. AMP is a target enrichment strategy for NGS that uses molecular-barcoded adapters and single gene-specific primers for amplification, permitting open-ended capture of DNA fragments from a single end. This approach enables flexible and strand-specific primer design to provide better coverage of ITD-containing regions of *FLT3* and GC-rich regions of *CEBPA*. To optimize *FLT3*-ITD detection, we developed a novel *de novo* sequence assembly algorithm based on > 2000 *in silico* ITD datasets.
Results: *In silico FLT3*-ITD datasets enabled optimization of the Archer Analysis ITD detection algorithm. This algorithm used in combination with the VariantPlex Core Myeloid assay enabled detection of *FLT3*-ITDs down to < 0.1% AF. Furthermore, we show 100% concordance of a VariantPlex Core AML assay with other methods commonly used to detect *FLT3*-ITDs from 25 blood and bone marrow samples. Importantly, we detected concomitant non-ITD variants in *FLT3* and NPM1 in some of these samples. Next, we show >1000x unique molecule coverage across *CEBPA* with 2M reads and 50ng input, enabling confident variant calling across the entire coding region. Finally, we demonstrate the concordance of the VariantPlex Myeloid assay with a DNA reference material containing 23 mutations that are covered by the Myeloid panel.
Conclusion: AMP provides NGS-based detection of complex mutation types that are relevant in AML, including *FLT3*-ITDs and *CEBPA* variants. Our results show that this approach is accurate as well as scalable, enabling simultaneous detection of multiple mutation types across multiple target genes in a single assay.

P23. Using the GeneReader NGS System and QIAact Lung All-in-One Assay to Detect Complex Mutations and Fusions
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Introduction: Lung cancer is the cause of 1 in 5 cancer deaths worldwide and is frequently driven by somatic mutations acquired in key genes. Although mutations in *EGFR* and *KRAS* are present in up to 50% of cases, several other genes play a critical role in tumor development. Targeted Next-Generation Sequencing (NGS) is a valuable tool for identifying mutations in these genes and has improved our understanding of disease progression. However, these mutations are not limited to just single nucleotide variants (SNV) or Insertion/Deletions (InDel) but also include complex chromosomal changes such as copy number variants (CNV) and fusions. Traditionally, multiple and different assays such as PCR, microarray or fluorescent *in-situ* hybridization (FISH) need to be deployed to detect these changes.The GeneRead QIAact Lung All-in-One Assay in combination with the QIAGEN GeneReader NGS System provides a single solution to simultaneously test for actionable mutations, saving sample material, shortening test time, and enabling simplification of lab operations.
Methods: The GeneRead QIAact Lung All-in-One Assay has been designed to include 549 DNA variants and 5 CNVs, plus 79 RNA fusions (including MET exon 14 skipping) known to be important in lung cancer. Samples used in this study were formalin-fixed paraffin embedded (FFPE) reference standards, CNV positive cell lines, and FFPE lung cancer samples. Following target enrichment of DNA and RNA using the GeneRead QIAact Lung All-in-One Assay the libraries were sequenced on the GeneReader and mutations assessed using QIAGEN Clinical Insight (QCI) Analyze.
Results: To confirm both DNA and RNA mutation detection, Horizon Discovery Reference Standards containing DNA (SNVs and InDels) and RNA (fusions)

mutations, typical of FFPE samples (Quantitative Multiplex and ALK-RET-ROS1 Fusion RNA Reference Standard), and well-characterized cell lines were used. All expected DNA (5% allele frequency threshold appropriate for FFPE DNA) and RNA mutations were repeatedly identified both within and between runs. Testing of clinical samples consistently detected the expected RNA fusions (EML4-ALK) and DNA mutations (*EGFR* and *KRAS* mutations) previously identified by FISH or quantitative PCR (QIAGEN *therascreen*), respectively.
Conclusions: These data showcase the performance reliability of the GeneRead QIAact All-in-One Assay on the GeneReader NGS System. This is the first study of its kind to systematically demonstrate the ability of a single assay to test for a wide range of mutations including complex chromosomal rearrangements. This allows a laboratory to accurately detect actionable lung cancer mutations with a single NGS assay in one seamless workflow, greatly improving efficiency and effectiveness.

P24. Using the GeneReader NGS System and QIAact Myeloid DNA UMI Panel to Detect Complex Mutations in Myeloid Leukemia

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Introduction: Myeloid leukemias are a group of different diseases such as myeloproliferative neoplasms (MPN), myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) driven by somatic mutations acquired in around 20-30 key genes. To assess all relevant genetic alterations for myeloid malignancies, multiple tests typically need to be performed which is time-consuming, requires large amounts of DNA input, and more effort for comparing and consolidating the results is necessary. This overall lengthens the time and increases the efforts from sample acquisition to final variant detection.
The QIAact Myeloid DNA UMI Panel in combination with the QIAGEN GeneReader NGS System provides a single solution to simultaneously test for actionable mutations, whilst also saving sample material (only 40ng DNA input required per sample), shortening test time and enabling simplification of lab operations.The QIAact Myeloid DNA UMI Panel is a 25-gene targeted sequencing panel for markers of known significance to clonal myeloid malignancies, allowing reliable and sensitive detection of single nucleotide variants (SNV) and large Insertion/Deletion (InDel) mutations.
Methods: Following the advice from key opinion leaders in the field of blood cancer disorders, the QIAact Myeloid DNA UMI Panel has been designed to detect complex mutations throughout the most informative genes linked to myeloid disease. A key feature of the panel is the addition of a unique molecular index (UMIs) to tag individual molecules prior to target enrichment by PCR, this enables sequencing and PCR bias corrections, allowing sensitive detection of mutations (e.g. below 1% MAF for *JAK2* (exon 12, 13, 14 & 15) & *KIT* (exon 8, 9, 10, 11 & 17)). To assess the assay performance, control samples (including reference standard and Coriell samples) and, blood and bone marrow samples were used. Following target enrichment, libraries were sequenced on the GeneReader NGS system and mutations analyzed using QIAGEN Clinical Insight (QCI) Analyze software suite.
Results: The samples used confirmed the ability of the assay to detect large indels, including the 52 bp deletion CALR type 1 variant. Limit of detection testing using the power of UMIs yielded sequencing results showing sufficient uniform amplification and sequencing coverage to consistently detect mutations with a 1% MAF and below for JAK2 and KIT and 5% for all the other genes covered by the panel.
Conclusion: The QIAact Myeloid DNA UMI Panel in combination with the QIAGEN GeneReader NGS System offer a fully integrated DNA to variant detection and interpretation solution. The optimized chemistry allows superior analytical sensitivity resulting in accurate and efficient mutation detection of highly relevant genetic alterations for myeloid malignancy research.

P25. Cardiovascular Disease (CVD) Patients with Hypertrophic Epicardial Adipose Tissue (EAT) Have a Microbiome Core Associated to Innate Immunity Activation

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Introduction: The epicardial adipose tissue (EAT) is a visceral fat surrounding myocardium with emergent role in heart metabolism due to its anatomical contiguity to cardiac organ. In case of EAT mass increase, dysfunctional adipocytes release pro-inflammatory adipokines and activate immune cells, including macrophages, developing a pro-inflammatory environment contributing to the severity and progression of cardiovascular diseases (CVDs). It is notable that endogenous alteration of the host tissue microbiome can promote innate immunity responses, participating in the amplification of local inflammatory environment. Due to the role of activated macrophages in tissue repair and anti-bacterial responses, our aim was to investigate in CVDs patients whether an increase in EAT mass can be associated with local microbiome host variation and with the activation of molecular patterns associated with innate immunity responses.
Methods: EAT biopsies were collected during open heart surgery from 23 CVDs patients. Patients were stratified according to an EAT cut off value of 7mm, as marker of hypertrophy (in: CVDs< 7mm and CVDs ≥7mm). Microarray assays were performed to evaluate the molecular patterns of EAT biopsies associated with macrophage activation and related-cytokine release. mRNA levels of CD14, CD163,CD163L, TLRs, NF-Kb, APC-1 mRNA as genes involved in macrophages

activation; and IL-1, IL-6, IL-12, IL-8 and TNFα as the main cytokines released by them, were measured. In all patients from each group, EAT microbiome composition was determined using next-generation sequencing technology.
Results: In EAT, mRNA expression of genes involved in macrophage activation and related pro-inflammatory cytokines were significantly higher in CVDs patients with hypertrophic EAT. A core of bacterial genera (*Acinectobacter* spp, *Chryseobacterium* spp, *Comamonas* spp, *Corynebacterium* spp, *Delftia* spp, *Flavobacterium* spp, *Kocuria* spp, *Methylobacterium* spp, *Paracoccus* spp, *Pelomonas* spp, *Propionibacterium* spp, *Pseudomonas* spp, *Sphingomonas* spp, *Staphylococcus* spp, *Streptococcus* spp) was identified in both CVDs group patients; interestingly CVDs with EAT ≥7mm presented different predominant opportunistic species than CVDs patients with physiological EAT thickness.
Conclusions: Our study demonstrated the presence of a preserved bacterial core directly in EA. We also verified that hypertrophic EAT biopsies presented more opportunistic pathogens species than biopsies with physiological EAT thickness. Furthermore, CVDs with EAT≥7mm presented higher expression of genes associated with macrophage activation and innate immunity responses. Our results suggest that the EAT local microbiome in CVDs patients changes when EAT mass increases and stimulates a pro-inflammatory environment through macrophage activation.

P26. Performance of Different Specimen Types and Quantitation on Ampliseq Cancer Hotspot Panel V2.0 Kit over Three Years

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Introduction: Using next generation sequencing (NGS) to identify somatic mutations in cancer is widely accepted in a routine clinical practice. Challenges remain with initial tumor specimen triages and the timely delivery of high quality NGS test results, which may impact successful test rate and turnaround time.
Methods: To address this issue, we investigated, in 2015-2017, the frequency of NGS test repeats of genomic DNAs derived from Formalin Fixed Paraffin Embedded (FFPE) samples verses samples from Fine Needle Aspiration (FNA). In 2015, we validated the AmpliSeq Cancer Hotspot Panel v2.0 kit on the Ion Torrent and then in May 2016 on the MiSeq. Samples, including amounts < 1 ng/mL on Qubit, are accepted, analyzed and not triaged since it is appreciated how these samples may be the only available tissue. A common assumption is that FNA and lung (LC) biopsies are at higher risk of failure due to insufficient quantity of DNA from limited tissue. The NGS repeat rates among various tumor types (colon [CC], LC, brain [BC], skin [SC], and others [OC]) were also studied. These results will benefit the laboratory's future workflow and data quality improvement. Logs that contained specimen type (FFPE/FNA/Cell block [CB]), cancer type and quantitative values were used. These logs documented if samples were repeated and the reason. Samples were excluded if ran for validation purposes. Specimens were included if analyzed on the Ion Torrent or MiSeq (2015-2017). Total number of samples were counted along with the number of FFPE/FNA/CB and cancer type (CC, LC, BC, SC, or OC). Samples repeated for uncommon variants or for machine failure were not considered as repeated but were included in total amount. Repeated specimens were counted and compared in each group. Total samples ran were: 911 on the Ion Torrent and 2429 on the MiSeq; 51 and 125 specimens were repeated respectively.
Results: For both FFPE and FNA, approximately 5% of samples needed to be repeated on both the Ion Torrent and MiSeq. When split into their cancer type, there was some variation. The Ion Torrent samples were composed of 77.5% LC, 10.1% CC, and 3.4% SC cases. No CC cases were repeated but 6.5% of SC and LC cases were repeated. For the MiSeq, samples were composed of 61.6% LC, 24.8% CC, 5.1% SC, and 6.9% BC; samples were repeated 5.8%, 3.5%, 4.9%, and 3.5% respectively. When chi-square analysis was performed, for FFPE vs. FNA and cancer types, no statistical significance was observed. Here we reviewed repeat results on samples that were not triaged by Qubit quantitation prior to preparing DNA libraries.
Conclusion: We demonstrated AmpliSeq Cancer Hotspot Panel is a robust assay in testing extracted DNA from FFPE and FNA specimens, with acceptable 5% test repeat rates without upfront input DNA triage. Despite the common impression that insufficient DNA may be received from FNA samples or LC biopsies, we show that the vast majority of FNA specimens may be successfully analyzed using NGS test, similar to FFPE specimens, across several major cancer types.

P27. Detection of EGFR Mutations in Circulating Cell-Free DNA (cfDNA) from Plasma Samples of Patients with Non-Small Cell Lung Cancer Using the Oncomine Lung cfDNA Assay

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Introduction: Analysis of cfDNA is increasingly used in management of patients with non-small cell lung cancer (NSCLC). This is commonly carried out by allele-specific PCR targeting limited mutations in the EGFR gene. We have evaluated the performance of a commercial molecular barcode based NGS approach developed to detect mutations of multiple lung cancer related genes in plasma at a sensitivity equivalent to allele-specific PCR methods.
Methods: Plasma samples were collected in Paxgene tubes from 26 NSCLC patients at diagnosis or relapse and cfDNA was extracted using MagMax DNA extraction kit or QiAamp circulating nucleic acid kit. The cfDNA samples were then analysed for mutations in 11 genes

including EGFR by NGS using Oncomine Lung cfDNA kit (Thermofisher Scientific). In addition 14 patients had samples available for in house testing by Therascreen EGFR assay (Qiagen) and 12 patients had samples analsyed by Cobas EGFR assay (Roche) in an external reference lab.

Results: Thirteen patients had matched FFPE tissues and cfDNA samples. Three were diagnostic samples and shown to carry sensitising EGFR mutations in the tissue samples. The same mutation was detected in plasma by Oncomine cfDNA assay in 2/3 cases and by therascreen assay in 1/3 cases. Four samples were collected from the patients at relapse following anti-EGFR therapies. The repeat biopsies of these four patients all carried both a primary sensitising EGFR mutation and the acquired EGFR T790M mutation. The Oncomine cfDNA assay detected the primary EGFR mutation in 4/4 cases and T790M in 3/4 cases whereas Therascreen assay detected the primary EGFR mutation in 3/4 and T790M in 1/4 samples. The remaining six cases were shown to be EGFR wild type in the matched FFPE tissues but carry mutations in TP53, BRAF or ERBB2 genes in 4/6 cases in the regions targeted by Oncomine cfDNA assay. As expected, no EGFR mutations were detected in cfDNA of these cases by Oncomine cfDNA assay. However, the same other gene mutations were detected in the plasma in 2/4 cases by this assay. From the 13 patients with plasma samples collected for EGFR T790M testing following relapse from anti-EGFR therapy and no matching tissue biopsies available, 12 samples were tested by both Oncomine cfDNA assay and cobas EGFR assay and the results were 100% concordant. The nine samples were tested by both Oncomine cfDNA assay and Therascreen assay. The results were concordant in 6/9 cases with three cases failed by therascreen assay due to inadequate DNA quality (2 cases) or the mutation being in an off-target region and hence not detected by the kit.

Conclusion: Our preliminary data showed that Oncomine Lung cfDNA assay appeared as sensitive as allele specific PCR method for detection of EGFR mutations from plasmas of NSCLC patients at both diagnosis and relapse. As the assay covers muti-gene targets, it is capable of detecting mutations in other lung cancer related genes, thus may be more suited to monitor disease progression.

P28. The Use of a Hybridization -Based Enrichment Approach to Achieve the Complete Coverage by Next-Generation Sequencing (NGS) of Difficult to Sequence Genes (CALR, CEBPA, FLT3) Associated with Myeloid Disorders

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Introduction: The application of short read next generation sequencing (NGS) for research into myeloid disorders such as myeloproliferative neoplasms (MPNs) and acute myeloid leukaemia (AML) has been hampered by the inability to fully sequence certain genes. These genes can harbour key mutations so it is desirable to improve the analysis of them. They include: *CALR* exon 9 insertions and deletions (up to 52 bp), *CEBPA* single nucleotide variants (SNVs) and *FLT3* Internal Tandem Duplications (ITDs) and SNVs. Each of these genes contains challenging regions which can impact the quality of the sequence data, e.g. large indels and low complexity regions (*CALR*), high GC content (75% on average for the whole gene with specific regions at 100%) and repetitive regions (*CEBPA*), and complex repetitive elements (*FLT3*).

Methods: To see if we could overcome these issues, we utilised a hybridization -based enrichment approach in combination with a SureSeq myPanel NGS Custom AML Panel. This panel includes many of the genes that are important in the research of myeloid disorders, and in particular the difficult to sequence genes mentioned above. We sequenced the library on a MiSeq using the 2x150 bp read length protocol. We assessed the minimum coverage and uniformity across the panel. We then looked for specific known mutations in each of these genes to see how well they were detected.

Results: The uniformity of coverage we obtained by this approach was >99% of the bases covered at >20% of the mean (after removal of PCR duplication). We obtained good depth across the entirety of each of these difficult to sequence genes in numerous research samples. Within some of these samples we were able to confidently identify and accurately size (including low allele frequency) insertions and deletions of up 52 bp in *CALR* (exon 9), SNVs throughout *CEBPA* (with a de-duplicated depth in excess of 2000x) as well as ITDs of between 24 and 126 bp in *FLT3*.

Conclusions: Excellent uniformity of coverage was obtained from the SureSeq myPanel NGS Custom AML Panel. High levels of uniformity were maintained across all genes permitting the reliable identification and accurate sizing of key *CALR* variants (including 52 bp deletions and 5 bp insertions), SNVs, indels and other in frame variants throughout *CEBPA* with a de-duplicated depth of greater than 2000x as well as ITDs of between 24 and 201 bp in *FLT3*.

P29. Speeding Clinical Reporting of Targeted Sequencing of Cancer Gene-Panels through Seamless Integration of Data Quality Control, Mutation Genomic and Clinical Annotations, and Drug Sensitivity Options

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Introduction: Sequencing of actionable cancer genes from tumors of patients allows identifying actionable cancer aberrations providing targeted therapy options. Despite its advantages, implementation of such tests has many challenges, from the selection assay format, deployment of the analysis pipeline, to the implementation of clinical reporting. Professional

guidelines should be followed, and analytical validation must be performed before offering the test.
Methods: To accelerate implementing precision oncology through tumor profiling, we have developed an end-to-end informatics platform that starts with raw sequence data and produces a fully compliant clinical pathology report. We leveraged technology from the Opal Clinical software, which is the leading platform to interpret genomes, exomes, and hereditary panels, and implemented features for the analysis and interpretation of somatic alterations complying with AMP guidelines.
Results: We show that Opal for Oncology can identify high quality somatic mutations, segregating these from germline variants, and provides relevant annotations for scoring following AMP guidelines, in a matter of hours. We also demonstrate that by using reference materials our software provides necessary metrics for analytical validation and accreditation.
Conclusions: Our platform allows clinical laboratories to quickly implement precision oncology tests by meeting guidelines and regulatory requirements, reducing implementation costs, and accelerating the test accreditation phase.

P30. Performance Evaluation of LymphoTrack Clonality Assays on Ion PGM and Ion S5 Platforms
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Introduction: Detection of clonal rearrangements within the immunoglobulin (Ig) and T-cell receptor (TCR) genes in clinical specimens is a finding used to assist in diagnosis of lymphoproliferative disease (LPD). Next-generation sequencing (NGS)-based clonality assays represent a major advance compared to the traditional capillary electrophoresis (CE)-based assays as the NGS assays can identify specific clonal sequences that can be tracked in follow-up testing. Here we evaluate the performance of LymphoTrack Dx (*IGH* (FR1/2/3), *IGK*, and *TRG*) assays on two Ion systems (Ion PGM and Ion S5).
Methods: LymphoTrack Dx Assays targeting the *IGH*, *IGK*, and *TRG* loci have been developed for the Ion systems. Consensus primers targeting V and J gene segments include specific adapter sequences and individual barcodes, so the PCR products from multiple independent samples and multiple clonality assays can be combined and sequenced together in a single NGS run. Individual master mixes were manufactured with 12 indices. Single step PCR amplification of 50 ng DNA input was followed by pooling of equimolar amounts of purified amplicons. Template preparations were performed with either the Ion OneTouch 2 system for Ion PGM or the Ion Chef system for Ion S5. Fastq data from both Ion PGM and Ion S5 were analyzed using LymphoTrack Dx Software, to generate frequency distributions, determine V-J usage, identify specific sequences for top sequencing reads, and determine the somatic hypermutation rate of *IGH* FR1 amplicons. Limit of detection (LoD), limit of blank (LoB) and linearity was evaluated by testing serial dilutions of contrived samples with V-J rearrangements in tonsil. Clinical samples were used to assess the clinical performance.
Results: All LymphoTrack Dx assays have demonstrated LoD to detect 5.0% clonality and excellent linearity for both Ion PGM and Ion S5 systems. Great concordance between Ion PGM and Ion S5 systems was demonstrated when testing clonality for all targets from clinical samples.
Conclusions: This study has demonstrated that the performance of LymphoTrack Dx Assays on Ion PGM and Ion S5 systems is comparable. The LymphoTrack Dx Assays have the potential to be utilized for routine Ig/TCR clonality detection in LPD diagnosis.

P31. Single-Step *IGHV* Next Generation Sequencing Detects Clonality and Somatic Hypermutation in Lymphoid Malignancies
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Introduction: Current gold standard established methods used to evaluate clonality and somatic hypermutation in lymphoid malignancies are multiplex PCR using consensus primers followed by capillary electrophoresis and Sanger sequencing. Recently, Next Generation Sequencing of immune receptor genes was suggested to be highly effective in detecting clonality with increased sensitivity. We designed a phase 3 diagnostic accuracy study to compare the value of the first commercially available Next Generation Sequencing approach testing for immunoglobulin heavy chain gene rearrangements with the existing gold standard methods.
Methods: Sixty eight samples were evaluated for IGH rearrangements with both traditional capillary electrophoresis and with the new NGS approach (*LymphoTrack IGH Assay, and LymphoTrack IGH Somatic Hypermutation Assay*), the latter two formatted and run on Illumina MiSeq instruments.
Results: The NGS clonality assay compared to conventional capillary based analysis had an overall diagnostic accuracy of 96% (63/66 cases). Sensitivity, specificity, positive predictive value, and negative predictive value of 95%, 100%, 100%, and 75%, respectively. In discrepant cases the NGS results were confirmed by a different set of primers that provided coverage of the *IGH* Leader sequence.
Conclusions: Conventional Sanger sequencing and Next Generation Sequencing based somatic hypermutation analyses gave overall comparable results. For future use in a routine diagnostic workflow, Next Generation Sequencing approaches should be evaluated prospectively and cost-effectiveness assessments should be performed.

P32. An Engineered DNA Ligase for Efficient Conversion of Input DNA During NGS Library Preparation

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Introduction: Molecular diagnostics next generation sequencing (NGS) assays are critically dependent upon efficient conversion of low-input DNA samples (*i.e.* cell-free DNA) to sequencing-capable library fragments during library preparation. Ligation-based library prep methods are the performance standard for low-input DNA samples, and their conversion efficiency is currently limited by the performance of the wild-type T4 DNA ligase used to attach adapters to both ends of end-repaired dsDNA fragments. The low efficiency of fragment conversion using wild-type T4 DNA ligase ultimately limits NGS assay sensitivity.
Methods: We have used directed evolution to develop an engineered DNA ligase optimized for NGS workflows. In this evolution program, ligase variants were assayed for performance under NGS-relevant conditions, using model end-repaired/A-tailed substrates and NGS adaptors, both present at low concentrations during screening.
Results: The engineered ligase achieved significantly improved conversion of A-tailed library fragments relative to wild-type T4 DNA ligase, under a variety of challenging ligation conditions, including low input DNA concentrations. Despite its higher specific activity, the engineered DNA ligase produced limited amounts of adapter dimer using T-tailed adapters.
Conclusions: The engineered ligase will be ideally suited for molecular diagnostics NGS applications, where its improved activity on dsDNA will improve conversion efficiency and, ultimately, assay sensitivity. In addition, the engineered ligase may find application under a variety of NGS preparation workflows, including microfluics or single-tube multi-enzyme workflows, due to its robust performance across the wide range of ligation conditions tested.
Keywords: molecular diagnostics, next generation sequencing, enzyme engineering

P33. Comprehensive Analysis of Genetic Variations in Patients with Acute Lymphoblastic Leukemia

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Introduction: Research on genetic variations in patients with B-cell and T- cell acute lymphoblastic leukemia(ALL) are ongoing, but a comprehensive analysis of genetic variations including chromosomal and genic copy number variations is rarely reported. We developed and applied next-generation sequencing (NGS)-based targeted gene panel and bioinformatics pipeline to profile the genetic changes in patients with acute lymphoblastic leukemia.
Methods: We analyzed 185 genes in 87 patients with B-cell ALL(n=72) and T-cell ALL(n=15). Thirty five adults and 53 pediatric patients were included.
Results: Among 72 patients with B-ALL, 33 has chromosomal abnormality such as hyperdiploidy. A total of 197 genetic variants were detected and 149 of these were deletion or duplication of genes(75.6%). *CDKN2A/B* and *IKZF1* genes were most frequently deleted. Among single nucleotide variants and small indels, those of *TP53, RAS* and *PTPN11* were most frequently detected. Among 15 patients with T-cell ALL, variations in *PTEN* and *PHF6* genes were frequently observed along with *CDKN2A* deletion. Deletions of genes indicating poor prognosis, such as *IKZF1* and *EBF1,* were more frequent in adult patients.
Conclusions: Using an NGS-based gene panel which can assess copy number variations as well as sequence variations, genetic variations of lymphoblastic leukemia could be better understood. Further study would be needed to connect the genetic variations to the clinical outcomes.

P34. Using the GeneReader NGS System to Identify Mutations in *BRCA1/2* Genes in Matched FFPE and Blood Samples

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Introduction: The *BRCA1* and *BRCA2* genes represent the best examples of the modern understanding of cancer molecular genetics. Supported by strong clinical evidence, testing for germline mutations in *BRCA1/2* in suspected familial breast cancer cases has gradually become common practice, especially in countries like the US. However, while less widely known and much harder to detect, somatic aberrations in these genes also play a critical role in cancer evolution, outcome and management.
Methods: In order to enable full investigation of mutations in *BRCA1/2* genes, we have developed the GeneRead QIAact BRCA Advanced UMI Panel that is compatible with a range of clinical samples. To provide a fully integrated solution enabling any laboratory to adopt the test, we used the GeneReader NGS System, combining wet bench sample processing, target sequence enrichment, and optimized bioinformatics for BRCA 1/2 gene analysis. The performance of this assay has been demonstrated by running a set of matched formalin fixed paraffin embedded (FFPE) and Blood samples harbouring *BRCA1/2* mutations.
Results: The assay, which was designed to cover the entire coding regions of both genes +/- 20bp flanking intronic sequences, in combination with a specifically designed bioinformatics pipeline, can detect both single nucleotide variants (SNVs) as well as Insertion/Deletions (InDels) in challenging real-world patient samples from both breast and ovarian tumor types. For sequencing, samples were pooled and run in a 8-plex (FFPE) or 12-plex (Blood) configuration per flowcell, which yielded

sequencing results with an average coverage of ≥300x unique molecular index (UMI) across all target regions. For FFPE and blood samples >95% and >99% of the target regions showed sufficient uniform amplification and sequencing coverage, to detect mutant alleles at a minimum 5% and 50% allele frequency, respectively. Mutational status was correctly confirmed in all matched samples, resulting in 100% concordance with an alternative testing technology.
Conclusions: These results support the use of the GeneReader NGS System to detect *BRCA1/2* mutations relevant to both germline and somatic cancers. This fully integrated workflow together with an optimized BRCA pipeline enables an easy implementation of the test by any molecular cancer research laboratory.

P35. A Comparative Study of PD-L1 Protein Expression Using the Ventana SP263 Diagnostic Assay and *PD-L1* mRNA Expression Using RNAscope in Formalin Fixed Paraffin Embedded Samples of Non-Small Cell Lung Cancer (NSCLC), Head and Neck Squamous Cell Carcinoma (HNSCC) and Urothelial Carcinoma (UC)

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Introduction: Numerous IHC diagnostic assays have been developed for tumor PD-L1 protein assessment, but concordance between IHC assays varies. mRNA detection by *in situ* hybridization (ISH) could be utilised as an alternative to protein detection. Additionally, detecting spatial changes in gene expression provides vital prognostic and diagnostic information, particularly in immune oncology where the phenotype, cellular infiltration and immune activity may be associated with survival. Translation of mRNA expression to a clinically relevant level is challenging due to assay variability and the detection of different analytes. This work aims to confirm the suitability of formalin fixed paraffin embedded (FFPE) tissue sections for use with RNA ISH. A comparison of mRNA expression and protein expression will inform the suitability of mRNA as a patient selection biomarker in a similar manner to IHC and provide evidence of a suitable scoring algorithm.
Methods: Thirty (30) clinical cases for each of NSCLC, HNSCC and UC, previously assessed using the VENTANA SP263 IHC assay were chosen to represent a wide range of percent tumor cell staining (TC_{IHC}). mRNA expression was assessed by ISH using the RNAScope 2.5 assay and probe CD274/PD-L1 (Advanced Cell Diagnostics) with appropriate positive and negative control probes. Brightfield whole slide images of tissues were captured. The percentage of tumor cells with PD-L1 mRNA expression ($\%TC_{mRNA}$) and level of expression (mean punctate dots/cell) were determined using image analysis. Differences between the TC_{IHC}≥25% and < 25% groups were assessed using t-tests. For each indication, a receiver-operating characteristic (ROC) analysis identified thresholds for patient classification using $\%TC_{mRNA}$ and dots/cell, with reference to TC_{IHC}≥25%.
Results: We successfully tested 86 samples; 3 failed due to insufficient control probe staining, 1 due to lack of tumor. The percent of TC_{mRNA} was significantly higher (at α=0.05) in the TC_{IHC}≥25% vs < 25% groups for NSCLC (38.7 vs 13.1% p=0.0014), HNSCC (35.1 vs 20.2% p=0.0040) and UC (31.4 vs 12.4% p=0.030). Dots/cell was significantly higher in the TC_{IHC}≥25% vs < 25% groups for NSCLC (0.99 vs 0.24 p=0.0083) and HNSCC (0.73 vs 0.32 p=0.0023) but not UC (1.0 vs 0.25 p=0.12).vFor $\%TC_{mRNA}$, ROC analysis identified thresholds of: NSCLC 18.0%, HNSCC 31.8%, UC 25.8%. For dots/cell: NSCLC 0.26, HNSCC 0.53, UC 0.45.
Conclusions: We conclude that FFPE tissue is suitable for determining PD-L1 mRNA expression using RNA ISH. Additionally, the PD-L1 mRNA extent and level is associated with PD-L1 status determined by IHC, and the detection of multiple markers in addition to PD-L1 in a multiplex assay may provide further insight into the expression profile of behaviour of tumors in response to immune therapy. Finally, this threshold optimisation for $\%TC_{mRNA}$ and mean dots/cell results in high specificity to IHC PD-L1 classification, but only moderate sensitivity.

P37. Evaluation of the Luminex ARIES HSV1&2 Assay for the Detection and Differentiation of HSV-1 and HSV-2 in Cerebrospinal Fluid Samples

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Introduction: Herpes simplex virus type 1 (HSV-1) causes approximately 10% of all cases of encephalitis and is the most common cause of fatal viral encephalitis worldwide. The mortality HSV-1 encephalitis is very high, and nearly all untreated survivors will suffer lifelong sequelae. Herpes simplex virus type 2 (HSV-2) CNS infections often appear as meningitis or meningoencephalitis. Neonatal infection with HSV-2 can result in disseminated disease in ~25% of cases. Rapid turn-around time for PCR testing of cerebrospinal fluid (CSF) samples, and initiation of antiviral therapy, is critical to ensure prompt and appropriate patient care. Luminex has launched the ARIES HSV1&2 Assay, based on their proprietary MultiCode technology, for direct detection and differentiation of HSV-1 and HSV-2 DNA in cutaneous or mucocutaneous lesions. Here we describe our evaluation of this test for the detection of HSV-1 and HSV-2 DNA directly from CSF samples.
Methods: Testing CSF samples with the ARIES HSV1&2 Assay was per the manufacturer's directions. Reproducibility and lower limit of detection (LLOD) studies were done using CSF spiked with stabilized HSV-1 and HSV-2 particles (ZeptoMetrix). Accuracy studies used a panel of 21 CSF samples that previously tested positive or negative for HSV-1 or HSV-2 with our laboratory developed test (LDT) based on TaqMan PCR chemistry, and analyzed on the Roche LightCycler 480 (LC480) platform.
Results: Reproducibility testing was done using normal

CSF with and without addition of HSV-1 or HSV-2 particles, tested in triplicate. Reproducibility of results was excellent with %CV's ranging from 0.21 to 2.06. The LLODs for both HSV-1 and HSV-2 were determined to be 500 DNA copies/mL of CSF. The accuracy panel consisted of 21 CSF samples with known positivity for HSV 1 and HSV-2 as tested by our LDT. Agreement between both the methods for HSV-1 and HSV-2 was 100% and 90.9% respectfully. The single HSV-2 discordant result was positive with the LDT but negative on ARIES®. The crossing threshold for that sample with the LDT was very high (41.57) indicating that the HSV-1 DNA target copy number was extremely low, and repeat testing on the ARIES may have been adversely affected by multiple freeze-thaw cycles.
Conclusions: The performance of the ARIES HSV 1&2 Assay compared very well to our HSV 1&2 LDT. The major advantage of the ARIES method is the elimination of all up front sample processing allowing testing of CSF samples as they arrive in the laboratory, providing rapid turnaround of test results to the ordering physician, resulting in improved patient care.

P38. An Open-Source End-to-End NGS Clinical Data Management System Used to Improve Variant Reporting and Data Mining

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Introduction: A next generation sequencing (NGS) laboratory with increasing volume and scale cannot function properly without the organization of large amounts of data through a support platform for review and interpretation. Recently, we clinically validated SIMPL, (System for Informatics in the Molecular Pathology Laboratory), a free, open-source LIS/LIMS system for academic and non-profit molecular pathology NGS laboratories, developed at the Genomics and Molecular Pathology division at the University of Chicago Medicine (UCM-GMP). SIMPL was designed as a modular end-to-end information system to handle all stages of the NGS laboratory workload from test order, patient tracking, to variant interpretation and reporting. Here we present how SIMPL has been applied in clinical service to improve variant interpretation through searchable archives of historical variants and interpretations for easy review of new cases. Our hope is that this system will help build a platform for future inter-laboratory data sharing, facilitating data interpretations. We also present the utility of such a system to mine genomic information from large numbers of patient samples.
Methods: SIMPL is a web-based Laboratory Information System (LIS) implemented largely in Python/Django. The post-analytic module of SIMPL, which has been designed for interpretation and reporting, starts with the storage of variants (and other result types) from the clinical assay performed, followed by auto-generation of editable reports for each patient test including patient and specimen details, results, interpretations and general information about the test. To demonstrate the power of mining such a system, we queried 2,500 patient samples stored in SIMPL across three clinical NGS assays for solid tumors and hematologic malignancies targeting 50, 55 and 147 genes.
Results: SIMPL is now clinically live at the molecular pathology laboratory at UCM-GMP. We show multiple examples of utility of historic interpretation data stored in SIMPL, and its effect on increase in efficiency of interpretations and reporting. Across a cohort of 2,500 patients, we also show the genomic trends by diagnosis and its consequence on research hypothesis generation, focusing on lung and colon adenocarcinomas and acute myeloid leukemia.
Conclusions: SIMPL has been designed and implemented as a useful and affordable option that other labs in the community may adopt to make tedious variant interpretations and clinical reporting easier. Data mining of this rich genomic data can be useful to study trends as well as hypothesis generation for research.

P39. Evaluation and Application of RNA Fusion Gene Panel for the Patients with Acute Leukemia

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Introduction: The assessment of gene fusions is essential in diagnosis and treatment of patients with acute leukemia, but currently used tests reverse transcription-PCR (RT-PCR) or fluorescence *in-situ* hybridization (FISH) have several limitations. The application of next-generation sequencing (NGS) technology would be helpful in this group of patients.
Methods: We have evaluated the clinical validity of two commercially available RNA fusion panels, the TruSight RNA fusion panel (Illumina, USA) and FusionPlex Pan-Heme Kit (ArcherDx, USA). The correlation with conventional methods and the limit of detection were confirmed. We applied these NGS-based RNA panels to 46 diagnostic samples.
Results: The two commercial RNA fusion panels showed superior clinical sensitivity to conventional tests, detecting *KMT2A-AFF1* gene fusion with unusual breakpoint and rare gene fusions such as *DDX3X-MLLT10* and *NUP98-HOXC1.* The limit of detection was somewhat unsatisfactory, ranging from 10^{-1} to 10^{-2}. Among 46 patients with acute leukemia, 13 had gene fusions. Four of the positive results were those cannot be detected by RT-PCR. Three were rare or novel gene fusions, *ETV6-EIF4B, USP42-RUNX1*, and *PAX5-CBFA2T3. P2RY8-CRLF2* gene fusion indicating Philadelphia-like ALL was detected in a patient with B-cell lymphoblastic leukemia.
Conclusions: RNA fusion panels showed superior clinical utility in detecting gene fusions in patients with acute leukemia. They were suitable for the initial diagnosis, however, for follow-up samples, conventional RT-PCR should be selected.

P40. Next Generation Sequencing on Ion Proton for Mutation Detection in Brain Tumors: Development of Molecular Pathology Assays in the Kingdom of Saudi Arabia

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Introduction: In the Kingdom of Saudi Arabia molecular pathology and molecular diagnostic laboratories are in evolving stages, especially in LDTs (Laboratory Developed Tests). Molecular diagnostic testing enhances the use of therapeutic products in a more precise manner to treat patients. Next Generation Sequencing (NGS) strategies provide a fast, precise and cost-effective molecular diagnostic tool to identify mutations in clinical samples. Hospitals usually send the patient samples for genetic analysis to companies outside the Kingdom. We plan to develop Molecular genetic tests for brain tumors in the Kingdom using NGS technologies that can be used for treating patient with targeted drugs based upon the mutations in specific genes.
Methods: DNA was isolated using QIAamp DNA FFPE kit, 10 ng of DNA was used for NGS analysis on Ion Proton. Libraries were prepared using Ion AmpliSeq Cancer Hotspot panel v2 primer pools. Ion AmpliSeq 2.0 library kit, and Ion PI Hi-Q-OT2 200 kit was used for libraries and templates preparation respectively. Sequencing was done using Ion PI Hi-Q Sequencing 200 kit, 16 libraries having Ion Express barcodes (1-16) were pooled and sequenced using Ion PI chip on Ion Proton instrument. Data were analyzed using Ion reporter v5.6 software.
Results: We have analyzed approximately 40 brain tumor samples by NGS. We have identified 11 exonic (8 synonymous and 3 missense), 8 intronic and one 3'-UTR variants in Epedymoma grade III. In this case missense mutations detected were in *KDR* c.1416A>T; p. Gln472His in exon 11, in *PIK3CA* c.1173A>G; p. Ile391Met in exon 7, and in *TP53* c.215C>G; p. Pro72Arg in exon 4 respectively. In grade I ependymoma we found 9 exonic (7 synonymous and 2 missense) variants, and 2 intronic and one 3'-UTR variants. Missense mutations detected were in *IDH1* p. Arg132His, in exon 4; and in *TP53* c.215C>G; p. Pro72Arg in exon 4 respectively. Coverage data showed, that 99.52% and 93.24% amplicons had at least 100 and 500 reads respectively. 99.61% targets had 100x coverage, and 93.16% targets had 500x coverage. In glioblastoma cases also, we found mutations in *TP53, IDH1, N-Ras, K-Ras, EGFR,* and *PIK3CA* genes. Missense mutations detected in other samples were in *CTNNB1* c.113G>A, p. Gly38Asp in exon 3; in *TP53* c.722C>T; p. Ser241Phe in exon 7, and c.393_395delCAA; p. Asn131del non-frameshift deletion in exon 5 respectively.
Conclusions: Developing NGS technologies in the Kingdom of Saudi Arabia may potentially be useful for patients as well as clinicians. The development and application of a genetic test for glioma markers at molecular level will have significant economic and social benefits for the Kingdom. If we develop these tests and train the technicians in specialty hospitals then there will be no need to send the samples abroad.

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P41. The Challenge of Whole Genome Sequencing in Mainstream Cancer Testing

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Introduction: A national network of 13 NHS Genomic Medicine Centres (GMCs), announced in December 2013, has integrated genomics into mainstream medicine in England. The 100,000 Genomes Project is an initiative to sequence 100,000 genomes from patients with rare disease and cancer. It has driven the implementation of a framework to enable the acquisition of tumor samples and clinical data of sufficient quality to enable whole genome sequencing (WGS) and accurate interpretation.
Methods: The cancer programme pilot phase identified difficulties with genomic testing of formalin-fixed paraffin-embedded tumor samples and led to the embedding of formalin-free tissue handling pathways from theatres to laboratories to ensure samples were WGS conducive. The ability to simultaneously detect small variants, copy number variants and structural variants for cancer patients provides opportunities e.g. new treatment options and accessibility to clinical trials, and is able to identify mutational signatures which is not possible without such a genome wide approach. Cancer patients are benefiting from the implementation of genomic medicine into mainstream care.
Results: The standardization of processes from sample handling, DNA extraction, quality control, validation and reporting has enabled the delivery of WGS and evidences the provision of genomic services within a national healthcare system is beneficial and deliverable for cancer. An external quality assessment has been integrated into all pathways from the outset to monitor and measure the quality of the service and has driven the improvement of patient care.
Conclusions: This approach has adopted innovative approaches to design the GMC genomic services, created genomic multi-disciplinary teams spanning a multitude of specialisms including pathology, driven creative workforce training and forward-thinking approaches to informatics.

P42. Detection of a Rare Deficient Allele of Alpha-1 Antitrypsin, M(Procida), Using Melt Curve Technology Variant Analysis for the F and I Alleles

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Introduction: α-1 antitrypsin (AAT) deficiency is an inherited disorder that predisposes to lung and liver disease due to decreased levels of AAT or impaired AAT antiproteolytic activity. More than 150 variants in the *SERPINA1* gene that encodes AAT have been described. The S and Z alleles account for the vast majority of cases; the F and I alleles are also important in clinical assessment.
Methods: n our clinical laboratory, melt curve analysis is used to interrogate these alleles. A rare SERPINA1 allele was detected aiding in the treatment of a patient. Genomic DNA was analyzed for four variants in SERPINA1 [RefSeq NM_001127701.1]. Targeted analysis was performed using the LightMix (TIB MOLBIOL, Berlin, Germany) melt curve assay to identify the S and Z alleles (c.863A>T and c.1096G>A, respectively) while LightSNiP (TIB MOLBIOL) melt curve assays was used to identify the F and I alleles (c.739C>T and c.187C>T, respectively). Reactions were performed on a LightCycler480 II (Roche Diagnostics, Indianapolis, IN). Melt curves outside the calculated melting temperatures of wild type, S, Z, F, and I alleles were subjected to PCR amplification followed by bi-directional Sanger sequencing of exons and flanking intronic regions. Sequenced data were compared with existing mutation databases including the Human Gene Mutation Database.
Results: Sanger sequencing detected a thymine to cytosine transition (c.194T>C) leading to missense mutation of leucine to proline (p.L65P). When this variant was investigated on ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), it was classified as the unusual variant M(Procida). M(Procida) AAT is associated with deficient serum levels of AAT, presumably related to intrahepatocye degradation. AAT deficiency accounted for ~2% of chronic obstructive pulmonary disease and 6% of lung transplantations from 1995-2012 in the U.S. Testing for the S and Z alleles has been standard of care for suspicion of α-1 antitrypsin deficiency. Though rare, F and I allele testing are clinically consequential; the F allele is associated with normal AAT serum levels but impaired neutrophil elastase binding and the I allele can predispose to emphysema in compound heterozygotes with the Z allele. Introducing these alleles to the test algorithm covered more areas in SERPINA1 enabling the detection of additional alleles by aberrant melt curves.
Conclusions: The allele, M(Procida) (c.194T>C), has been characterized as having both decreased neutrophil elastase functionality and levels of AAT due to intracellular degradation, resulting in increased risk of emphysema when inherited in the homozygous state or in combination with a deficient or null allele. In this patient, M(Procida) was detected because the c.194T>C is molecularly adjacent to the site of the I allele (c.187C>T) leading to decreased binding of the probe and an early 'melting' curve. Detection of the severe deficiency allele M(Procida) impacted the clinical care of the patient by prompting close serial pulmonary follow up.

P43. Detection of 17 Targets in a Single PCR Tube by a Novel Probe System Combining Melting Curves and Taqman Probes

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Introduction: Multiplex PCR has become an increasingly popular method to provide more, clinically relevant answers from the same sample. In many clinical settings, achieving multiplex answers from the same sample provide benefits both in terms of cost, speed, and added clinical value as well as preservation of limited samples. In cases such as cancer susceptibility testing, sepsis, RSV-testing, gastrointestinal testing and many others, a broad spectrum of agents are relevant for testing to assess possible infections. However, PCR readout is commonly limited to the current maximum of 4-5 fluorophores on most instruments. We have developed a homogenous assay method to allow read-out of more than 20 answers from a single PCR reaction. The method is applicable to most PCR-based diagnostic platforms.
Methods: The method utilizes a system of target-specific labelled probes allowing each to be read out by subsequent melting curve analysis, allowing more than 5 probes per fluorophore channel, thereby greatly increasing the level of multiplexing achievable. By utilizing meltcurve readout of modified probes - one for each target - rather than only of the PCR amplicons, the system also adds an extra level of specificity to meltcurve analysis. Reaction and melting analysis is performed without adding reagents during analysis and hence without the need to re-open PCR reaction tubes.
Results: To test the system, we designed probes based on previously designed TaqMan probes targeting 17 different hemorrhagic fever viruses and tested them against artificial DNA targets. Testing was performed in single wells containing all probes labeled with different flourophors including FAM, Cal570, Cal610 and Quasar705 and was able to separately detect all 17 targets. PCR was performed on a standard Bio-Rad CFX instrument with 4-colour read-out, a MIC PCR cycler as well as the Agilent AriaMX. This successfully demonstrated its relevance in routine clinical testing.
Conclusions: The method comprise a robust, high-multiplex, homogeneous system to provide 20+ readouts per PCR reaction on most PCR platforms in use in current clinical diagnostics.

P44. Implementation of the GeneReader NGS System in a Molecular Pathology Laboratory

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Objectives: The goal of this study was to evaluate and implement a next generation sequencing (NGS) System, the GeneReader, in a routine molecular laboratory, compare its performance to other methodologies, and use the system in the identification of key tumor mutations and application of personalized cancer care.
Methods: Reference standards, formalin fixed paraffin embedded (FFPE) tumor and liquid biopsy samples with known mutations in various cancer driver genes (*BRAF, EGFR, KRAS and NRAS*) were used in this study . All samples were processed following the manufacturer's instructions, and their mutational status compared with previous results. Furthermore, interpretation of the variant findings was performed using the QIAGEN Clinical Insight Interpret (QCII) software, to provide actionable recommendations for each sample based on its mutational profile.
Results: All previously known mutations were correctly confirmed with the GeneReader NGS workflow, making it a reliable system for routine cancer mutational analysis. Importantly, in 6% (3/50) of the samples, NGS identified additional pathogenic mutations previously missed by single-analyte Pyrosequencing. These results suggest an NGS approach can more sensitively and accurately detect mutations and potentially broaden clinical options. Furthermore, 50% (10/20) of liquid biopsies harbored pathogenic mutations, 30% (3/10) of which were not found in tumor mass analysis from the same individuals. As such, liquid biopsy not only provides an additional sample source for analysis, but also additional insights into full cancer mutational profile.
Conclusions: The GeneReader NGS system provides a solution for cancer testing with low hands-on time and high accuracy. Such NGS approach also allows a more sensitive way of detecting critical cancer mutations. Additionally, the application of this technology in liquid biopsy enables a more accurate and comprehensive understanding of the cancer molecular profile, informing individualized clinical decisions.

P45. Anchored Multiplex PCR Enables Sensitive NGS-Based Mutation Detection in the Context of Large Primer Panels

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Introduction: Next-generation sequencing (NGS) enables nucleotide resolution detection of variants for comprehensive and accurate tumor characterization. As a result, the mutational landscape of a vast array of both pediatric and adult cancers is changing at a rapid pace, with large-scale whole genome and whole exome sequencing studies continually being published. The comprehensive nature of sequencing whole genomes or whole transcriptomes, however, results in low sensitivity and high cost, rendering these methods impractical for routine use. Target-enrichment strategies enhance NGS-based detection sensitivity, as target regions are sequenced to a greater depth. Traditional amplicon-based techniques utilize opposing primer sets to amplify a panel of specific target regions prior to sequencing. However, these approaches lack the flexibility to adapt to changing mutational landscapes, as each new addition of a target to existing panels often requires redesign of the entire panel, costing time and money, and expansion of panel size risks reducing the sensitivity of variant detection. Here, we describe a target enrichment method for NGS, Anchored Multiplex PCR (AMP), which enables sensitive detection of variants regardless of panel size while permitting flexibility in panel design.
Methods: AMP is a target enrichment strategy that uses unidirectional gene-specific primers and molecular barcoded (MBC) adapters ligated to DNA ends for amplification. Because MBC adapters contain a universal primer-binding site for amplification, optimization of a large panel of targets is simplified in comparison to having multiple sets of unique opposing primers. We designed the Archer VariantPlex NGS assays to detect single nucleotide variants (SNVs), insertions and deletions (indels) and copy number variants (CNVs) with a customizable set of panel targets.
Results: We assess the performance of the VariantPlex Solid Tumor Focus panel using reference materials titrated from 50ng down to 1ng total input. We show that this panel is able to detect CNVs, as well as known SNVs and indels with allele frequencies down to 5%. Furthermore, we compare per variant sensitivity and CNV sensitivity across input amounts for this panel alone and in the context of additional content that substantially increases the total targeted genomic sequence. Our data show that these VariantPlex assays maintain similar abilities to detect variants in small and large panels, indicating that this technology allows for flexible, modular panel design.
Conclusions: Our results demonstrate that AMP-based NGS assays are sensitive while maintaining flexibility in panel design. These assays are compatible with input amounts down to 1ng, can detect mutations with allele frequencies down to 5%, and have can be modified to accommodate new discoveries as needed without sacrificing sensitivity in variant or CNV detection.

P46. Analytical Validation of Digital Spatial Profiling: A Novel Approach for Multiplexed Characterization of Protein Distribution and Abundance in FFPE Tissue Sections

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Introduction: Characterization of the spatial distribution and abundance of key proteins within tissues enables a better understanding of biological systems in many research areas, including immunology and oncology. However, it has proven difficult to perform such studies in

a highly-multiplexed manner on FFPE tissue sections. To address this unmet need, we have developed a novel imaging platform, Digital Spatial Profiling (DSP), designed to simultaneously analyze 10's to 100's of proteins from discrete regions by detecting oligos conjugated to antibodies that can be released via a UV-cleavable linker. Here we describe the validation of the oligo-conjugated antibodies used with this new approach to highly multiplexed protein analysis.
Methods: Various antibodies targeting important immuno-oncology proteins (CD3, CD4, CD8, CD45, PD1, PD-L1, etc.) were tested for specificity and sensitivity. Immunohistochemistry was performed on formalin fixed paraffin embedded (FFPE) human tissues including tonsil and tumor samples, as well as human cell line pellets to evaluate binding specificity in both unconjugated and oligo-conjugated antibodies. The sensitivity and dynamic range of oligo-conjugated antibodies were tested using FFPE cell pellets with target-specific positive and negative cells at different ratios (0:100, 5:95, 20:80, 50:50, 100:0) and specific limits-of-detection (LODs) were determined. An interaction screen was performed to evaluate potential deleterious effects of multiplexing antibodies. Finally, a human tissue microarray (TMA) containing normal and cancer tissues was employed to assess assay robustness.
Results: Immunohistochemical analysis of antibodies integrated into the cocktail displayed indistinguishable staining patterns on control tissues and cell lines for both unconjugated and oligo-conjugated antibodies. Mixed-proportion cell pellet assays revealed strong correlations between observed counts above background and positive cell numbers in a region of interest, allowing us to accurately calculate LODs. For example, CD3 displayed a LOD of 4% when assayed using a cell pellet mixture containing increasing numbers of $CD3^+$ CCRF-CEM cells with $CD3^-$ HEK293T cells. Antibody interaction studies showed similar count values for antibodies alone or in combination ($R^2 > 0.8$). Finally, TMA hierarchical clustering analysis demonstrated expected patterns for immune cells and tumor cells across normal and disease tissue types.
Conclusions: These results demonstrate that indexing oligo conjugation does not interfere with antibody specificity and that these conjugated antibodies are robust reagents for quantification of protein abundance. Continued work on the DSP platform will expand the library of antibodies accessible for profiling.

P47. How Variabilities in Tumor Liquid Biopsy Workflows Can Be Tackled with cfDNA Reference Standards

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Introduction: The detection of mutations in circulating cell-free DNA (cfDNA) from the blood has become a valuable tool to guide cancer therapy and to monitor treatment response. Current technologies face the challenge to detect those variations from small amounts of highly fragmented DNA at low allelic frequencies (AFs). Assays are pushed to and beyond their sensitivity limits to detect those mutations, and sample processing is constantly optimised to extract as much cfDNA as possible from the patient sample.
Methods: To support laboratories in optimising and validating their NGS and PCR-based workflows, Horizon has developed a range of cell line-derived cfDNA Reference Standards that accurately mimic clinical samples. Our controls cover a range of relevant mutations (e.g. EGFR T790M) and allelic frequencies (5%, 1%, 0.1%, and wild type), fragmented to an average size of 160/170 bp, enabling testing of assay specificity, sensitivity and limit of detection (LOD).
Results: Here we demonstrate the use of our Reference Standards for validation and routine monitoring on a number of different platforms including the Roche Cobas qPCR, targeted NGS and Bio-Rad ddPCR assay.
Conclusions: Our cfDNA in synthetic plasma control reduces the risk of variability and contamination, assuring long-term stability, when compared to real human plasma, supporting the development and validation of the 'whole' tumor liquid biopsy workflow including DNA extraction.

P49. Comprehensive Sequencing Analysis Performed Routinely on Clinical Samples Can Provide Unexpected and Clinically Significant Findings: A Case Example

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Introduction: Comprehensive next generation sequencing (NGS) approaches, including integrative analyses that incorporate structural, copy number, and single nucleotide/indel variant detection, can offer an unbiased molecular evaluation of challenging surgical pathology cases. Here, we present a solid tumor case study that demonstrates the diagnostic and clinical utility of this approach. The patient, a 21-year-old female, presented to St. Jude Children's Research Hospital with an outside diagnosis of refractory metastatic malignant melanoma involving the soft tissue, lymph nodes, and brain. She had failed therapy with interferon and pembrolizumab and on arrival at our center received ipilimumab and TVEC. A representative lesion from her left axilla was submitted for histopathologic review. The tumor was composed of sheets of highly pleomorphic cells that were positive for melanocytic markers by immunohistochemistry including S100-protein, HMB-45, and Melan-A. An initial confirmatory diagnosis of metastatic malignant melanoma was made.
Methods: DNA and RNA extracted from the patient's tumor and germline samples were used to construct libraries for whole genome (WGS), whole exome (WES) and transcriptome (RNAseq) sequencing, using Illumina

TruSeq kits. All libraries were sequenced using a 2x125 paired-end protocol on the Illumina HiSeq at ≥45x coverage. The assay pipeline integrates genetic lesions detected by all 3 NGS platforms to cross-validate and characterize single nucleotide variations, short insertions and deletions, and structural variations including translocations. A parallel Rapid RNA-Seq pipeline separately reports fusion transcripts within 10 to 15 days after sample collection.
Results: Rapid RNA-Seq analysis was positive for the *EWSR1-ATF1* fusion transcript, and confirmatory FISH testing showed a positive rearrangement of *EWSR1*. Based on these findings, the diagnosis was updated from metastatic melanoma to diffusely metastatic clear cell sarcoma of soft tissue (CCS). The integrative genome sequencing report cross-validated the t(12;22)(q13;q12) event as part of a complex karyotype. The patient was switched to a therapy that included ifosfamide with clinical signs of disease improvement.
Conclusion: Clear cell sarcoma (CCS) of soft tissue has significant morphologic and immunohistochemical overlap with primary malignant melanoma, with both lesions being positive for classic melanocytic markers. As seen in this case, characteristic morphologic features of CCS can be masked in metastatic sites, in advanced disease, and in post-therapy patients. Comprehensive sequencing approaches can be used to identify unsuspected molecular abnormalities that have diagnostic relevance. Using a tiered approach where RNA-Seq data can be reported both separately and in tandem with WGS and WES, relevant known and novel fusion information can be provided in a diagnostic time-frame, while still allowing comprehensive reporting by day 30.

P50. ISO Accreditation of a Complete Next Generation (NGS) Sequencing Workflow for BRCA1/2 Analysis

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Introduction: Molecular diagnostic tests for germline pathogenic variants in the tumor suppressor genes *BRCA1* and *BRCA2* allows identification of patients with hereditary forms of breast (BC) and ovarian cancer (EOC), as well as assessment of individual risk for developing such cancers. Identification of *BRCA1/2* mutation is of great clinical importance as affected individuals are referred to genetic counseling, vigilant surveillance, and potentially life-saving preventative treatments. Furthermore, with recent development and approval of PARP inhibitors, the mutational status of *BRCA1/2* is also used to drive targeted therapy decisions.
Methods: In our laboratory we sought to establish a Next-Generation Sequencing (NGS) test to sequence full-length coding regions of the *BRCA1/2* genes. The complexity in the mutations and their clinical implications necessitates a diagnostic tool that offers a robust and easy workflow, combined with accurate and reproducible results as well as up-to-date interpretation for variant findings. In this abstract we describe the process to set up, validate, quality control and accredit an NGS workflow.
Results: Based on the GeneReader platform, the Center for Biomedical Analysis and Clinical Genomics (CABGeC) at Department of Oncology, IRCCS-"Mario Negri" Institute for Pharmacological Research. Milan Italy has been recently recognized as a certified laboratory (UNI EN ISO 9001:2015) for the identification of germline pathogenic variants in the *BRCA1/2* genes. Preliminary inter run and intra run tests were performed to confirm panel reproducibility in the correct variants identification and calling (100%). Over the last six months of activity, 42 cases of EOC have been analyzed, and five pathogenic germline variants (two cases were mutated in *BRCA1* and three in *BRCA2*) were identified. Orthogonal Sanger sequence validation confirmed identified mutations.
Conclusions: We have successfully brought live an NGS test for *BRCA 1/2* using the GeneReader NGS System. We established sample and process control from extraction through variant annotation and interpretation. Our experience can be leveraged by other laboratories new to NGS or BRCA testing.

P51. The Analysis of FFPE Samples by Next-Generation Sequencing (NGS) of Key Genes for Research into Breast and Ovarian Cancers

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Introduction: Next-Generation Sequencing (NGS) has enabled the simultaneous study of multiple mutations in high-penetrance cancer predisposition genes. However, tissue biopsies are typically archived as formalin-fixed, paraffin embedded (FFPE) blocks which can significantly compromise the quality and amount of nucleic acids available for genomics research. Is NGS a suitable and reliable approach for this research area?
Methods: To see if we could overcome these issues, we compared a PCR-based enrichment approach to the SureSeq hybridization -based NGS enrichment panel, the SureSeq Ovarian Cancer Panel when combined with the SureSeq FFPE DNA Repair Mix. The panel targets key research genes for both breast and ovarian cancer (*BRCA1, BRCA2, ATM, TP53, ATR, NF1 and PTEN*). We looked at minimum coverage and uniformity across the panel and compared this to the DNA integrity number (DIN) of the FFPE sample.
Results: The use of the SureSeq FFPE DNA Repair Mix generated higher de-duplicated (increased complexity) depths of coverage and also permitted the use of lower amounts of starting DNA material (down to 50 ng). The improved NGS library yields enable the generation of highly uniform coverage that allowed the detection of low allele fraction single nucleotide variants as well as insertions and deletions. The uniformity of coverage (expressed as the percentage of target bases that are

covered at >20% of the mean target coverage) was significantly better for the hybridization -based enrichment approach when compared to the PCR-enrichment approach. The data presented here are generated from a variety of different quality and input amounts.
Conclusions: By repairing the DNA prior to NGS library preparation we have confidently detected variants in the key targeted genes that are of particular interest in our studies/research. These truncating variants are likely to have deleterious effects to tumor suppressor genes (e.g. *TP53*) and DNA repair mechanisms (*BRCA1 & 2*). The utility of this approach and panel permits the analysis of somatic variation in these key DNA repair genes associated with ovarian and breast cancers but can also be used for research into many other cancers including prostate, pancreatic and melanoma.

P52. Allele-Specific Real Time Polymerase Chain Reaction (PCR) Versus Peptide Nucleic Acid Clamping for Low Copy Epidermal Growth Factor Receptor (*EGFR*) Mutation Detection in Liquid Biopsy Samples
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Introduction: Clinical data indicate that advanced non-small cell lung cancer (aNSCLC) patients harboring *EGFR*-activating mutations exhibit good response and prolonged progression-free survival when treated with anti-EGFR Tyrosine Kinase Inhibitors. A surrogate tissue, such as plasma, which contains circulating free tumor DNA, can be used to detect *EGFR* mutations reliably. Current guidelines recommend *EGFR* mutation testing of NSCLC patient plasma, making it critical that the assay accurately detect low copy number mutations. There are several EGFR test kits. This study compares the performance of Roche's cobas EGFR Mutation Test v2 and PANAGene's PANAMutyper R EGFR Kit. The PANAGene test consists of six 20µl reaction mixes, including 1 Internal Control (IC) reaction mix, to which 5 µl of sample is added; the cobas test consists of three 25ul reaction mixes, all containing an IC, to which 25 ul of sample is added. Both kits cover the same groups of *EGFR* mutations and claim similar analytical sensitivity of ≤ 0.1% mutant allele frequency.
Methods: Contrived cell line DNA spiked with plasmids containing eight different *EGFR* mutations at 5%, 1%, 0.5% and 0.1% mutation frequency, as well as healthy donor plasma samples spiked with 250, 100 and 50 mutant copies/mL mutant plasmid DNA were tested. DNA was extracted using cobas cfDNA Sample Preparation Kit for both tests. The assays were then performed in duplicate according to manufacturer's instructions.
Results: *Cell line DNA samples*: The PANAGene kit detected all samples at 5%, G719A was missed at ≤ 1%, L858R was missed at 0.1% and L861Q was missed at ≤0.5%; Ex19 deletion, Ex20 insertion, S768I and T790M mutations were detected at all input levels. The cobas test detected all mutations down to 0.1%.
Plasma samples: The PANAGene kit missed G719A at all copy levels tested. L861Q detection dropped out at ≤100 copies/mL and T790M was not detected at 50 copies/mL plasma. The Ex19Del, S768I, L858R, Ex20InsA mutations and one replicate of Ex20InsB mutant were detected at 50 copies/ml. The cobas kit detected all mutant copies except one replicate of G719A at 250 copies/mL and one replicate of L858R at 50 copies/mL demonstrating 0.1% analytical sensitivity for 17 of 18 mutations tested.
The PANAGene IC, a single independent reaction, failed for 3 of 43 samples tested; the cobas IC, incorporated into each of the 3 reaction mixes, did not fail for any samples. In general, the PANAGene workflow involves more handling steps, lower throughput, and the analysis requires skilled user evaluation. The cobas EGFR Mutation assay has fewer steps, higher throughput, and the results analysis is fully automated; it also provides a Semi-Quantitative Index (SQI; not available in all countries) which reflects the amount of mutant DNA in the blood.
Conclusions: Performance of the PANAGene kit was less robust relative to the cobas EGFR Mutation Test v2; sensitivity was not as high as the product claims with contrived samples.

P53. Rapid, Multi-Gene Mutation Detection Panel for Metastatic Colorectal Cancer
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Introduction: We have developed a real-time PCR assay for the detection of common metastatic colorectal cancer (mCRC) mutations in 5 oncogenes, including *KRAS* and *NRAS* (codon 12, 13, 59, 61, 117, 146); *BRAF* (codon 600), *PIK3CA* (codon 542, 545, 1047), and *AKT1* (codon 17), using DNA isolated from formalin-fixed, paraffin-embedded (FFPE) colorectal cancer (CRC) samples. The purpose of this study was to validate performance of this kit by evaluating limit of detection (LoD) and accuracy. We also determined concordance between the prevalence of *KRAS, NRAS, BRAF, PIK3CA*, and *AKT* mutations in CRC patients reported in the literature and results attained using the kit.
Methods: For LoD assessment, broad-range dilutions of mutant DNA (isolated from FFPE specimens or gBlocks Gene Fragments-based controls) at an internal control (IC) Ct of 29 were made using wild-type human colon FFPE DNA to yield 10%, 5%, 2.5%, 1%, and 0.5% mutant allelic burden over wild-type background. The assay was performed 3 times in duplicates on an ABI 7500 Fast instrument. Based on the results, narrow-range serial dilutions (in 0.5% increments) were made from the lowest detectable broad-range dilution to establish a more precise LoD. Assay accuracy was tested using 120 FFPE DNA samples isolated from patient CRC tumor specimens. The DNA samples were screened for relevant mutations with a commercial mutation detection kit to determine mutation status. Concordance between the Colorectal Cancer Mutation Detection Panel and the commercial kit was measured as the overall percent agreement, percent positive

agreement, and percent negative agreement for all valid samples (within an IC Ct range of 26-29). For a comparison of the prevalence of CRC mutations reported in the literature and detection with the kit, the assay was applied to 686 DNA samples extracted from FFPE CRC tumors.
Results: The panel detects 78% (39/50) of mutations at or below a LoD of 2.5%. 92% (46/50) of mutations are detected at or below a LoD of 5%. For assay accuracy, a comparison of mutation status calls between the Colorectal Cancer Mutation Detection Panel and the commercial kit shows 95.19%, 99.02%, 100%, 100%, 100%, and 99.10% overall percent agreement for reactions 1-6, respectively. Of the 686 CRC samples tested for assay concordance with the reported occurrence of mutations in patient samples from the literature, 35 (5.10%) did not meet the input requirement for the assay and gave an invalid result. Of the 651 valid samples, 391 (60.06%) presented a single mutation, while 51 (7.83%) had double mutations occurring concurrently. When grouped by oncogene, the incidence of observed single mutations was comparable to what has been described in the literature.
Conclusions: The CRC Mutation Detection Panel can accurately recognize single and double mutations in 5 different oncogenes with high sensitivity. Furthermore, the occurrence of the mutations detected is in line with what has been reported in the literature.

P54. Clinicopathological Features of Colorectal Carcinoma in Ukrainian Patients with MSI-H and MSS Status

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Introduction: To date, there are several molecular subtypes of colorectal cancer (CRC), including microsatellite-unstable, canonical, metabolic and mesenchymal types. Identification of molecular characteristics of CRC, especially microsatellite instability (MSI) status and RAS mutations is of significant clinical importance in terms of Lynch syndrome, prediction of efficacy to resistance to therapy and prognosis. In this study we assessed the relations between molecular and clinicopathological characteristics of CRC in the Ukrainian population.
Methods: This retrospective study included investigation of clinical data, histological characteristics, and molecular peculiarities in 177 patients (88 females and 89 males) with sporadic CRC. For identification of molecular profile of CRC, MSI was assessed by immunohistochemistry with antibodies to MLH1, MSH2, MSH6 and PMS2. In addition molecular tests for detecting *KRAS, NRAS* and *BRAF* mutations were performed. There were also 26 cases with panel of 65 genes sequencing. Statistical analysis was performed using MedCalc.
Results: The overall incidence of MSI-H status was 14.7% among observed patients with prevalence of MSI detection among men - 25.35% comparing with 10% in females (P = 0.0369). MSI-H status was tightly associated with tumor location in the proximal part of the large intestine (P < 0.0001) and found often among men younger than 50 yo (P=0.002), whereas MSS status was associated with advanced age and rectosigmoid cancer. MSI was more often associated with special histological types: medullary or mucinous (P = 0.000767), and most of the cases with such histology was found in males. The vast majority of MSI-H (76.9%) were identified in CRC of grade 2 and 3 of CRC (P < 0.0001) regardless of sex. Overall 50% MSI-H status was in CRC of Grade 3. In most cases, MSI was due to lack of MLH1 and PMS2 expression (64%), MLH1 deficiency was higher in men than in women. In contrast, women demonstrated a lack of MSH2+MSH6 more often. Lack of MSH2+MSH6, as well as isolated block of PMS2 expression, were associated with the highest tumor grade (P < 0.0001). Despite relation to the high tumor grade, MSI-H CRC demonstrated significantly lower rate of metastatic disease (P < 0.0001) comparing with MS-stable (MSS) tumors. MSS status CRC more likely had high grade histology (P = 0.0407) and was associated with high rate of RAS mutations: KRAS mutations were found in 47.2% cases (mostly in codons 12 and 13), NRAS - in 3.4% and BRAF in 7.5% patients.
Conclusions: Thus, in the Ukrainian population overall, the frequency of MSI-H CRC is 14.7%. MSI status was associated with male sex, younger age, local stage of disease, proximal location of tumor and high grade. MSS CRC was associated with high frequency RAS mutations and showed higher rate of advanced stage and metastasis.

P55. Rational "Error Elimination" Approach to Evaluating Molecular Barcodes Containing Next-Generation Sequencing Data Identifies Low-Frequency Mutations in Hematologic Malignancies

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Introduction: The emergence of highly sensitive molecular diagnostic approaches such as droplet digital PCR (ddPCR) has allowed the accurate identification of low-frequency variants in clinical specimens; however, the multiplex capabilities of ddPCR for variants detection are inadequate. The incorporation of molecular barcodes or unique IDs into next-generation sequencing (NGS) libraries through PCR has enabled the detection of low-frequency variant alleles across multiple genomic regions. However, rational library preparation and sequencing data analytical strategies that integrate molecular barcodes in clinical settings are very limited in number.
Methods: In this study, we evaluated the parameters that are critical to molecular barcodes containing NGS of hematologic malignancy patients' samples.
Results: We found that the uniform incorporation of molecular barcodes into the DNA templates through PCR

is critical and that the extent of uniformity is governed by multiple interdependent variables. We developed an error elimination strategy for removing sequencing background errors as an alternative to the traditional error correction approach by using molecular barcode sequence information. We successfully used this approach to identify low-frequency mutations, as low as 0.15%, and were able to unravel the clonal heterogeneity of hematologic malignancies.
Conclusions: In summary, our findings have implications for elucidating tumor heterogeneity, temporal and spatial clonal evolution of tumors, evaluations of tumor responses to therapy, and monitoring for disease relapse in hematologic malignancies.

P56. Clinical Correlation between Translocation Variant and Outcome in Philadelphia Chromosome-Positive Chronic Myeloid Leukemia and Acute Lymphoblastic Leukemia Patients
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Introduction: BCR/ABL fusion gene usually occurs as a result of Philadelphia (Ph) translocation between chromosomes 9 and 22 in Chronic Myeloid leukemia (CML) as well as in Acute Lymphoblastic Leukemia (ALL). This rearrangement results in the formation a chimeric BCR/ABL fusion gene on the derivative chromosome 22. Fluorescence *in-situ* hybridization (FISH) analysis using dual color BCR/ABL translocation probes allows the visualization of BCR/ABL rearrangements in both interphase and metaphase cells, and the presence of the BCR/ABL fusion gene on chromosomes 22 has been reported in substantial subset of these patients. The pattern of rearrangement may be classical, variable or mixed. Only the classical pattern has been reported to have good prognosis with lesser disease progression and good response to tyrosine kinase inhibitors.
Methods: The incidence of both classical and variable BCR/ABL gene rearrangement was determined in all the patients suspected of CML and ALL using dual fusion fluorescence *in situ* hybridization (D-FISH) probes. A minimum of 200 nuclei were scored where possible.
Results: This study investigated 860 patients of CML and ALL between January 2016 and September 2016 at the Aga Khan University Hospital. Out of 860 patients 775(90%) were diagnosed as CML and 85 cases (10%) were diagnosed as ALL. About 659 cases (76%) of both CML and ALL patients displayed the classical DF-FISH signal pattern and 201(24%) of CML and ALL showed variable DF-FISH signal pattern. In variable DF-FISH signal, various different patterns were analyzed: 1F1G1R is 31%, 1F2G1R is 27%, 1F2G2R is 26% and 1F1G2R is 14% were observed in both CML (22%) and ALL (2%). The rare combination of classical and variable was observed in around 2% of cases with CML.
Conclusions: The classical pattern was the most common pattern identified in both CML and ALL, predicting a good prognosis. Variable patterns were identified in equal proportion except for 1F1G2R which was the least common.

P58. Molecular Profiling in Colorectal Carcinoma: A Tertiary Centre Experience from Eastern India
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Introduction: Colorectal cancer (CRC) is a major cause of morbidity and mortality around the world and is the third most common cancer type worldwide. *KRAS* and *BRAF* mutation analysis is very crucial for targeted therapy management in advanced cases. Oncogenic activation of *KRAS* and *BRAF* is mutually exclusive and occurs in approximately 40% and 10% of all CRCs respectively. Several other mutations are also gaining significance in CRC as they can be potential for alternative targeted therapies or prognostic markers that could ultimately affect therapeutic outcome. The aim the study was to determine the frequency of various mutations in CRC at our centre and its co-relation with clinicopathological findings.
Methods: A retrospective analysis of 96 CRC patients referred for KRAS/NRAS/BRAF testing over a period of 2 year was done. Exon 2, 3, and 4 of *KRAS/NRAS* gene (n=71) and exon 15 of *BRAF* (n=55) gene were analyzed by Sanger sequencing. The remaining 25 cases were evaluated for 13 common genes (*KRAS, NRAS, TP53, BRAF, APC, CDKN2A, PIK3CA, EGFR, STK11, SMAD4, KIT, PGFRA, ERBB2)* using Ampliseq Cancer Hotspot Panel V2 on the Ion Torrent platform.
Results: All the cases were of advanced CRC (mean age-61.4 yrs, M:F ratio-1.34:1).Of the 96 cases evaluated, 44.79% (n=43) had *KRAS* mutations. These included exon 2 (n=39), exon 3(n=2) and exon 4 (n=2). BRAF mutations were positive in 6/80(7.5%). Target sequencing by Ion Torrent platform lead to the identification of frequent mutations in *TP53* (64%)*, KRAS* (52%) *and APC* (12%)*, CDKN2A* (8%)*, PIK3CA* (8%)*. BRAF, EGFR, STK11, SMAD4, KIT, PDGFRA, ERBB2* were mutated at a frequency of 4%. Though mutually exclusive, concurrent *KRAS* and *BRAF* mutations were seen in three cases. Not a single case of *NRAS* mutation was seen in this cohort of patients.
Conclusion: In the era of precision medicine and personalized oncology, it is very important to identify underlying variations in CRC cancers as such understanding can potentially affect treatment and prognosis. Although our sample size analysed by Ion Torrent panel was small, the results of mutation prevalence at our centre co-relate well with studies reported in the literature.

P59. Identification of a Novel ATM Missense Mutation by Next Generation Sequencing in Choroid Plexus Papilloma

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Introduction: Mutation of the ATM [ataxia telangiectasia (A-T) mutated] gene is the main cause of A-T a rare autosomal recessive disease. This protein is a member of a unique family of large proteins, which show sequence homology to the catalytic domain of PIK3CA. Choroid plexus papilloma (CPP) is a rare benign tumor of the central nervous system and it is usually confined to the ventricle system. Choroid plexus papilloma corresponds to WHO grade I brain tumor, atypical CPP (a-CPP) can behave more aggressively and is considered WHO grade II, occasionally, there can be malignant transformation to a choroid plexus carcinoma (WHO grade III) also. Somatic ATM mutations or deletions are usually found in lymphoid malignancies, as well as a variety of solid tumors. However, a causal relationship between ATM and brain tumors is not known so far. We performed the DNA analysis by next generation sequencing (NGS) in the tissue from a 3-year-old patient with a-CPP.

Methods: Routine radiological and immune-histological investigations were performed. Formalin fixed paraffin embedded (FFPE) tumor tissue was obtained after surgical excision, and the DNA was isolated using QIAamp DNA FFPE kit for NGS analysis. Ion AmpliSeq cancer panel primer pool was used in library construction. Sequencing was done using Ion PI chip on Ion Proton instrument. Data were analyzed using Ion reporter v5.6 software.

Results: Brain CT without contrast revealed a fairly large cystic mass lesion in the right cerebral hemisphere containing a rounded solid component. Moderate supratentorial hydrocephalus and peritumoral edema present, with a shift of midline structures to left side. Histological examination showed a fibrovascular papillary projections of choroid plexus lined by columnar epithelium with crowding. Areas of cribriform formation and focal solid growth pattern are also seen. Immunhistochemistry revealed positive for S-100, Ki67, beta-catenin, and E-cadherin staining, and synaptophysin staining was focally positive. Immunhistochemistry staining was negative for *EGFR, GFAP*, and *EMA*. Next generation sequencing (NGS) data analysis identified a novel missense mutation in *ATM* gene c.5808A>T; (p. Leu1936Phe) in exon 39. Also, two novel synonymous mutations were identified in in exon 21 of *PIK3CA* c.3144T>C; p. His1048His, and c.2307G>T; p. Leu769Leu in exon 13 of *SMAD4* respectively. Four known synonymous mutations were identified in *FGFR3, PDGFRA, APC* and *RET* genes. Coverage data showed, that 97.88% amplicons had at least 500 reads. Target base coverage at 500x was 98.38%.

Conclusion: We have identified a novel missense mutation in exon 39 of the *ATM* gene, which was not reported in any data bases so far. Developing NGS technologies in the Kingdom of Saudi Arabia may potentially be useful for patients as well as clinicians.

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