EXECUTIVE SUMMARY

TITLE: Automation of the PowerSeq™ mtDNA Control Region NGS System: Internal Validation

PURPOSE:

The following studies were performed by the FBI Laboratory to internally validate the automation scripts and laboratory information management system (LIMS) integration of the Promega PowerSeq® CRM Nested System. The PowerSeq CRM Nested System includes the Promega PowerSeq CRM assay for mitochondrial DNA (mtDNA) control region (CR) library preparation, the PowerSeq Quant MS System for library normalization, the Illumina MiSeq™ instrument for sequencing, and the GeneMarker® HTS (High Throughput Sequencing) software package for mtDNA data analysis. The workflow was previously validated for manual laboratory processing. Validation of the automated workflow included validation of the robotic scripts used for laboratory processing, and integration of the entire system in the LIMS, STACs. Overall, these studies serve to establish the reliability of the automated PowerSeq™ mtDNA CR Next Generation Sequencing (NGS) System for mtDNA CR typing.

INTRODUCTION:

The DNA Casework Unit (DCU) currently uses four amplification strategies and traditional capillary electrophoresis-based Sanger sequencing for mtDNA analysis of casework evidence. NGS offers the capability to simplify and standardize workflows, while at the same time generating more complete, and therefore more informative, mtDNA profiles. Given these benefits, the DNA Support Unit (DSU) tested, evaluated and validated the PowerSeq CRM Nested assay according to the Scientific Working Group on DNA Methods (SWGDAM) Validation Guidelines for DNA Analysis Methods. Prior to implementation of the assay in operational casework, however, automation of the laboratory steps and integration of LIMS tracking of the entire mtDNA workflow was required.

The work described here serves as the internal validation of the automated PowerSeq™ mtDNA CR NGS System by the DSU. The validation was performed according to the SWGDAM Validation Guidelines, and in accordance with the FBI's Quality Assurance Standards (QAS) for Forensic DNA Testing Laboratories.

SUPPORTING DOCUMENTATION:

Validation (Val2020) Chemistry Validation Documents_2018 - OneDrive (sharepoint.us)

Bead purification material modification 2023 (BPMM2023) (Final Files-OneDrive-Bead Purification material mod 2023 and approval.pdf)

VALIDATION STRATEGY:

The validation experiments followed the protocol finalized in the manual validation of the PowerSeq mtDNA CRM assay (VAL2020), but with adjustments to the amplicon purification steps, as detailed in the bead purification material modification (BPMM2023). The automated workflow validated herein involves amplification set-up on a Tecan Evo and post-PCR steps on a Tecan Fluent. Data analysis for all experiments was initially performed using parameters established during the manual validation. However, some analysis parameters (detailed in the study summaries) were changed following initial analysis of the data to accommodate the higher background contamination levels observed with the automated system.

ACCURACY, REPEATABILITY, REPRODUCIBILITY AND PRECISION:

Accuracy, repeatability, reproducibility and precision were tested via three experiments in which the same known samples were repeatedly typed by the same scientist and by different scientists on the same automation equipment and Illumina MiSeq instrument. The experiments used 24 samples with known mtDNA CR profiles representing 24 distinct haplotypes. More than 79,400 total base pairs (bp) of CR data were analyzed across the three experiments. All 675 expected variants of the 24 known haplotypes were correctly obtained, barring length heteroplasmies that are known to vary across different extracts, amplifications and sequencing reactions. The only discrepancy was a point heteroplasmy observed in a single sample in all three experiments. Upon analyst review of the data, the heteroplasmy was found to be the result of incomplete GeneMarker HTS primer trimming. Manual adjustment of the primer trimming parameter corrected the issue. While a similar incomplete trimming could potentially occur with CR haplotypes not represented in these studies, the issue was easy to both recognize and manually correct. Following review of the data and manual correction by the analyst, all expected variants for the 24 known samples were accurately represented in each of the replicate PowerSeq profiles.

The experimental results indicated very high precision (both repeatability and reproducibility). The PowerSeq™ variant calls were identical across all sample replicates regardless of the scientist performing the typing. Variant frequency percentages reported by the GeneMarker® HTS software were compared in pairwise fashion across runs and revealed highly consistent results. Regardless of whether the process was performed by the same individual or different individuals, the differences in variant frequency percentages were less than 0.7% in over 75% of the comparisons for substitutions and point heteroplasmies (PHPs), averaging only 0.61%. Variant frequencies were generally more variable for length heteroplasmies and insertion/deletion (indel) polymorphisms, as expected due to sequencing and alignment issues generally associated with these types of variants. Nevertheless, differences in these variant frequency values across runs still averaged only 1.19% for length heteroplasmies (LHPs).

SENSITIVITY:

Sensitivity of the automated PowerSeq System was evaluated using a known reference buccal swab tested at ten DNA inputs: 20,000 copies, 10,000 copies, 5,000 copies, 2,500 copies, 1,250 copies, 625 copies, 312 copies, 156 copies, 78 copies and 39 copies. Overall, the automated workflow produced complete CR profiles down to DNA inputs as low as 156 mtDNA copies. At the 200 variant and read count thresholds recommended based on the results of all studies in this validation, the correct mtDNA profile was produced at all inputs tested. One hundred percent of the control region was obtained at inputs down to 156 mtDNA copies. At 78 copies, 85% of the control region was recovered, and at 39 copies, 56% of the control region was recovered.

CASE-TYPE SAMPLES:

The performance of the automated PowerSeq™ mtDNA CR NGS System on sample types regularly encountered in mtDNA casework was evaluated by typing 5 hair and 11 calcified tissue DNA extracts. Three of the five hair samples produced full CR haplotypes that were concordant with known Sanger CR data. These samples were amplified with mtDNA inputs ranging from 1,100-6,200 copies and had low degradation indices under 1. The remaining two hair samples yielded no results, likely due to the age and condition of the hairs. MtDNA qPCR values for the two samples were 0 cn/ul (0 copies input in amp) and 4 cn/ul (50 copies input in amp). Though partial profiles were recovered from 39 copies in the sensitivity data, the DI of the sensitivity sample was low, at less than 1. The two hair extracts that failed to produce results both had undetermined DIs, indicating extreme degradation. All eleven of the calcified tissue samples produced full control region

sequence data with inputs ranging from ~3,000 copies to 25,000 copies. For those samples with reference data available, the profiles developed by the automated system were consistent with the known profiles. One sample, a tooth, was processed in replicate with approximately 3,000 copies of input. Both replicates yielded sequence results with numerous mixed positions, suggesting mixture, contamination and/or damage. The degradation index for this sample was over five.

Overall, when degraded samples similar to those encountered in mtDNA casework were tested, the control region haplotypes recovered from three hair and eleven bone samples were consistent with profiles from replicate extracts/amplifications or previously generated Sanger or NGS data. In the remaining two instances for which data were not recovered, the samples tested likely did not meet the lower limits of detection of the system, based on their low mtDNA copy numbers and high degradation indices.

When combined with data from the sensitivity experiments, the results from the case-type samples demonstrate that the automated PowerSeq™ System will successfully produce correct, entire mtDNA CR haplotypes for multiple sample types down to DNA inputs in the range of ~150-1000 mtDNA copies. This represents a sensitivity increase of ~40-2000x times as compared to current mtDNA copy number guidelines for full CR amplification. Thus, use of the automated PowerSeq™ System should substantially increase the number of samples for which full CR haplotypes can be produced.

CONTAMINATION:

Contamination was assessed by examining amplification negative controls (NCs) and reagent blanks (RBs). Amplification negative controls and RBs processed in the absence of samples exhibited some evidence of low-level contamination with a low average read depth of approximately 8 across the 50 controls evaluated. However, when controls processed in the presence of multiple samples were tested, observed contamination levels were substantially higher, with controls exhibiting average read depths of approximately 200. To address this, known sample extracts were diluted 1:20 and 1:50 prior to being loaded on the Tecan deck, and the controls associated with those dilutions were evaluated. Contamination levels were most improved when the 1:50 sample dilutions were used. Further examination of 49 controls associated with 1:50 diluted samples showed that average read counts in the controls were consistently reduced when 1:50 dilutions of high quality/quantity samples are used. It is therefore recommended that high quantity/quality samples be diluted 1:50 following extraction and both the neat and diluted sample should be quantified. Based on the quantification values, the most appropriate sample to be carried forward – diluted or undiluted – can then be selected to balance recovery of control region data with minimization of contamination across the run.

Assuming this dilution scheme, the 129 controls processed in the validation that reflected the workflow were analyzed for contamination. Analysis of all positions across the controls resulted in an average read depth + 3 SD of 205.94, supporting the use of a 200 read depth threshold. Overall, only 2.7% of the 148,221 control positions had read depths greater than 200.

CONCLUSION:

In summary, the experiments performed here demonstrate the accuracy, reproducibility, sensitivity, and reliability of the automated PowerSeq™ mtDNA CR NGS System. In addition, they serve to define the conditions under which reliable and reproducible mtDNA sequence data can be obtained from the range of sample types, quantities and qualities typically encountered in mtDNA casework. The internal validation studies performed support the recommendation to implement the automated PowerSeq™ mtDNA CR NGS System for the FBI's mtDNA casework.