

ST002. Analytical and Clinical Validation of a Droplet Digital Polymerase Chain Reaction (ddPCR) Assay for Detection of O-6-Methylguanine-DNA Methyltransferase (MGMT) Promoter Methylation

M. Isaacson, D. Milosevic, A. Schneider, V. Pazdernik, S. Kizilbash, M. Maqbool, D. Lachance, C. Ida
Mayo Clinic, Rochester, MN.

Introduction: MGMT promoter methylation is a well-established molecular biomarker for patients with glioblastoma, isocitrate dehydrogenase (IDH)-wild-type (glioblastoma (GBM), IDH-wt). Testing is primarily performed using bisulfite conversion, a chemically harsh process resulting in considerable DNA loss. Therefore, a method with high sensitivity such as droplet digital PCR (ddPCR) is of special interest. ddPCR also allows for absolute quantitation of methylated (Mt) and unmethylated copies and calculation of fraction abundance (FA) as a measurement of methylation level. We describe the validation of a ddPCR assay for detection of MGMT promoter methylation. **Methods:** Custom TaqMan assays covering downstream CpG sites 75 to 80 and 84 to 87 were designed (Integrated DNA Technologies, Coralville, IA) to test bisulfite converted DNA using the EZ DNA Methylation-Lightning kit (Zymo Research, Irvine, CA) by ddPCR (Bio-Rad, Hercules, CA). Analytical experiments included precision, accuracy, reportable range, reference range, sensitivity, and specificity. Two types of DNA extraction were tested: An in-house Tris-EDTA (TE) digest-based and a modified version of Designed Solution for PCR (DSP) extraction (Qiagen, Hilden, Germany). Samples with available patient outcomes (n = 203) were utilized for clinical validation studies. Optimized cutoffs were statistically defined to distinguish results associated with high risk of death ("unmethylated") versus low risk of death ("methylated"). **Results:** A "methylated" result was defined by a combination of 2 cutoffs: ≥ 25 Mt copies, which was the lower limit of quantitation, and $\geq 6.50\%$ FA, which was above the upper 95% CI for 95% quantile for non-neoplastic brain tissue (5.7% and 5% FA for DSP and TE DNA, respectively). Concordance of $\geq 95\%$ with other methods including an in-house analytically validated real-time PCR (95%; 194/205), methylation array (97%; 38/39), and CAP (College of American Pathologists) proficiency testing (100%; 13/13) was achieved. This combined cutoff was statistically associated with risk of death, with a hazard ratio for an "unmethylated" result of 2.01 (95% CI, 1.47 to 2.74; $p < 0.0001$), after adjusting for age. Among 180 (of 203) patients with available clinical data, after accounting for multiple parameters including age, extent of resection, and treatment, the independent impact of an "unmethylated" result remained statistically significant with a hazard ratio of 2.46 (95% CI, 1.73 to 3.50; $p < 0.001$). Three intra- and inter-assay replicates were 100% concordant. Dilution experiments indicated a limit of detection of 20% tumor estimated based on TERT promoter mutation level and 25ng of DNA prior to bisulfite conversion. **Conclusions:** We analytically and clinically validated a ddPCR assay for detection of MGMT promoter methylation in patients with GBM, IDH-wt that is suitable for clinical testing.

TT045. Direct Microbial Cell-Free DNA (mcfDNA) Detection from Plasma by Nanopore Sequencing

R. Majumdar, M. Wolf, R. Patel, S. Murphy, A. Norgan
Mayo Clinic, Rochester, MN.

Introduction: Conventional pathogen detection from blood relies on culture-based (e.g., bacterial, fungal) and targeted molecular (e.g., viral) techniques. Metagenomic sequencing for the detection and characterization of microbial cell-free DNA (mcfDNA) is an emerging technology with potential to detect infections throughout the body in an agnostic way. Here, Oxford Nanopore Technology (ONT) Singleplex human cell-free DNA (cfDNA) sequencing was used to identify and characterize mcfDNA in plasma. **Methods:** cfDNA was extracted from 1 mL plasma, derived from EDTA (ethylenediaminetetraacetic acid) whole blood collected from 15 individuals, using the MagNA Pure 96 DNA kit (Roche Diagnostics) with an output volume of 100 mL. The extracted DNA was concentrated and size-selected with SPRISelect (Beckman Coulter) paramagnetic beads. DNA concentration and size were measured using the Qubit HS assay and TapeStation analysis. The cfDNA samples, which included 3 negative and 12 positive samples (as determined by reference metagenomic sequencing and real-time PCR methods), were sequenced using the ONT-CF-Singleplex sequencing protocol (ONT). Libraries were constructed using the ligation sequencing SQK-LSK114 kit, loaded onto PromethION R10.4.1 flow cells, and sequenced on PromethION for 48 to 72 hours. Bioinformatic analysis was

conducted using Kraken 2 (<https://ccb.jhu.edu/software/kraken2>). Results: Sequencing runs produced on average 220 million reads per sample with more than 99.9% of these reads aligned to host nucleic acid, which was subtracted bioinformatically. There was a 100% concordance in pathogen detection between Nanopore sequencing and the reference methods. The reference method was a composite of results obtained from an internal research metagenomics assay with Illumina sequencing and/or commercial metagenomic sequencing with Illumina sequencing (i.e., Karius). Ten of the 15 plasma samples were tested by both methods. Reads corresponding to 4 bacterial species (*K. michiganensis*, *P. aeruginosa*, *E. chaffeensis*, and *P. maltophilia*) were identified in 4 samples, whereas reads corresponding to viruses (mastadenovirus, parvovirus, HSV-6beta, EBV, and betapolyomavirus) were identified in the remaining samples using the ONT method. **Conclusions:** Results from the pilot study support the successful identification of target reads in clinical samples using ONT sequencing with minimal up-front sample processing from EDTA whole blood. Despite the overall abundance of host DNA in this setting, deep sequencing of plasma samples demonstrated sufficient recovery of pathogen DNA reads to achieve diagnostic concordance with an optimized research metagenomics method and a leading commercial plasma mcfDNA assay.

ID032. Impact of Recentrifugation on Epstein-Barr Virus DNA Viral Load Reproducibility in Patient Plasma

E. Hallock¹, K. Kasabwala², E. Slattery², D. Flick², H. Wang², Z. Yetmar²
¹Cleveland Clinic Foundation, Cuyahoga Falls, OH; ²Cleveland Clinic Foundation, Cleveland, OH.

Introduction: Epstein-Barr virus (EBV) viral load testing is used in the diagnosis and management of EBV-related disorders in transplant patients. Many labs use the FDA (US Food and Drug Administration)-approved cobas EBV quantitative PCR assay (Roche) for this purpose. In our lab, inferior assay precision was observed when retesting patient plasma as compared to package insert claims, whereas plasma spiked with whole virus control performed as expected. We hypothesized that the observed imprecision in patient plasma was due to inadequate separation of lymphocytes. This study aimed to determine how a second centrifugation step immediately prior to testing would impact assay results and precision. **Methods:** Residual EDTA (ethylenediaminetetraacetic acid) plasma inside plasma preparation tubes or aliquot tubes from 2 clinical runs were retested at 24, 72, and 120 hours (T24, T72, T120) after the initial run using the cobas EBV assay on the cobas 8800 system (Roche). The initial (T0) runs were conducted per standard procedures, in which the specimen arrived to the lab with plasma already separated from red blood cells and were not re-centrifuged. For the "No respin" batch (N = 72), the repeat tests were performed without additional centrifugation steps. For the "With respin" batch (N = 80), the repeat tests were performed with an additional centrifugation at 1,900 relative centrifugal force for 10 minutes immediately prior to each repeat test. Precision measurements and descriptive statistics were compared between the 2 groups. Results: In the "With respin" batch, median EBV load interquartile range (IQR) of 0.00 (0.00 to 0.00) log IU/mL at T24 was significantly reduced compared to T0 time point 0.00 (0.00 to 2.30) log IU/mL ($p < 0.001$). Among initially EBV-Detected samples, there was a median 100-fold decrease in viral load when testing was repeated after centrifugation at T24. In contrast, no such difference was seen in the "No respin" batch, which had similar median EBV loads (IQR of 0.00 [0.00 to 1.66] log IU/mL at T24 compared to 0.00 (0.00 to 2.13) log IU/mL at T0 ($p = 0.38$)). In the "With respin" batch, 21% (15/72) of samples went from Detected to Not detected, with none going the other direction. In the "No respin" batch, 10% (8/80) went from Detected to Not detected, and 11% (9/80) went from Not detected to Detected. The proportion of samples with all replicates at the T24, T72, and T120 repeat test time points measuring within 0.5 log IU/mL of each other was significantly higher in the "With respin" batch compared to the "No respin" batch (94% vs. 68%, $p < 0.001$). **Conclusions:** Repeat centrifugation immediately prior to plasma EBV viral load testing on the cobas 8800 resulted in significantly decreased viral loads and improved assay precision. The findings support the hypothesis that EBV viral load results and imprecision in clinical specimens are impacted by pre-analytic variables like centrifugation.