MODIFICATIONS TO CYCLE SEQUENCING PURIFICATION PROTOCOLS TO ALLEVIATE THE NEED FOR RESEQUENCING OF mtDNA ON THE ABI 3100 CAPILLARY ELECTROPHORESIS PLATFORM

Scott C Schroeder, BS; Suzanne M Barritt, MS, Col Brion C Smith, Timothy P McMahon, PhD
Armed Forces DNA Identification Laboratory, Armed Forces Institute of Pathology, Rockville, MD

An accepted strategy within the forensic community to aid in the identification of ancient skeletal remains is the isolation and sequencing of the mitochondrial DNA (mtDNA) hypervariable regions one and two (HV1 and HV2). The high copy number of mtDNA genomes contained within the cytoplasm allows for identification of individuals where little to no nuclear DNA would be isolated. This strategy allows the mtDNA section of the Armed Forces DNA Identification Laboratory (AFDIL) to accomplish its primary mission to aid the Central Identification Laboratory, Hawaii, (CILHI) in the identification of human skeletal remains recovered from past military conflicts dating back to WWII. Currently amplified product is Centricon purified and cycle-sequenced using BigDye version 1.0 cycle sequencing chemistry. The sequenced product is then purified using EDGE's Performa DTR gel filtration cartridges and analyzed on the ABI 377 Sequencer (Applied Biosystems, Foster City, CA). In experiments described below, we utilized advances in purification and capillary electrophoresis technology to aid in validating new procedures that will allow AFDIL to process mtDNA case samples on the ABI 3100 Genetic Analyzer.

Currently both the Performa DTR 96 well standard filtration plate (EDGE Biosystems, Gaithersburg, MD) and the Performa DTR 96 well short filtration plate are validated only for purification of high-throughput database samples (amplified blood extracts). To validate these plates for use in processing mtDNA case samples, the upper and lower purification thresholds for both plates were determined by serially diluting amplified positive control DNA, then cycle-sequencing in duplicate with either full strength BigDye version 1.1 (ABI, Foster City, CA) or half-reaction BigDye version 1.1 containing dGTP. The duplicate plates were then purified using either the standard or short plate and analyzed on the ABI 3100 Genetic Analyzer using the 36cm default run conditions (3Kv, 15 sec injection and ~1 hour run time). Results demonstrated that the standard block purified sequencing products produced consistent quality data with little to no background or "dye blobs" (un-incorporated labeled ddNTP's) for all dilutions sequenced. However, sequencing data obtained from the short block purification was of a lower quality in that there was an increased presence of background and dye blobs. Interestingly, it was observed that the Relative Fluorescent Units (RFUs) for samples purified through standard plates were almost half the RFUs for the corresponding samples purified through the short plate. To balance the RFUs between the two plates, an additional recovery spin was added to the standard plate where the plate was rotated 180-degrees between spins.

Next, it was observed that when 5ul of undiluted product was sequenced and purified with either plate the RFUs exceeded 4000 at the standard injection time, which is above the dynamic range of the 3100 Genetic Analyzer. Instead of re-sequencing samples that were above AFDIL's 3500 RFU cut off, they were re-injected for 7-seconds and 3-seconds to save time and money. The 7-second and 3-second injection times were chosen to test whether there is a linear relationship between reduced injection time and reduced RFUs. Initial injection experiments utilized a non-probative case sample that had RFU's above 3500 at the 15-second injection. Results demonstrated that as the injection time decreased from 15 seconds to 7-seconds the RFUs decreased by approximately one-half and the data was now below the 3500 RFU cut off. On the other hand, data from the 3-second injection was of lower quality than the 7-second injection due to loss of peak definition. In another set of experiments, the control DNA amplicon sequenced with 2ul and exhibiting RFUs exceeding 3500 at the 15-second injection was re-injected for 5 seconds. As seen above, the reduction in RFUs observed at the 5-second injection was linear, being roughly one-third of those observed at the 15-second injection time. These results support that there is a linear relationship between the reduction of RFU's and the reduction of injection time.

In conclusion, the extra time needed for the manipulation of the standard plates is offset by the higher quality and reproducibility of the sequencing data obtained. Even though the RFU values were lower, they

fell within the acceptable range of 25-3500. Likewise, once the dynamic range is defined for the 3100 Genetic Analyzer being used, it is more cost effective to use shorter injection times to handle samples that exceed the upper dynamic range than it is to re-sequence.

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