7.1 PURPOSE:

7.1.1 Samples are subjected to cycle sequencing, gel filtration, and drying in preparation for electrophoresis using the ABI PRISM™ 3130 Genetic Analyzer.

7.2 RESPONSIBILITY:

7.2.1 Forensic Science Examiners from the CT DESPP Division of Scientific Services who have been trained in the discipline of Mitochondrial DNA Analysis according to the Mitochondrial DNA Section Training Manual.

7.3 SAFETY:

7.3.1 Use appropriate measures for the proper handling of physical evidence following the Mitochondrial DNA General Evidence Procedures (mtDNA SOP-01) and according to the Laboratory Safety Manual.

7.4 Cycle Sequencing Reaction (BigDye chemistry)

7.4.1 Cycle sequencing can be performed using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit. The reaction is carried out by combining the ready reaction mix, mtDNA template and a single sequencing primer in a 0.2 mL microcentrifuge tube. The reaction mixture is subjected to a series of controlled temperature changes in an ABI 9700 thermal cycler. The amount of mtDNA template used for sequencing should be approximately 8ng. However, this range is not absolute, and lesser or greater amounts of template can be used as required.

7.4.2 The primer sequences used for sequencing are identical to those used in amplification (see mtDNA SOP-05). The primers A4 and B4 are used on samples that contain a homopolymeric region from positions 16184-16194. They are designed to bind near the homopolymeric region and prime the sequencing reaction on their respective strands. Primer A4 is used to sequence the HV1B region. Primer B4 is used to sequence the HV1A region. Primer C4 (317f) can be used to sequence samples that exhibit significant length heteroplasmy in the 303-310 region.

A4: (L 16209) 5'-CCC CAT GCT TAC AAG CAA GT-3' (HV1B)

B4: (H 16164) 5'-TTT GAT GTG GAT TGG GTT T-3' (HV1A)

C4: (L 317) 5'-CCC CCC CTC CCC CCG C-3' (HV2B)

7.4.2.1 Add 8.0 µL of sequencing ready reaction mix to each tube.

7.4.2.2 Add 5.0 µL of the appropriate 0.7 µM primer to each sample tube.

7.4.2.3 To prepare purified mtDNA template, dilute if necessary (based upon the quantity determined by CE quantitation) to approximately 8 ng mtDNA per 7 µL of added template.

Note: For known samples processed using the EZ1 in a batch and amplified in a set, the RB's volume will reflect the largest volume used for a sample in the set. If necessary, dH_20 will be added up to 7µL.

7.4.2.4 Add 7 µL of the mtDNA template to the appropriate tubes.

7.4.2.5 Place the tubes into the ABI 9700 thermal cycler, close and clamp the cover.

7.4.2.6 Run program with the following parameters:

96°C for 1 minute
25 cycles:
a. 96°C for 15 seconds
b. 50°C for 1 second
c. 60°C for 1 minute
4°C indefinite hold

7.5 **Preparing Samples for Electrophoresis using Spin Columns (Qiagen DyeEx 2.0 Spin Kit)**

7.5.1 Qiagen DyeEx 2.0™ spin columns are prepared for removal of unincorporated fluorescent terminators from the completed sequencing reaction.

7.5.2 Gently vortex the spin column to re-suspend the pre-hydrated resin.

7.5.3 Place the appropriate number of wash tubes in a microcentrifuge.

7.5.4 Loosen the cap of the spin column a quarter turn, snap off the bottom closure of the spin column, and place the spin columns into the wash tubes.

7.5.5 Spin at a setting of ~750g (2900 rpm) for 3 minutes, or as required.

7.5.6 While the columns are spinning, label the collection tubes with the appropriate sample designation.

7.5.7 Remove the columns from the wash tubes and place them into the appropriately labeled sample collection tubes. Discard the wash tubes. Remove and discard the cap of the spin column. Note: columns must be used as soon as possible.

7.5.8 When the cycle sequencing is complete, remove the entire reaction mixture from each reaction tube and carefully load it onto the top of the gel material. It is important to dispense the sample slowly onto the center of the gel without touching the sides of the column or disturbing the surface of the gel.

7.5.9 Place the collection tubes with spin columns in a centrifuge and spin at ~750g (2900 rpm) for 3 minutes. Discard the spin columns.

7.5.10 Place the collection tubes containing purified sequencing fragments into a vacuum centrifuge and spin until dry (~20 minutes). Dried samples can be stored at–20 °C for up to one week.

7.5.11 See mtDNA WI-08 and WI-09 for further instructions on cycle sequencing and using Qiagen columns.