

**Department of Forensic Science**

**MITOCHONDRIAL DNA SECTION  
PROCEDURES MANUAL**

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## 1 INTRODUCTION AND SAMPLE REQUIREMENTS

### 1.1 Introduction

Mitochondria are cellular organelles responsible for oxidative phosphorylation (energy production) and are unique in that they have their own genome existing separate and outside the nucleus of the cell. The circular genome, comprised of approximately 16,569 base pairs, is polymorphic and inherited maternally. Mitochondria are abundant within the cytoplasm of cells with an average of 500 mitochondria per cell with an average of 5 copies of mitochondrial DNA (mtDNA) per mitochondrion. This is nearly 1200 copies of mtDNA for each copy of nuclear DNA found within a cell. Mitochondria are inherited maternally, meaning all maternal relatives have the same mtDNA (mother, sister, aunt, grandmother, etc.). It has a heavy (H) strand base paired to a light (L) strand, defined by the predominance of heavy versus light base compositions. The coding region of the genome contains 37 genes involved in cellular oxidative phosphorylation with very few non-coding bases in between the genes. Replication of the mitochondrial genome originates in the Displacement Loop (D-loop), where the primary source of polymorphism is found. This non-coding region of the DNA consists of approximately 1,122 base pairs and is referred to as the Control Region. The mutation rate observed in mtDNA is approximately 10 times that of nuclear DNA which gives rise to the distinguishing polymorphisms. Because of its circular nature and its relative abundance over nuclear DNA, mitochondrial DNA analysis is helpful in special circumstances for forensic comparison.

Mitochondrial DNA analysis of hair, bone and teeth is particularly successful in part due to the encapsulation of DNA by the exterior of the tissue and protection of mtDNA within layers of keratin (hair) and hydroxyapatite (bone and teeth). However, mtDNA is susceptible to the same type of environmental degradation as nuclear DNA. Fragmentation and degradation of the DNA can complicate analysis. Additional complications include contamination by laboratory or environmental sources of DNA not associated with the tissue being analyzed. For this reason, contamination prevention in the laboratory is maximized. Sample segregation is observed at the outset of evidence analysis in the laboratory. Evidence samples are examined in a separate laboratory from reference samples. Individual items of evidence are handled separately from each other with careful attention to cleaning the work area and implements between each item/sample.

Mitochondrial DNA analysis relies upon a strategy of polymerase chain reaction (PCR) amplifications that focus on the Control Region or smaller regions of interest within the Control Region. Primarily these regions of interest have been described as hyper variable region I (HVI), hyper variable region II (HVII), and hyper variable region III (HVIII) which contain a large majority of the polymorphisms. A primer set strategy of PCR amplification isolates the control region with overlapping regions by creating specific amplicons that can be sequenced. The sequencing reactions are carried out in both the forward and reverse (5' to 3' with respect to the light strand) directions creating redundant DNA sequence data. This sequence data is then compared to the Revised Cambridge Reference Sequence (rCRS) to produce a summary of the polymorphisms or differences in sequence from the reference. The cumulative information from these sequences is the mitochondrial haplotype for the sample. The Department's mtDNA Section amplification and sequencing strategy is depicted in Figure 1 shown below. Known samples or evidence samples in which high quality DNA would be expected to be obtained can be processed with control region amplifications. Evidence samples in which degraded or low concentration of DNA is expected to be obtained can be processed with primer set amplifications.

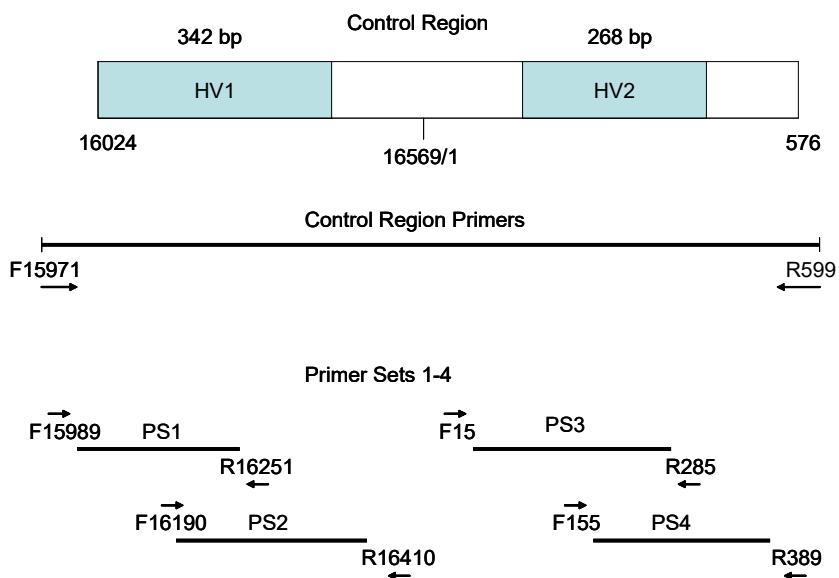


Figure 1 Primer Set Strategy

To the maximum extent possible:

- Evidence samples will be processed separately from reference samples.
- Evidence samples will be processed prior to the reference samples for the same case.
- Evidence sample consensus sequences will be determined before reference sample consensus sequences for the same case.
- Evidence sequence data will be interpreted prior to the interpretation of reference sample sequence data for the same case.

## 1.2 Case Type Requirements

The results obtained from mtDNA analysis are much less discriminating than nuclear STR DNA results; therefore, an effort to obtain nuclear STR DNA results should be considered before the sample is submitted for mtDNA testing.

### 1.2.1 Body Identifications

A reference sample from a putative maternal relative or a direct reference sample/alternate known from the presumed to be (PTB) must be submitted.

### 1.2.2 Forensic Comparison Cases

For a forensic comparison case to be accepted:

- Any sample to be submitted must show no indication that it is from more than one individual (e.g., DNA mixture from mixed body fluids).
- Any evidence samples to be submitted have been previously processed for nuclear DNA and have exhibited no result or limited results.
  - Nuclear DNA extracts will not be examined.
- All pertinent references (suspect and victim) must be submitted.

### 1.2.3 Unidentified Human Remains

Analysis can be conducted on bones, hair, teeth, and blood/tissue samples.

### 1.3 Sample Type Requirements

Samples must be of sufficient quantity to perform testing. In some instances, the remaining portion of a sample analyzed for nuclear DNA will be consumed for analysis.

#### 1.3.1 Acceptable Evidentiary Samples

- Hairs/hair fragments of at least two centimeters in length, including those previously analyzed for nuclear DNA and those determined to be unsuitable for nuclear DNA analysis
  - All hair examination requests will be conducted prior to analysis.
- Bones/bone fragments
- Blood/tissue samples (including toenails)/teeth

#### 1.3.2 Acceptable Reference Samples

- Buccal swabs
- Blood samples
- Hair samples (minimum of 5) – may be required for sequence comparison in certain cases
  - Hairs from a known hair standard may be combined and processed as a single reference sample.
- Alternate knowns (e.g., toothbrush, razor)

## 2 DNA ISOLATION

When the mtDNA analysis is complete, the remaining evidence samples, extracts, and associated reagent blanks will be returned to the submitting agency. (Refer to Appendix D for the procedure.) If the sample is consumed during the analysis the cutting, if possible, will also be returned to the submitting agency. Amplified DNA product and cycle sequencing product will not be retained or returned to the submitting agency.

Unless a reference sample was consumed, a majority of the reference sample was used to obtain a result the first time, or the reference sample is degraded and the analyst believes the best chance of obtaining a result in possible future retesting is from the extract, the return of the DNA extract from a reference sample and its associated reagent blank is optional.

Chelex® may be used for extraction of DNA from reference specimens containing ample amounts of high quality DNA (e.g., a whole blood or buccal sample from a victim or suspect). If a Chelex® extraction does not yield suitable DNA, then an organic extraction will be attempted.

Organic extractions are used for all extractions of DNA from evidence. Organic extractions may also be used for reference samples, including alternate knowns.

Extracts created following mitochondrial DNA specific organic extraction procedures may also be used for nuclear DNA amplifications.

### Special Precautions

- Extraction of reference samples is conducted in a different laboratory than evidence samples. Evidentiary samples which are believed to contain high quantity/quality DNA may be extracted in the reference laboratory.
- Dedicated extraction instruments are used for reference samples.
- Dedicated extraction instruments are used for evidence samples.
- Disposable gloves, surgical mask, hair net, lab coat and sleeves will be used. Fresh 10% bleach will be applied to the gloves and sleeves between handling samples, or gloves/sleeves will be changed.
- Scissors and tweezers, if used, will be thoroughly cleaned with fresh 10% bleach. Subsequently, isopropanol or ethanol is used to remove any residual bleach from the surfaces of the implements.
- A sterile disposable scalpel may also be used to cut each item/stain.
- Aerosol-resistant pipette tips will be changed between samples.
- Extraction procedures are performed in a dedicated laminar flow or chemical fume hood.
- Laminar hoods are UV irradiated prior to use.
- Tubes, racks, and designated extraction reagents are UV irradiated prior to use.
- All work surfaces and pipettes will be thoroughly cleaned with fresh 10% bleach. Isopropanol or ethanol may be used to remove any residual bleach from the surfaces.
- Reagent blanks will be included and are processed with each sample or batch of samples.
- Batching may be performed as described in the general guidelines below.
- The samples will be listed on the case worksheets in the order in which they were processed/handled.
- Appropriate labeling of sample tubes will include at a minimum the case number and the item number.

## General Guidelines for Batching Samples

- A batch consists of up to 4 samples and associated reagent blanks or 4 reference samples and associated reagent blank. (If batching reference samples from multiple cases, unless enough of the reference samples remain for re-analysis, a reagent blank is used with each case).
- Batches of samples will be handled in the following order and will maintain this ordering throughout analysis.

<u>Evidence Samples</u>	<u>Reference Samples</u>
Sample 1	Known 1
Reagent Blank 1	Known 2
Sample 2	Known 3
Reagent Blank 2	Known 4
Sample 3	Reagent Blank
Reagent Blank 3	
Sample 4	
Reagent Blank 4	

### 2.1 Equipment

- Beaker
- Centrifuge (including fixed angle or swinging bucket rotor)
- Chisel
- Compound Microscope
- Dremel Tool
- Forceps
- Freezers (-20°C, -30°C)
- Hammer
- Heat Block
- Hood
- Incubator, 56°C
- Magnetic Stirring/Hot Plate
- Microcentrifuge
- Nutator
- Pipettes
- Racks, Tubes
- Refrigerator, 4°C
- Safety Glasses
- Scissors
- Stereomicroscope
- Stir bar
- Ultrasonic Cleaner
- Ultraviolet Crosslinker
- Vortex
- Waring Blender

### 2.2 Materials

- Amicon® Ultra-4 Concentrators
- Boileezers
- Cutting Disk
- Gloves
- Kim-Wipes
- Laboratory Coat
- Mask, Surgical
- Microcentrifuge tubes, screw-top and flip-cap

- Microcon® 30 Concentrators
- Sanding/grinding bits
- Scalpel
- Sleeve Protectors
- Spin-EASE™ Tubes
- Tips, Aerosol-Resistant (for pipettes)
- Tissue Grinders
- Waste Containers
- Weigh Boats

### **2.3 Reagents**

- Bleach, 10% Commercial
- Chelex® 100 (Bio-Rad) in Ultra-Pure Water
- Demineralization Buffer
- DTT
- Ethanol, Absolute
- Ethanol
- Extraction Buffer
- Isopropanol
- Liquinox, 10%
- n-Butanol
- Phenol/Chloroform/Isoamyl Alcohol (PCIAA) (25:24:1)
- Proteinase K (20 mg/mL)
- TE Buffer
- Terg-a-zyme™
- Water, Ultra-Pure
- Xylene

### **2.4 Chelex® Extraction Method for Reference Bloodstains**

- 2.4.1 Pipette 1.0 mL of ultra-pure water into the appropriately labeled tubes, including a reagent blank, which must be initiated at this point.
- 2.4.2 Add an appropriately sized piece (approximately 3 mm square) of blood stained material (including FTA cards) to the tube.
- 2.4.3 Vortex at high speed for 10 seconds.
- 2.4.4 Incubate at room temperature (or 95°C for samples on FTA cards) for a minimum of 15 minutes, vortexing several times during the incubation.
- 2.4.5 Centrifuge for 3 minutes at approximately 10,000 x g.
- 2.4.6 Discard all but 20-30 µL of the supernatant, leaving the substrate and pelleted material in the tube.
- 2.4.7 Proceed to 2.6.

### **2.5 Chelex® Extraction Method for Reference Buccal Swabs**

- 2.5.1 Using a sterile, disposable scalpel, add an appropriately sized piece of sample (approximately 1/3 of a swab tip) to the lower portion of an appropriately labeled tube or Spin-EASE™ unit.
- 2.5.2 Add 1.0 mL ultra-pure water to the tube and initiate a reagent blank.

- 2.5.3 Vortex at high speed for 10 seconds.
- 2.5.4 Incubate at room temperature for 30 minutes.
- 2.5.5 Centrifuge for 3-15 minutes at approximately 10,000 x g.
- 2.5.6 Remove and discard the top 0.5 mL of supernatant.
- 2.5.7 Transfer the cutting into the basket portion of a Spin-EASE™ tube or similar device and replace the basket in the lower portion of the Spin-EASE™ tube.
- 2.5.8 Centrifuge for 3 minutes at approximately 10,000 x g, remove the basket and dry and repackage, if necessary.
  - 2.5.8.1 Alternatively, sterile forceps can be used to remove the cutting(s) from the water in the tube and squeezed to remove excess water from the cutting(s). Dry and repackage the cutting(s), if necessary.
- 2.5.9 Remove and discard all but 50 µL of supernatant.
- 2.5.10 Proceed to 2.6.

## 2.6 Chelex® Isolation of DNA

- 2.6.1 Add 5% Chelex® to a final volume of 200 µL and vortex gently to re-suspend the pellet.

**NOTE:** When pipetting Chelex® solutions, the resin beads must be distributed evenly in solution.

- 2.6.2 Incubate at 56°C for 30 minutes.

- 2.6.3 Vortex briefly.

- 2.6.4 Incubate in a boiling water bath for 8 minutes.

- 2.6.5 Vortex briefly.

- 2.6.6 Centrifuge for 3 minutes at approximately 10,000 x g.

- 2.6.7 Transfer the supernatant from the Chelex® beads to a sterile, appropriately labeled tube and store at -20°C.

## 2.7 Organic Extraction Method for Loose Hairs

- 2.7.1 Clean micro tissue grinders with 10% bleach, then water, then ethanol and allow to dry completely before use.

- 2.7.2 Irradiate the micro tissue grinders in the UV crosslinker (Refer to Appendix B).

- 2.7.3 Carefully remove approximately 2 cm of hair shaft material from the proximal end of the hair and place in an appropriately labeled sterile tube.

**NOTE:** A stereo or compound microscope may be used in making the determination of the proximal end of the hair by observing the root end (if present) or the directionality of the scales of the hair or hair fragment. (Refer to Appendix A, if necessary).

- 2.7.4 Add 1.0 mL of 5% Terg-a-zyme™ solution and place the tube in the ultrasonic cleaner for 20 minutes.

- 2.7.5 Remove the tube from the ultrasonic cleaner and carefully remove the 5% Terg-a-zyme™ solution.

- 2.7.6 Repeat the Terg-a-zyme™ wash process three times for a total of four washes.
- 2.7.7 Add 1.0 mL absolute ethanol, recap the tube, and gently agitate several times.
- 2.7.8 Remove the absolute ethanol, add 1.0 mL ultra-pure water, recap the tube, and gently agitate several times.
- 2.7.9 Remove the water.
- 2.7.10 Prepare a reagent blank as follows:
  - 2.7.10.1 Add 187 µL of extraction buffer to a clean, irradiated micro tissue grinder and briefly simulate grinding.
  - 2.7.10.2 Transfer the extraction buffer to a labeled tube (this is the reagent blank and will be processed last for the remaining steps of the extraction).
- 2.7.11 To the same micro tissue grinder used to create the reagent blank, add 130 µL of extraction buffer and add the washed/prepared hair shaft(s).
- 2.7.12 Grind until fragments of hair are no longer visible.
- 2.7.13 Transfer the solution into a labeled tube.
- 2.7.14 Add an additional 57 µL extraction buffer to the micro tissue grinder to rinse and transfer it to the tube with the sample.
- 2.7.15 Add 5 µL Proteinase K and 8 µL DTT, vortex, and pulse spin.
- 2.7.16 Incubate at 56°C for a minimum of 2 hours.
- 2.7.17 Add 200 µL PCIAA, vortex thoroughly, and centrifuge for 2 minutes at approximately 10,000 x g.
- 2.7.18 Transfer upper aqueous layer to an appropriately labeled sterile tube.
- 2.7.19 Dispose of the lower layer (phenol waste) in the appropriate waste container.
- 2.7.20 If necessary, repeat extraction with PCIAA until the interface is clean.
- 2.7.21 To the aqueous layer, add 200 µL n-butanol.
- 2.7.22 Vortex thoroughly and centrifuge for 2 minutes at approximately 10,000 x g.
- 2.7.23 Remove and discard into an appropriate waste container the upper n-butanol layer.
- 2.7.24 Label a sufficient number of pre-assembled irradiated Microcon® 30 concentrators.
- 2.7.25 Add 300 µL TE buffer to each sample reservoir of the Microcon® 30 concentrators.
- 2.7.26 Transfer the lower aqueous layer to the sample reservoir of the Microcon® 30 concentrators, avoiding the pipetting of any residual n-butanol.
- 2.7.27 Centrifuge the concentrator at a maximum of 1,000 x g for 15-30 minutes or until the sample has spun through.
- 2.7.28 Discard the filtrate.

- 2.7.29 Add 300 µL TE buffer to the sample reservoir of the Microcon® 30 concentrators.
- 2.7.30 Centrifuge the concentrator at a maximum of 1,000 x g for 15-30 minutes or until the sample has spun through.
- 2.7.31 Add 60 µL TE buffer to the filter side of the sample reservoir of the Microcon® 30 concentrators.
- 2.7.32 Place a retentate cup on the top of each concentrator and briefly vortex with the retentate cup pointing upward.
- 2.7.33 Invert each concentrator sample reservoir with its retentate cup and centrifuge at approximately 10,000 x g for 3 minutes.
- 2.7.34 Discard the concentrators, and measure the volume of the retentate with a pipette.
- 2.7.35 Add TE buffer, if necessary, to bring the retentate volume up to 100 µL.
- 2.7.36 Transfer the 100 µL of retentate to an appropriately labeled sterile tube.

## 2.8 Organic Extraction Method for Bone

**NOTE:** It is acceptable, based upon the age and condition of the submitted bone, to replace the steps outlined in 2.8.1 through 2.8.12 below with those outlined in Chapter 9 of the FB PM Screening and Collection for DNA Analysis. The age of the submitted bone will be the primary factor in making this determination.

- 2.8.1 In a hood, sand the exposed surfaces of the bone with a clean sanding bit fitted to a rotary tool.
- 2.8.2 Obtain approximately 1.0 g of bone using a cutting disc, if necessary.

**NOTE:** Clean hood appropriately and change bits/discs, gloves, and disposable sleeves between each specimen.
- 2.8.3 Clean the sample of powdered debris by placing the sanded sample into a 50 mL conical tube containing approximately 25 mL of ultra-pure water, shaking back and forth several times, and decanting into a waste container.
- 2.8.4 Repeat the above ultra-pure water wash twice.
- 2.8.5 Cover the sample in the conical tube with absolute ethanol, shake back and forth several times, and decant into a waste container.
- 2.8.6 Repeat the above absolute ethanol wash twice.
- 2.8.7 Remove the sample from the conical tube, place it in a labeled weigh boat, and allow it to air dry in the laminar flow hood.
- 2.8.8 If not already prepared, clean an appropriate number of Waring blender cups and lids as follows:
  - 2.8.8.1 Fill each cup approximately 1/3 full with 10% Liquinox, attach lid, and run blender for 10-20 seconds.
  - 2.8.8.2 Remove lid and rinse both the lid and cup with water, then 10% bleach, then water, then ethanol.
  - 2.8.8.3 Drain excess ethanol and allow surfaces to dry.
  - 2.8.8.4 Irradiate in a UV crosslinker for the same amount of time required for a 50 mL conical tube.

- 2.8.9 Initiate a reagent blank by swabbing the inside surfaces of a clean (see above) Waring blender cup and lid. The reagent blank will be the last sample processed for the remaining steps of the extraction.
- 2.8.10 Place the sample into the same Waring blender cup used to initiate the reagent blank, attach the lid, and run the blender until the sample is finely ground.

**NOTES:** When starting the blender, the sample may become wedged between a blade and the cup wall. If this occurs, shut off the power, remove the cup and dislodge the sample by tapping or rotating the blade spindle from below.

If sample is not completely ground after one minute, shut off the power, tap the container and repeat.

- 2.8.11 Place powder into a clean weigh boat or funnel for transfer to an appropriately labeled sterile pre-weighed or tared 15 mL conical tube.
- 2.8.12 Determine the weight of the sample in the conical tube. Approximately 0.2 g of powdered bone is needed for extraction.

**NOTE:** The extraction procedure may be stopped at this point for the day, if desired, by storing the sample in the -20°C freezer.

- 2.8.13 Add 3 mL demineralization buffer and 200 µL Proteinase K to the sample and reagent blank, making sure that the sample is thoroughly suspended in the reagents.
- 2.8.14 Wrap the conical tubes tightly with parafilm to ensure the liquid does not leak.
- 2.8.15 Incubate overnight at 56°C on a nutator.

**NOTE:** The parafilm may crack in the heat. Check the tubes and parafilm prior to leaving the samples overnight.

- 2.8.16 Add 3 mL PCIAA, vortex thoroughly, and centrifuge for 10 minutes at approximately 10,000 x g using a fixed angle rotor OR at 3,270 x g using a swinging bucket rotor centrifuge.
- 2.8.17 Transfer the upper aqueous layer to an appropriately labeled sterile tube.
- 2.8.18 Dispose of the lower layer (phenol waste) in the appropriate waste container.
- 2.8.19 Repeat extraction with PCIAA until the interface is clean, disposing of waste in the appropriate waste container.
- 2.8.20 To the aqueous layer, add 3 mL n-butanol.
- 2.8.21 Vortex thoroughly and centrifuge for 10 minutes at approximately 10,000 x g using a fixed angle rotor OR at 3,270 x g using a swinging bucket rotor centrifuge.
- 2.8.22 Remove and discard into an appropriate waste container the upper n-butanol layer.
- 2.8.23 Label a sufficient number of pre-assembled irradiated Amicon® Ultra-4 concentrators.
- 2.8.24 Transfer the lower aqueous layer to the sample reservoir of the Amicon® Ultra-4 concentrator, avoiding the pipetting of any residual n-butanol.
- 2.8.25 Centrifuge for approximately 10-30 minutes at 2,000 x g using a swinging bucket rotor and discard filtrate.

- 2.8.26 Add 2 mL of TE buffer to the sample reservoir, centrifuge for approximately 10-30 minutes at 2,000 x g using a swinging bucket rotor, and discard filtrate.
- 2.8.27 Repeat the above TE buffer wash at least once.
- 2.8.28 Taking extreme care in not allowing the pipette to touch the sides of the sample reservoir, pipette the retentate directly from the sample reservoir and transfer it to an appropriately labeled sterile tube.
- 2.8.29 Measure the volume of the retentate with a pipette, and add TE buffer, as necessary, to bring the volume up to 100 µL.
- 2.8.30 The samples may be stored at 4°C if amplified within 3 weeks or used routinely. Store at -20°C for long-term storage.

## 2.9 Organic Extraction Method for Other Evidence Samples, Reference Samples, and Alternate Knowns

- 2.9.1 Cut an appropriately sized portion of the stain (3mm square or larger, if necessary), swab, or sample and place into an appropriately labeled sterile tube.
- 2.9.2 Add to the sample and to a tube for the reagent blank:

400 µL extraction buffer  
10 µL of 20 mg/mL Proteinase K
- 2.9.3 Vortex, pulse spin, and incubate at 56°C for a minimum of 2 hours.
- 2.9.4 Transfer the cutting to a Spin-EASE™ basket or similar device, place the basket in the tube and close the lid. Centrifuge for 3 minutes at approximately 10,000 x g to remove the excess liquid from the cutting.
  - 2.9.4.1 Alternatively, sterile forceps may be used to remove the cutting from the buffer in the tube, squeeze the cutting to remove excess buffer, and then remove the cutting from the tube.
- 2.9.5 Retain the cutting, if necessary, to be dried and repackaged.
- 2.9.6 Add 400 µL PCIAA, vortex thoroughly, and centrifuge for 2 minutes at approximately 10,000 x g.
- 2.9.7 Transfer upper aqueous layer to an appropriately labeled sterile tube.
- 2.9.8 Dispose of the lower layer (phenol waste) in the appropriate waste container.
- 2.9.9 If necessary, repeat extraction with PCIAA until the interface is clean.
- 2.9.10 To the aqueous layer, add 400 µL n-butanol.
- 2.9.11 Vortex thoroughly and centrifuge for 2 minutes at approximately 10,000 x g.
- 2.9.12 Remove and discard the upper n-butanol layer into an appropriate waste container.
- 2.9.13 Label a sufficient number of pre-assembled irradiated Microcon® 30 concentrators.
- 2.9.14 Add 100 µL TE buffer to each sample reservoir of the Microcon® 30 concentrators.
- 2.9.15 Transfer the lower aqueous layer to the sample reservoir of the Microcon® 30 concentrators, avoiding the pipetting of any residual n-butanol.
- 2.9.16 Centrifuge the concentrator at a maximum of 1,000 x g for 15-30 minutes or until the sample has spun through.

- 2.9.17 Discard the filtrate.
- 2.9.18 Add 400 µL TE buffer to the sample reservoir of the Microcon® 30 concentrators.
- 2.9.19 Centrifuge the concentrator at a maximum of 1,000 x g for 15-30 minutes or until the sample has spun through.
- 2.9.20 Discard the filtrate, and repeat the 400 µL TE buffer wash.
- 2.9.21 Add 60 µL TE buffer to the filter side of the sample reservoir of the Microcon® 30 concentrator.
- 2.9.22 Place a retentate cup on the top of each concentrator and briefly vortex with the retentate cup pointing upward.
- 2.9.23 Invert each concentrator sample reservoir with its retentate cup and centrifuge at approximately 10,000 x g for 3 minutes.
- 2.9.24 Discard the concentrators, and measure the volume of the retentate with a pipette.
- 2.9.25 Add TE buffer, if necessary, to bring the retentate volume up to 100 µL.
- 2.9.26 Transfer the 100 µL of retentate to an appropriately labeled sterile tube.

### 3 MITOCHONDRIAL DNA AMPLIFICATION

Amplification of mtDNA using primer set primers is effective for subsequent cycle sequencing of samples which may contain low levels of or degraded mtDNA. Control region primers may be used for reference samples and for evidence samples which potentially have an abundant amount of mtDNA.

Evidence samples will be amplified with Primer Set 2 first when sample quality/quantity may be insufficient to determine if results would be obtained with the subsequent primer sets.

No more than 4 samples (evidence or reference) may be amplified together in a batch.

Batches of samples will be amplified in the following order and will maintain this ordering through cycle sequencing:

<u>Evidence samples</u>	<u>Reference Samples</u>
Negative Control	Negative Control
Sample 1	Known 1
Reagent Blank 1	Known 2
Sample 2	Known 3
Reagent Blank 2	Known 4
Sample 3	Reagent Blank
Reagent Blank 3	Positive Control
Sample 4	
Reagent Blank 4	
Positive Control	

#### Special Precautions

- Dedicated lab coats are worn in the mtDNA isolation laboratory and replaced at least weekly.
- Personnel entering the mtDNA isolation laboratory will step onto adhesive floor mats, top layer changed when no longer effective.
- The setup of mtDNA amplification of samples will be performed in a dedicated laminar flow hood, separate from the DNA extraction hood(s).
- Disposable gloves, surgical mask, lab coat and sterile sleeves will be used. Fresh 10% bleach will be applied to the gloves before beginning amplification set up.
- Aerosol-resistant pipette tips will be changed between samples.
- Negative amplification controls will be included with each amplification reaction.
- Laminar hoods are UV irradiated prior to use.
- Tubes and racks are UV irradiated prior to use.
- All work surfaces and pipettes will be thoroughly cleaned with fresh 10% bleach. Isopropanol or ethanol may be used to remove any residual bleach from the surfaces.
- Evidence samples will be amplified separately from known reference samples.
- The samples will be listed on the case worksheets in the order in which they were processed/handled.

#### 3.1 Equipment

- Freezers (-20°C, -30°C)
- Laminar flow hood
- Microcentrifuge
- Pipettes
- Racks, tube
- Refrigerator, 4°C
- Safety glasses
- Thermal cycler GeneAmp® PCR Systems 9700
- Ultraviolet crosslinker
- Vortex

### 3.2 Materials

- Gloves
- Kim-wipes
- Laboratory coat
- Mask
- Sleeves, disposable
- Tips, aerosol-resistant (for pipettes)
- Tubes, microcentrifuge
- Tubes, thin-walled PCR
- Waste containers

### 3.3 Reagents

- Bleach, 10% commercial
- Bovine Serum Albumin (BSA) (0.625 µg/µl)
- Deoxynucleotide triphosphate (dNTP) mix
- PCR buffer, 10X
- Positive control DNA
- Primers, 10 µM (see full listing below)
- Taq Gold™ DNA Polymerase
- Water, ultra-pure

Primer pairs typically used for samples with high levels of mtDNA that are of good quality (Reference & Evidence Samples)

Control Region (CR)      F15971/ R599

Primer pairs typically used for samples with low levels of mtDNA that may be degraded (Evidence Samples)

Primer Set 1 (PS1)	F15989/R16251
Primer Set 2 (PS2)	F16190/R16410-m19
Primer Set 3 (PS3)	F15/R285
Primer Set 4 (PS4)	F155/R389

Primer sequences

F15971	5' TTA ACT CCA CCA TTA GCA CC 3'
F15989	5' CCC AAA GCT AAG ATT CTA AT 3'
F16190	5' CCC CAT GCT TAC AAG CAA GT 3'
R16251	5' GGA GTT GCA GTT GAT GT 3'
R16410-m19	5' GAG GAT GGT GGT CAA GGG A 3'
F15	5' CAC CCT ATT AAC CAC TCA CG 3'
F155	5' TAT TTA TCG CAC CTA CGT TC 3'
R285	5' GTT ATG ATG TCT GTG TGG AA 3'
R389	5' CTG GTT AGG CTG GTG TTA GG 3'
R599	5' TTG AGG AGG TAA GCT ACA TA 3'

**NOTE:** Primers are labeled with respect to the numbering system of Anderson et al. Numbering begins at the 5' end of each primer.

### 3.4 Amplification of mtDNA for Control Region and Primer Set Sequencing

3.4.1 Prepare the master mix in a UV irradiated sterile tube, as follows:

Component	Volume	Volume / Reaction
10X PCR Buffer	(n+1) X 5µL	5 µL
2.5 mM dNTPs	(n+1) X 4 µL	4 µL
0.625µg/ µl BSA	(n+1) X 2 µL	2 µL
Forward primer (10 µM)	(n+1) X 2 µL	2 µL
Reverse primer (10 µM)	(n+1) X 2 µL	2 µL
Taq Gold™ (5 units/ µL)	(n+1) X 1 µL	1 µL
ultra-pure water	(n+1) X 24 µL	24 µL
<b>Total Volume</b>	<b>(n+1) X 40 µL</b>	<b>40 µL</b>

**NOTE:** n represents the total number of samples

- 3.4.2 Aliquot 40 µL master mix into each appropriately labeled UV irradiated sterile thin walled PCR tube.
- 3.4.3 Pipette the following sample and control volumes (as appropriate) to the corresponding PCR tubes, one at a time:

Volume	Component
10 µL, ultra-pure water	Amplification Negative Control
1-10 µL of sample qs to 10 µL with ultra-pure water	Sample DNA extracts
10 µL HL60 (200pg /10 µL primer sets) 10 µL HL60 (500pg/10 µL control region)	Amplification Positive Control
1-10 µL of the Reagent blank qs to 10 µL with ultra-pure water	Reagent Blank volume is equal to the maximum sample volume added

**NOTE:** The total reaction volume is 50 µL per PCR tube.

- 3.4.4 Vortex the PCR tubes briefly and pulse spin prior to placing the tubes in the thermal cycler.
- 3.4.5 Start the appropriate PCR program listed below:

Control region reaction in a 9700 Thermal Cycler

- 96°C for 10min
- 36 cycles of 94°C for 30sec, 56°C for 30sec, 72°C for 60sec
- 72°C for 7min
- 4°C soak

Primer set reaction in a 9700 Thermal Cycler

- 96°C for 10min
- 38 cycles of 94°C for 20sec, 56°C for 20sec, 72°C for 30sec
- 4°C soak

## 4 MITOCHONDRIAL DNA AMPLIFICATION PRODUCT EVALUATION

The product gel is used to determine the success of the amplification process and for assessing the concentration of amplified mitochondrial DNA (mtDNA) that should be used for sequencing. SYBR Green is used to detect DNA by staining. It intercalates with the double stranded DNA molecule and fluoresces under UV light. A UV transilluminator, at a wavelength of 302 nm, is used to visualize the fluorescent reaction. The DNA Molecular Weight Marker XIV ladder consists of double stranded DNA fragments ranging in length from 100 to 2642 bp. The fragments corresponding to the following sizes also correspond to known concentrations.

Size bp	[~DNA] ng/4 $\mu$ L	[~DNA] ng/2 $\mu$ L
2642	468	234
1500	24	12
1000	113	56
500	105	52
400	39	19
300	29	14
200	19	9
100	26	13

### Special Precautions

- Product gels are run in the post-amplified DNA laboratory.
- Dedicated lab coats are worn in the post-amplified DNA laboratory, replace at least weekly.
- Personnel entering post-amplified DNA laboratory will step onto adhesive floor mats, top layer changed when no longer effective.
- All equipment and supplies used in the post-amplified DNA laboratory are dedicated to the post-amplified DNA laboratory and will not be used in the DNA isolation laboratory.
- All work surfaces and pipettes will be thoroughly cleaned with fresh 10% bleach. Isopropanol or ethanol may be used to remove any residual bleach from the surfaces.
- Evidence samples will be processed separately from known reference samples.
- The samples will be listed on the case worksheets in the order in which they were processed/handled.
- Aerosol-resistant pipette tips will be changed between samples.
- Discard gloves when leaving the work area to avoid transport of amplified DNA from the Post-PCR work area.

### 4.1 Equipment

- Balance
- DC power supply
- Flask, Erlenmeyer or flat-bottom boiling
- Fotodyne FOTO/Analyst® Apprentice-UV system
- Gel beds
- Gel lane combs
- Gel tank, cover and electrodes
- Graduated Cylinder
- Microwave oven
- Orbital platform shaker
- Pipettes
- Racks, tube
- Safety glasses
- UV Transilluminator

### 4.2 Materials

- Gloves

- Laboratory coat
- Microtiter plate
- Tips, aerosol-resistant (for pipettes)
- Weigh boat
- Ziploc bag

#### 4.3 Reagents

- 10X TAE buffer
- 5X Loading buffer
- DNA MW Marker XIV
- Ethanol
- Nusieve® 3:1 agarose
- SYBR® Safe DNA gel stain
- Water

#### 4.4 Gel and Buffer Preparation

- 4.4.1 Make up on day of use at least 600 mL 1X TAE buffer by making a 1:10 dilution of the stock 10X TAE solution (e.g., 60 mL of stock 10X TAE into 540 mL water for a total of 600 mL).
- 4.4.2 Each 3% (w/v) gel contains 1.5 g Nusieve® 3:1 agarose in 50 mL 1X TAE buffer. Calculate the weight and volume required for a maximum of 4 gels.
- 4.4.3 Measure the volume (50 mL per gel) of 1X TAE buffer with a graduated cylinder and add to the Erlenmeyer or flat-bottom boiling flask.
- 4.4.4 Add the appropriate amount of Nusieve® agarose to the flask slowly while swirling the buffer to avoid clumping of the agarose.
- 4.4.5 Place the weigh boat upside down on the top of the flask to use as a lid and heat in the microwave oven.

**NOTE:** Actual heating time will depend upon the volume and number of flasks, as well as the characteristics of the microwave oven.

- 4.4.5.1 Suggested time for 50 mL gel is 1 minute at 50% power.
- 4.4.5.2 Remove and swirl.
- 4.4.5.3 Repeat 1 minute at 50% power.
- 4.4.5.4 Place on an orbital shaker and rotate for 5 minutes. If necessary, return the flask to the microwave and heat to completely dissolve the agarose (approximately 30-45 seconds).
- 4.4.6 Pulse spin the SYBR® Safe DNA gel stain 10,000X concentrate in a microcentrifuge.
- 4.4.7 Stop the orbital shaker and add 5 µL of the concentrate per 50 mL of agarose from the stock solution, then restart the orbital shaker.
- 4.4.8 Assemble the gel bed(s) in either the casting tray or by placing the gel bed sideways in the electrophoresis tank.
- 4.4.9 When the flask is cool enough to handle (approximately 5-10 minutes at room temperature), the agarose gel can be poured into the prepared gel beds.

- 4.4.10 Ensure the gel bed is level and pour the molten agarose gel into the center of the gel bed. Remove any bubbles with a pipette tip.
- 4.4.11 Place the comb(s) in position, and allow the gel to solidify for approximately 15 minutes at room temperature.
- 4.4.12 Once the gel has solidified and the comb(s) have been removed, the gel is ready to be used.
- 4.4.13 If the gel will not be used on the same day of preparation, the gel may be stored as follows:
  - 4.4.13.1 Place the gel, while still on the gel bed, into a plastic Ziploc bag along with a moistened Kim-wipe and refrigerate at 4°C.

## 4.5 Loading and Running the Gel

- 4.5.1 Ensure the gel tank is situated so that the red (positive) electrode is farthest from the loading wells.
- 4.5.2 Load the product gel into the tank and add a sufficient volume of 1X TAE buffer to the tank to cover the product gel at least 1 mm.
- 4.5.3 Prepare the DNA MW Marker XIV by adding 4 µL of 5X loading buffer to the appropriate microtiter plate wells for each (1:2) standard to be run.
- 4.5.4 Add 2 µL of 5X loading buffer into the wells of the microtiter plate to correspond to the amplified samples that will be loaded into the product gel.
- 4.5.5 Add 2 µL of DNA MW Marker XIV to the appropriate well of the microtiter plate containing 5X loading buffer.
- 4.5.6 Add 4 µL of the amplified sample to the appropriate well of the microtiter plate containing 5X loading buffer and store the remainder of the sample at 4°C.
- 4.5.7 Begin each origin with a DNA MW Marker XIV standard.
- 4.5.8 Load the entire amount of sample from the microtiter plate into the designated well of the gel, continuing until all of the samples have been loaded.
- 4.5.9 Place the cover on the gel tank.
- 4.5.10 Plug the red (positive) electrode into the positive plug of the power supply; then, plug the black (negative) electrode into the negative plug of the power supply.
- 4.5.11 Turn on the power supply and set the voltage to 150 volts.
- 4.5.12 Run the gel for a minimum of 45 minutes, or until the loading buffer moves approximately 4 cm.
- 4.5.13 When electrophoresis is complete, slide the gel onto the UV transilluminator and place the digital camera/gel hood over the transilluminator.

**NOTE:** UV light is hazardous and may cause damage to eyes. Ensure proper safety glasses are in place prior to turning on UV light.

- 4.5.14 Turn on the UV transilluminator and, while using the macro setting on the camera, zoom into the gel to take the photo.
- 4.5.15 Turn the UV transilluminator off and dispose of the gel.

4.5.16 Once the photo is taken, it can be transferred to the computer using the proper cable and photo software.

#### 4.6 Evaluation of the Gel Results

The product gel will be used to define and quantify contamination in the analytical procedure.

The concentration of amplified DNA determined from the product gel will be used to determine the volume of input DNA required for cycle sequencing.

Examiners must use case specific knowledge and discretion in determining when and/or how to proceed with cycle sequencing.

4.6.1 Evaluate the quality and quantity of amplified product by comparing the samples and controls to the DNA Molecular Weight Marker XIV.

- If product is visible in a Reagent Blank lane, the corresponding sample(s) will be reextracted.
- If product is visible in the Negative Control lane, the corresponding samples will be reamplified.
- Primer Set amplification products should display a single band between the 200 bp and 300 bp marker bands.
- Control Region amplification products should display a single band between the 1000 bp and 1500 bp marker bands.
- If an amplified sample is not observed on the product gel for a Control Region amplification, no further cycle sequencing is conducted.
- If an amplified sample is not observed on the product gel for a Primer Set amplification, it will be cycle sequenced.
- Based on case information, sample availability, and examiner discretion, samples may be re-extracted or re-amplified.

## 5 PURIFICATION/SEQUENCING OF MITOCHONDRIAL DNA

### **Special Precautions**

- Dedicated lab coats are worn in the post-amplified DNA laboratory, replace at least weekly.
- Personnel entering post-amplified DNA laboratory will step onto adhesive floor mats, top layer changed when no longer effective.
- All equipment and supplies used in the post-amplified DNA laboratory are dedicated to the post-amplified DNA laboratory and will not be used in the DNA isolation laboratory.
- Discard gloves when leaving the work area to avoid transport of amplified DNA from the Post-PCR work area.
- All work surfaces and pipettes will be thoroughly cleaned with fresh 10% bleach. Isopropanol or ethanol may be used to remove any residual bleach from the surfaces.
- Evidence samples will be cycle sequenced separately from known reference samples.
- The samples will be listed on the case worksheets in the order in which they were processed/handled.
- Aerosol-resistant pipette tips will be changed between samples.

### **5.1 Equipment**

- Centrifuge, 96 well micro-plate
- Freezers (-20°C, -30°C)
- Microcentrifuge
- Pipettes
- Racks, tube
- Refrigerator, 4°C
- Safety glasses
- Speedvac concentrator, Savant ISS110
- Thermal cycler GeneAmp® PCR Systems 9700

### **5.2 Materials**

- Gloves
- Kim-wipes
- Laboratory coat
- MicroAMP™ clear adhesive film
- Optical plate, 96 well
- Performa® DTR Gel Filtration cartridge
- Tips, aerosol-resistant (for pipettes)
- Tubes, microcentrifuge
- Tubes, thin-walled PCR
- Waste containers

### **5.3 Reagents**

- BigDye® Dilution Buffer
- BigDye® Terminator 1.1 Ready Reaction Mix
- Bleach, 10% commercial
- dGTP BigDye® Terminator Ready Reaction Mix
- Ethanol
- ExoSAP-IT®
- ExoSAP-IT® Dilution Buffer
- Hi-Di™ Formamide
- Sequencing Primers (10 µM – see list below)
- Water, ultra-pure

Any of the below listed primers may be used for sequencing:

F15971	5' TTA ACT CCA CCA TTA GCA CC 3'
F15989	5' CCC AAA GCT AAG ATT CTA AT 3'
F16190	5' CCC CAT GCT TAC AAG CAA GT 3'
R16251	5' GGA GTT GCA GTT GAT GT 3'
R16410-m19	5' GAG GAT GGT GGT CAA GGG A 3'
F15	5' CAC CCT ATT AAC CAC TCA CG 3'
F155	5' TAT TTA TCG CAC CTA CGT TC 3'
R285	5' GTT ATG ATG TCT GTG TGG AA 3'
R389	5' CTG GTT AGG CTG GTG TTA GG 3'
R599	5' TTG AGG AGG TAA GCT ACA TA 3'

#### 5.4 Enzymatic Clean Up of PCR Amplification Reaction Product

- 5.4.1 Pulse spin the PCR product prior to opening the tube.
- 5.4.2 Prepare a master mix of ExoSAP-IT® and ExoSAP-IT® dilution buffer as follows:

Volume per reaction	Component
1.5 µL	ExoSAP-IT®
18.5 µL	ExoSAP-IT® dilution buffer

- 5.4.3 Add 20 µL of the master mix to each sample.
- 5.4.4 Briefly vortex and pulse spin the PCR tubes, and then place them into the thermal cycler.
- 5.4.5 Select and start the following program on the thermal cycler:
  - 37°C for 30 min
  - 85°C for 15 min
  - 4°C soak

#### 5.5 Sequencing mtDNA PCR Product

- 5.5.1 Prepare a sequencing reaction master mix as follows:

Component	VOLUME	VOLUME PER RXN
Sequencing Primer <sup>a</sup>	(n+1) X 1.0 µL	1.0 µL
BigDye® Terminator 1.1 RR Mix	(n+1) X 3.6 µL	3.6 µL
dGTP BigDye® Terminator RR Mix	(n+1) X 0.4 µL	0.4 µL
BigDye® Dilution Buffer	(n+1) X 2.0 µL	2.0 µL
Total Volume	(n+1) X 7 µL	7 µL

<sup>a</sup>Any of the primers previously listed may be used for sequencing

- 5.5.2 Add 7 µL of master mix to each appropriately labeled PCR tube.
- 5.5.3 Use the product gel DNA estimates to determine the amount DNA to be sequenced from the samples and controls using the following table as a guide:

Volume	Component
1-13 µL of the Reagent Blank and Amplification Negative Control qs to 13 µL with ultra-pure water <sup>b</sup>	Reagent Blank and Amplification Negative Control volume is equal to the maximum sample volume added.
1-13 µL of sample or positive control qs to 13 µL with ultra-pure water <sup>b</sup>	Optimum Sample and Positive Control PCR product concentration should be between 5-20 ng total.

<sup>b</sup>When sequencing reference samples, the maximum volume used for sequencing is 7 µL if sequencing all eight primers at once.

- 5.5.4 Add the samples and controls to the corresponding PCR tubes one at a time.

**NOTE:** Ensure that the total reaction volume is 20 µL per PCR tube.

- 5.5.5 Briefly vortex and pulse spin the PCR tubes, and then place them into the thermal cycler.

- 5.5.6 Select and start the following program on the thermal cycler:

- 96°C for 1min
- 25 cycles of 96°C for 10sec, 50°C for 5sec, 60°C for 4min
- 4°C soak

## 5.6 Purification of mtDNA Sequencing PCR Product

When handling samples, ensure they remain in the proper order outlined in Chapter 3 Mitochondrial DNA Amplification of this manual. Ensure a full injection column (three columns total) is skipped for reference samples when loading evidence and reference samples onto the same plate for capillary electrophoresis.

- 5.6.1 Spin the Performa® DTR Gel Filtration cartridge for 1 minute at 750 x g.
- 5.6.2 Transfer cartridge to the provided 1.5 mL tube and add the sample to the packed column, ensuring that the fluid is placed in the center of the gel.
- 5.6.3 Close the cap and spin at 750 x g for 2 minutes.
- 5.6.4 Remove and discard the cartridge from each tube.
- 5.6.5 Transfer the sample(s) to a 96 well optical plate.
- 5.6.6 Spin the plate in a speedvac until dry.
- 5.6.7 Pipette 10 µL of Hi-Di™ Formamide into any well of the optical plate that contains the dried sequence product.
- 5.6.8 Pipette 10 µL of Hi-Di™ Formamide into any unused wells of the plate that are in a set of three columns in which samples will be injected to prevent the injection of air by the instrument.
- 5.6.9 Place a 96 well septa on the plate and vortex to help re-suspend the samples.
- 5.6.10 Pulse spin the plate.

## 6 CAPILLARY ELECTROPHORESIS OF THE SEQUENCING PRODUCT

The Applied Biosystems 3500xL Genetic Analyzer is a fluorescent capillary electrophoresis instrument with a 24 capillary array which can be used for a wide variety of DNA sequencing and fragment analysis applications. This includes DNA sequencing and resequencing (mutational profiling), as well as STR (Short Tandem Repeat) analysis and SNP (Single Nucleotide Polymorphism) analysis. The capillary length and polymer type will vary based on the individual application. The use of RFID (Radio Frequency Identification) tags on instrument consumables enables the instrument to track lot and serial numbers, expiration dates, as well as usage. Sample handling and injection is automated by use of the auto sampler, which holds two 96 or 384 well plates. The Polymer Delivery Pump (PDP) automates the replenishment of polymer between injections so that the instrument is capable of fully automated operation. Fluorescent detection is accomplished by utilizing a solid-state 505 nm laser to excite fluorescent dyes from both top and bottom. A cooled CCD array records the fluorescence emitted from each capillary into 20 zones to allow 20-color data. The 20-color data is converted into multi-dye data for the individual application. Four-color data is used for sequence analysis.

For the purpose of this manual, the following definitions apply:

**Injection** - all samples that were separated via CE at a single time. Thus, for one injection on the 3500xL, up to 24 samples can be separated at a single time.

**Run** - all samples that were defined on a single plate and loaded onto the instrument at a single time for separation, regardless of the number of injections utilized to separate each sample contained on the plate. Thus, a run could contain up to 96 samples for separation on a single plate.

### 6.1 Equipment

- Applied Biosystems 3500xL Genetic Analyzer
- 96-well plate centrifuge
- Heat block
- 96-well plate retainer and base (Applied Biosystems, Inc. P/N 4409530)
- Freezer block
- Pipettes

### 6.2 Materials

- 36-cm capillary array (Applied Biosystems, Inc. P/N 4404687 (24-capillary))
- MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, Inc. P/N N801-0560)
- 96-well plate septum (Applied Biosystems, Inc. P/N 4315933)
- Cathode buffer septa (Applied Biosystems, Inc. P/N 4410715)
- PDP Cleaning kit
- Microcentrifuge tubes
- 50 mL conical tube
- Sterile ART Tips, aerosol-resistant (for pipettes)

### 6.3 Reagents

- Hi-Di Formamide (Applied Biosystems, Inc. P/N 4311320)
- POP-6™ Polymer for 3500/3500xL (Applied Biosystems, Inc. P/N A26071 (96 samples), P/N 4393717 (384 samples), P/N 4393712 (960 samples))
- Anode buffer container (Applied Biosystems, Inc. P/N 4393925)
- Cathode buffer container (Applied Biosystems, Inc. P/N 4408256)
- Conditioning Reagent (Applied Biosystems, Inc. P/N 4393718)
- 3500/3500xL Sequencing Standards, BigDye™ Terminator v1.1 (Applied Biosystems, Inc. P/N 4404314)
- Sterile water

## 6.4 Safety Considerations

The Applied Biosystems 3500xL Genetic Analyzer uses a single-line 505nm solid-state laser. All instrument panels must be in place on the instrument while the instrument is operating. When all panels are installed, there is no detectable radiation present. If any panel is removed when the laser is operating, (during service with safety interlocks disabled) you may be exposed to laser emissions in excess of the Class 3B rating. Do not remove safety labels or disable safety interlocks.

Performance Optimized Polymer (POP) is an irritant and a neurotoxin. Always wear gloves when handling or cleaning liquid or dried polymer. It may cause skin, eye, and respiratory tract irritation.

Exposure to Hi-Di formamide causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing and gloves.

## 6.5 Instrument Preparation

- 6.5.1 With the 3500xL turned off, turn on the computer, but do NOT log on.
- 6.5.2 Power on the 3500xL instrument. Wait for the solid green light indicating the initialization process is complete.
- 6.5.3 Log onto the computer - the Server Monitor/Daemon will launch automatically. Do NOT launch the 3500 Series Data Collection Software until the “3500 Services Loaded” at the lower right corner of the screen is completely loaded and indicated with the green check mark.
- 6.5.4 Double-click on the 3500 instrument icon to launch the Data Collection Software, which will launch the Dashboard screen.
- 6.5.5 Select “Start Pre-Heat” at least 30 minutes prior to the first injection of the day to preheat the oven to 55 °C.
- 6.5.6 Click “Refresh” in the “Consumables Information” window. The readings/gauges will be updated once the Radio Frequency Identification (RFID) tags are read.
- 6.5.7 If the capillary array needs to be changed, follow the directions given in the Install a Capillary Array wizard. The wizard should take approximately 15-45 minutes. Otherwise, continue to 6.5.8.
- 6.5.8 If the anode buffer container (ABC) and cathode buffer container (CBC) have been on the instrument longer than 14 days, they will need to be changed. Otherwise, continue to 6.5.9.

### 6.5.8.1 Changing the ABC:

- 6.5.8.1.1 Verify that the buffer level is at or above the fill line and check that the seal is intact.
- 6.5.8.1.2 Invert the ABC, then tilt it slightly to make sure most of the buffer is in the larger side of the container. There should be less than 1 mL of buffer remaining on the smaller side of the container.
- 6.5.8.1.3 Peel off the seal at the top of the ABC and place the ABC into the anode end of the instrument.
- 6.5.8.1.4 Click “Refresh” on the dashboard to update the screen.

### 6.5.8.2 Changing the CBC:

- 6.5.8.2.1 Press the TRAY button on the instrument to bring the old CBC forward.
  - 6.5.8.2.2 Discard the container and septa.
  - 6.5.8.2.3 Verify that the buffer level is at or above the fill line and check that the seal is intact. Tilt the CBC back and forth gently to ensure that the buffer is evenly distributed across the baffles.
  - 6.5.8.2.4 Peel off the seal at the top of the CBC. Wipe off any buffer on the top of the CBC to make sure the container is dry.
  - 6.5.8.2.5 Align and place the appropriate septa on both sides. Push the septa lightly into the holes to start and then push firmly to seat the septa.
  - 6.5.8.2.6 Install the CBC on the autosampler.
  - 6.5.8.2.7 Close the instrument door and wait for the green light.
  - 6.5.8.2.8 Click “Refresh” on the dashboard to update the screen.
- 6.5.9 Verify that enough polymer remains in the pouch (# of samples remaining/#injections remaining). This information is available in the dashboard window. If the polymer has been on the instrument longer than 14 days and/or it has expired, and/or the amount is insufficient for the run, the polymer pouch will need to be changed. Otherwise, continue to 6.5.10.

### 6.5.9.1 Changing the polymer pouch:

- 6.5.9.1.1 Verify that the new pouch to be installed has not expired.
  - 6.5.9.1.2 Allow the new pouch to equilibrate to room temperature.
  - 6.5.9.1.3 Select the Replenish Polymer Wizard. Follow the directions given. The wizard will take 10 to 20 minutes to complete.
- 6.5.9.1.4 Click “Refresh” on the dashboard to update the screen.

### 6.5.10 Prior to the first run on each day on which the instrument will be used:

- 6.5.10.1 Visually inspect the level of fluid inside the ABC and CBC. The fluid must line up with the fill line.
- 6.5.10.2 Ensure the CBC septa are positioned on the plate deck properly and securely.
- 6.5.10.3 Check for bubbles in the pump block and channels.
  - 6.5.10.3.1 If bubbles are observed, use the Remove Bubble Wizard.
- 6.5.10.4 Check the loading-end header to ensure that the capillary tips are not crushed or damaged. If crushed or damaged, the array will need to be changed.
- 6.5.10.5 Check for leaks and dried residue around the buffer-pin valve, check valve, and array locking lever. If leaks persist, the manufacturer will need to be contacted.

## 6.6 Sample Plate Preparation

- 6.6.1 Place the 96-well plate loaded in 5.6.5 into a blue plate base and snap the white plate retainer over the top, taking care to align the tabs on both ends.
- 6.6.2 Press the TRAY button on the 3500xL to bring the autosampler tray forward.
- 6.6.3 Open the instrument door and place the plate assembly into an open position (A or B) with the labeled end of the white retainer facing the analyst. The plate can only be placed in one orientation.
- 6.6.4 Close the instrument door.

## 6.7 Creation of an Injection Plate File

### 6.7.1 Manual Creation of Injection Plate File

- 6.7.1.1 In the dashboard, click on “Create Plate from Template”.
- 6.7.1.2 Select “MitoSequencingTemplate” and enter the plate name. Entering the Owner as DFS is optional.
- 6.7.1.2.1 The following parameters are provided in this template:
  - Number of wells: 96 (default)
  - Plate Type: Sequencing
  - Capillary Length: 36 cm (default)
  - Polymer: POP6 (default)
- 6.7.1.3 Click on “Assign Plate Contents”.
- 6.7.1.4 Using either the Plate View or the Table View, enter the sample names in the appropriate wells.
  - 6.7.1.4.1 Sample Names will, at a minimum, include the following:
    - Case number
    - Item number and type of sample/control
    - E followed by the extraction number
    - A followed by the amplification number
    - Direction of sequencing primer/amplification primer
    - Injection time
  - 6.7.1.5 Under “Assays”, select one for each assigned well.
  - 6.7.1.6 Under “File Name Conventions”, select Casework for all assigned wells.
    - 6.7.1.6.1 At a minimum the Sample Name, and Date of Run should be included to differentiate each sample from similar samples on different runs.
  - 6.7.1.7 Under “Results Groups”, select the appropriate Results Group from the list provided.
 

**NOTE:** To set up a new Results Group, refer to the Applied Biosystem’s 3500xL Genetic Analyzer User Guide, as needed.
  - 6.7.1.8 Using the drop down menu under “Sample Type”, if desired, the positive and negative controls may be designated.

- 6.7.1.9 Review the parameters for each well and change, if necessary.
- 6.7.1.10 Save the plate file and proceed to 6.8.
- 6.7.2 Importing an Injection Plate File
 

**NOTE:** The same Sample Name, File Name, and Results Group naming conventions, as described in Section 6.7.1, will be followed.

  - 6.7.2.1 If the plate file is not first imported into the Library:
    - 6.7.2.1.1 Under Workflow, select “New Plate” and then select “Create new plate from a standard format file.”
    - 6.7.2.1.2 Click Import. Use the drop down menu to select “\*.xls”, browse to find the appropriate run file and double click to import the file. Click OK after verifying the correct file was imported.
    - 6.7.2.1.3 Select the plate from the list and select “edit.”
    - 6.7.2.1.4 Follow steps 6.7.1.5 through 6.7.1.10 above, as applicable.
  - 6.7.2.2 If the plate file is imported into the Library:
    - 6.7.2.2.1 In the navigation pane, click Library → Plates.
    - 6.7.2.2.2 Click Import. Use the drop down menu to select “\*.xls”, browse to find the appropriate run file and double click to import the file. Click OK after verifying the correct file was imported.
    - 6.7.2.2.3 Select the plate from the list and select “edit.”
    - 6.7.2.2.4 Follow steps 6.7.1.5 through 6.7.1.10 above, as applicable.

## 6.8 Linking a Plate File for a Run

- 6.8.1 In the navigation pane, click Load Plates for Run.
  - 6.8.2 Plate(s) must be loaded onto the autosampler tray before linking for a run.
  - 6.8.3 Click Link Plate in the appropriate position (A or B) and browse in the pop-up window to find the appropriate plate file. Double click on the plate file to select it and then close the window.
  - 6.8.4 Click on Create Injection List and refer to the Injection List Details to review the selected injection parameters and/or to move selected injections up or down to change the order in which they will occur. Once the order is as desired, click Start Run.
- NOTE:** The order of any remaining injections may also be adjusted during a run.

- 6.8.5 Once Start Run has been clicked, the instrument will notify the user if there are any instrument or reagent issues with the run. If an issue is detected, the run will not begin until the issue is resolved. Expired reagents may not be used for casework. However, a capillary array may be used past the manufacturer’s recommended use/expiration date as long as the quality of the data obtained does not decline. If this is the reason for the notification, click on “Use” to continue.

**NOTE:** Once the run has ended, the instrument will “pause” to allow for reinjections. If no reinjections are necessary, terminate the run to officially complete the run for the software.

## 6.9 Pausing or Aborting a Run

6.9.1 To pause during a run, click Pause and the run will pause after the current injection is completed.

6.9.1.1 To terminate the instrument run at this point, click Terminate Injection List.

6.9.1.2 To resume the run instead, click Resume.

6.9.2 To abort a run, click Abort.

6.9.2.1 Verify that the current injection is to be aborted by clicking “yes” or “no” in the comment box.

**NOTES:** Choosing “yes” will terminate the current injection. The option to resume at this point will resume at the NEXT injection. The option to terminate injection list will abort all remaining injections, including the current one.

Choosing “no” will cause the instrument to pause. The option to resume at this point will resume the CURRENT injection. The option to terminate injection list will abort all remaining injections, including the current one.

6.9.2.2 To terminate the instrument run at this point, click Terminate Injection List.

6.9.2.3 To resume the run instead, click Resume.

## 6.10 Creating Reinjections

Reinjections may be created either the same day as the plate set up or on the day following the plate set up.

6.10.1 Creating reinjections within the same plate file when the “pause” at the end of the run has been enabled

Following the last injection of an instrument run, the instrument will “pause” if that feature is enabled. The oven will stay heated for two hours following the end of the run as well.

During this time, reinjections can be created either within the same plate file or as a separate plate file.

Re-injections with the same injection time created in this way are denoted in the full file name with “.2” at the end after the Date of Run.

If not created during this pause time, reinjections will have to be created as a separate plate file – see 6.10.2.

6.10.1.1 Click on “Monitor Run” and highlight the well(s) or injection to be reinjected.

6.10.1.2 If the sample(s) or injection is to be reinjected at the same parameters, click “Duplicate” (at the top of the screen).

6.10.1.3 If the sample(s) or injection is to be reinjected under different parameters, click “Re-inject”.

6.10.1.3.1 In the “Re-injection” dialog box, click on “Reuse a protocol in the library” and select the desired Instrument Protocol from the drop down menu. Also select “Following all injections” or “After original injection” and click OK.

6.10.1.3.2 In the “Setup and Instrument Protocol” dialog box, click “Apply to Assay” at the bottom, but DO NOT click on “Save to Library”.

**NOTE:** On the “Monitor Run” screen, the selected reinjection(s) will reflect the original selected Assay name with a suffix of “\_#”; however, the correct reinjection time will be reflected under the column “Instrument Protocol”. The sample file name(s) for the re-injected sample(s) will reflect the correct reinjection time.

- 6.10.1.4 The injection parameters will be designated for selected wells of the plate. Review and change if necessary.
- 6.10.1.5 If reinjections are created during the instrument pause, click “Resume” to continue the instrument run with the newly created reinjections.
- 6.10.2 Creating reinjections as a separate plate file
  - 6.10.2.1 If the reinjections are created as a separate plate file, but during the instrument pause, click “Terminate Injection List” to terminate the pause. Otherwise, proceed to 6.10.2.2.
  - 6.10.2.2 Under “New Plate”, click on “Create New Plate from Existing Plate”, select the original plate file and name the new plate file.
  - 6.10.2.3 Deselect and unassign all samples with previously designated injection parameters that were initially run. Highlight the desired well(s) or injection and select the desired Instrument Protocol.
  - 6.10.2.4 Link and run the newly created reinjection plate file.

## 7 SEQUENCE ASSEMBLY AND ANALYSIS WITH SEQUENCHER™ SOFTWARE

Effective mitochondrial DNA (mtDNA) sequencing requires multiple overlapping regions of the mtDNA genome to be amplified and sequenced. Typically the length of the sequenced product will vary based on the quality of the DNA. Reference samples can have much longer amplification fragments and longer subsequent sequence fragments, whereas environmentally challenged samples may require multiple amplifications of smaller regions and subsequent short sequence fragments.

The Sequencher™ software is used to edit and assemble these sequences to produce a contiguous consensus sequence (contig). The consensus sequence can then be compared to the Revised Cambridge Reference Sequence (rCRS) to report the differences to the reference.

Each sample is edited and assembled from its sequence fragment(s) to contig with respect to the rCRS.

Evidence and control contigs will be evaluated for sequence concordance.

- Sequence concordance is defined as having a common base at each position. In order of preference, the following can be used to confirm base concordance:
  - Both light (L) and heavy (H) strand sequencing reactions
  - Two forward strands (L) or two reverse strands (H) from separate primer set amplifications
  - Two forward strands (L) or two reverse strands (H) from the same control region amplification, but from different sequencing amplifications
- Data not confirmed to be concordant (e.g., single stranded data) will not be interpreted.

Contigs for all single source samples, as well as the negative control, reagent blank(s) and positive control, will be independently assembled and edited in the same order by a second Forensic Scientist or Forensic Lab Specialist.

- Separate Sequencher™ Variance Table Reports and Contig Maps are generated, printed, and initialed by each Forensic Scientist or Forensic Lab Specialist.

Independent assembly of contigs by two analysts is not required for:

- samples that are analyzed solely for quality control purposes.
- samples that are deemed to be a mixture by the original Forensic Scientist assembling a contig.
  - Samples exhibiting three or more areas of apparent heteroplasmy within the control region may be considered as mixtures.
  - Samples deemed to be a mixture by the original Forensic Scientist assembling the contig will be evaluated during technical review to ensure agreement that the sample is, indeed, a mixture sample.

### 7.1 Equipment

- PC Computer
- Color Printer
- Sequence Scanner v1.0 or higher
- Sequencher™ software v4.8 or higher

### 7.2 Printing/Displaying Electropherogram Data

7.2.1 Open the Sequence Scanner software.

7.2.2 Click on the “File” menu and select “Import Traces”.

7.2.2.1 Click on the desired trace file or folder of trace files and click on the “Add Selected Traces” button.

**NOTE:** The traces to be added will appear in the right side window and can be cleared individually or in groups.

7.2.2.2 To clear or change the traces which will be imported, select them and click on “Clear”.

- 7.2.2.3 Click on the “OK” button to import all the traces listed in the traces to add window.
- 7.2.3 From the “Trace Manager” tab the imported traces can be selected to be viewed or printed.
- 7.2.3.1 To view the traces, double click the trace listed in the details window.
- NOTE:** Opening multiple traces will show each displayed in a different tab.
- 7.2.3.2 To print the trace, ensure that it is highlighted in the details window and select “Print” from the “File” menu.
- 7.2.4 Additional helpful information can be viewed in the Reports section, if desired.
- 7.2.4.1 Click on the “Reports” tab to view the reports.
- There are three very useful reports for sequencing which can either be printed or exported to a data file:
- NOTE:** Printing or exporting these reports is optional.
- The QC report displays the quality score and continuous read length (CRL) of each trace imported.
  - The Plate report displays the score for each sample as they relate to each other within the plate (if the samples are from the same plate).
  - The Signal strength report displays graphically the average raw signal intensity for all imported samples relative to each other.
- 7.2.4.2 Select the desired report and click “Print” from the “File” menu to print the report.
- 7.2.4.3 Click on “Export Reports” from the reports tab menu and check on each report to include in the export file, name the file and click “Save” to save the report as an electronic file.

### 7.3 Creating a Sequencher™ Project

- 7.3.1 Open the Sequencher™ software.
- 7.3.2 If an untitled project window doesn’t appear with the rCRS DNA reference, create a new one.
- 7.3.2.1 Click on the File menu and select “New Project from template” and choose “rCRS” as the template.
- 7.3.3 Add DNA sequence fragments to the project by selecting “Import” from the file menu.
- 7.3.4 Select “Sequences” to select individual sequences to add to the project.
- 7.3.5 Select “Folder of Sequences” to add an entire folder (or run) of samples to the project.

### 7.4 Assembling a Contig

- 7.4.1 Select the reference and sequence fragments to include in the contig by using the control key and clicking on each fragment.
- 7.4.2 With the mouse over the selected fragments, right click and choose “Assemble Contig” from the menu.
- 7.4.3 Select “Assemble to Reference” or “Assemble to Reference by Name” if you have the “Assemble by Name” option active.

7.4.4 Ensure that the contig is named with at least the FS Lab number and Item number.

7.4.4.1 This is the name that will appear as the title in subsequent printouts of the data associated with this contig.

7.4.4.2 To change the name, right click on the newly assembled contig name text and select “Rename Contig”.

## 7.5 Editing Assembled Contigs

7.5.1 Nomenclature Rules

Differences in the consensus sequence, as compared to the rCRS, are noted by stating the nucleotide position followed by the code for the polymorphic base (e.g., 263G).

Nucleotide base positions are designated according to the standard nomenclature set by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry and Molecular Biology (IUPAC-IUBMB) Joint Commission on Biochemical Nomenclature and listed in the tables below:

CODE	DESIGNATION
A	Adenine
B	C, G, or T
C	Cytosine
D	A, G, or T
G	Guanine
H	A, C, or T
K	G or T (Keto)
M	A or C (aMino)
N	aNy base
R	A or G (puRine)
S	G or C (Strong H-bonds)
T	Thymine
V	A, C or G
W	A or T (Weak H-bonds)
Y	C or T (pYrimidine)

Other	DESIGNATION
-	Deletion
X.1 (.2, .3, ...)	Insertion after position X

Profiles are characterized such that known patterns of polymorphisms (i.e., known phylogenetic alignments) are maintained (e.g., maintain deletions at positions 249, 290 and/or 291 when present).

The least number of differences from the rCRS are maintained, unless this violates known patterns of polymorphisms.

Homopolymeric C-Stretches in HV1 should be interpreted with a 16189C when the otherwise anchored T at position 16189 is not present. Length variation in the short A-tract preceding 16184 should be noted as transversions.

Homopolymeric C-Stretches in HVII should be interpreted with a 310C when the otherwise anchored T at position 310 is not present. C-stretches should be interpreted with a 311T when the anchored T at position 310 is followed by a second T.

The AC repeat motif in HVIII region from np 515-525 is maintained.  
Substitutions are preferred to insertions/deletions (indels).

Transitions are preferred to transversions, unless this conflicts with maintaining known patterns of polymorphisms (i.e., known phylogenetic alignments).

Indels are placed contiguously when possible.

Indels are placed on the 3' end of the light strand.

Insertions are described by noting the site immediately prior to the insertion with respect to the light strand of the rCRS followed by a point and a '1' for the first inserted base, with sequential numbering for each inserted base thereafter (e.g., 315.1C).

### 7.5.2 Evaluation of and Notation for Sequences with Length Heteroplasmy

Length heteroplasmy must be observed above the level of background in both strands of data.

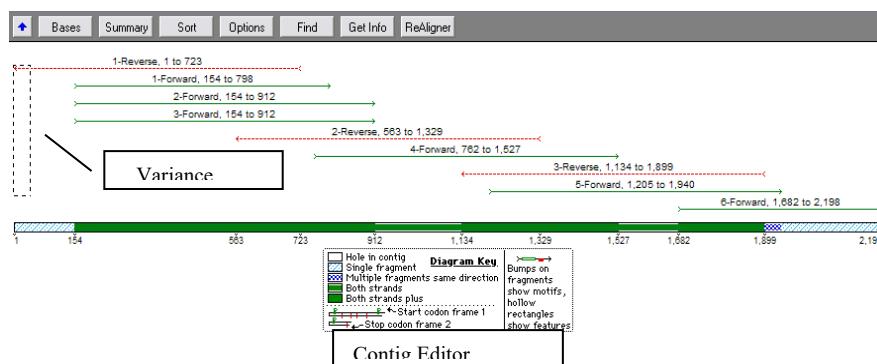
Length heteroplasmy in HVI most commonly arises when there is a substitution of a C for a T at position 16189. The reference type in HVI is C TC<sub>5</sub><sup>4</sup>. HVI length heteroplasmy will not be recorded in casework samples. Rather, the sequence reported will be the first 10 cytosines observed beginning at position 16184.

The number of C nucleotides exhibited in samples with HVII length heteroplasmy (nucleotide positions 303-309) is highly variable and care must be exercised when making comparisons. Sequence concordance within HVII is defined as a common length variant between samples being compared. Differences in sequences due to the absence of a common length variant are not exclusionary. Length variants in HVII are commonly observed in the number of C residues preceding a T residue at position 310. It is often possible to determine unambiguously the dominant length variant in this region, as well as some or all of the minor length variants, and the sequence is notated to reflect these variants. The reference type in HVII is C TC<sub>7</sub><sup>5</sup>. A 309.1C insertion in HVII will be annotated C TC<sub>8</sub><sup>5</sup>, and the insertions 309.1C, 315.1C will be annotated as C TC<sub>8</sub><sup>5</sup><sub>6</sub>.

### 7.5.3 General Sorting and Editing

The Contig Editor provides tools for assisting review and editing of sequences from each electropherogram within the assembled contig.

The overview display is shown below.



#### 7.5.3.1 To sort and organize the fragments, choose any of the following options:

- Click on the “Sort” button to select the sort method of choice, and the fragments will be sorted based on that criterion.

- Use the grab function by pressing the control key and using the mouse to click and drag the fragments.

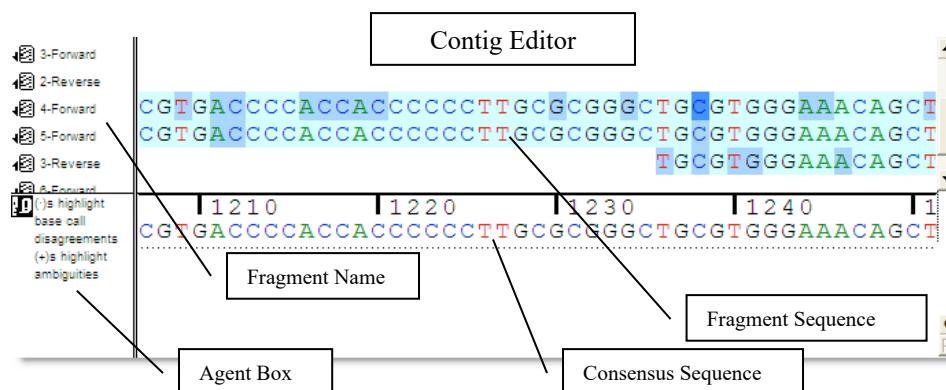
**NOTE:** Moving the fragments this way may change their alignment to the reference.

- Click on the fragment name while pressing the control key within the fragment name box.

7.5.3.2 To edit the bases, move the Selection Marquee to choose the region to edit and click on the “Bases” button.

7.5.3.3 To return to the previous view of all the fragments, click on the “Overview” button.

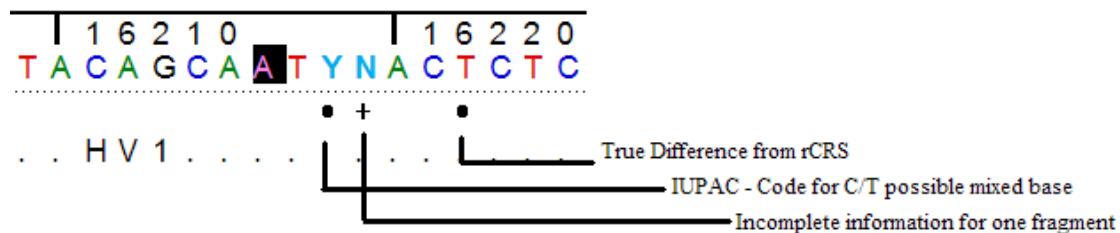
The contig editor (shown below) is organized into 4 quadrants including the fragment name, the agent box, the fragment sequences, and the consensus sequence.



The Agent Box contains descriptive information regarding the sequence and selected areas.

The Consensus Sequence area contains the consensus sequence as well as discrepancies between fragments (designated with “+”) and disagreements from the reference (designated with “•”).

T A C A G C A A T C A A A C C C T C  
T A C A G C A A T C A A A C T C T C  
T A C A G C A A T C A A A C T C T C  
T N A C T C T C



**NOTE:** Ambiguous (mixed) bases are indicated by their appropriate code as detailed in 7.5.1.

The colon “:” is used as a placeholder within the fragment or reference sequence and indicates a missing base at that position.

- The colon can represent:
  - a deletion if placed in the consensus
  - OR
  - an insertion if placed in the reference sequence.
- The colon placeholder is automatically placed during assembly of the contig and may be edited, as appropriate.

#### 7.5.4 Editing bases in the Contig Editor

**NOTE:** Edited bases will be reflected as lowercase letters.

- 7.5.4.1 Select a base on either the consensus sequence or a fragment sequence to navigate the contig, click on “Select”, and choose from the options listed.

- 7.5.4.2 To repeat the selection, use the spacebar.

- 7.5.4.2.1 To find the next base designated “N”, click on “Next ambiguous base”.

- 7.5.4.2.2 To find the next base with disagrees with the reference sequence, click on “Next contig disagree”.

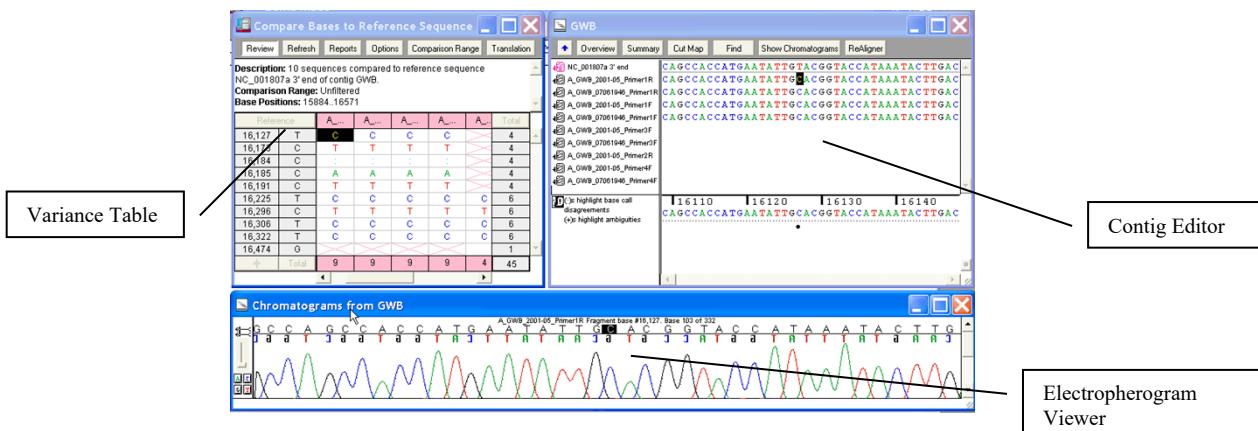
- 7.5.4.2.3 To find the next base which has been edited previously, click on “Next edited base”.

- 7.5.4.2.4 To find the next base which has a low quality score, click on “Next low confidence base”.

- 7.5.4.3 To view the electropherogram for selected base(s), click the “Show Electropherogram” button.

- 7.5.4.3.1 Scroll through the electropherogram by using the arrow keys or scroll bar.

- 7.5.4.3.2 To view peaks within the electropherogram corresponding to the bases by type, toggle the bases on or off using the **A**, **C**, **G**, and **T** buttons.



- 7.5.4.4 To edit a base while in the electropherogram view window, click on the base and type a base letter.

7.5.4.5 To add or subtract a base, select the base or an adjacent base, click “Sequencing”, and select the appropriate option.

7.5.4.5.1 To delete bases, click “Delete Bases” and “fill void from right” or “fill void from left”.

**NOTE:** The selection between “fill void from right” and “fill void from left” will affect the remaining alignment for that fragment.

7.5.4.5.2 To insert a colon “:”, click “Insert Gap or Move Bases and move the bases by clicking “left” or “right”.

**NOTE:** The selection between “left” and “right” will affect the remaining alignment for that fragment.

7.5.4.5.3 To remove the sequencing primer bases which remain on a complementary fragment (e.g., remove the forward primer bases which are sequenced on a reverse sequence fragment), use the “Delete Bases” function.

7.5.5 To optimize the sequence fragments’ alignment to the reference sequence, click the “ReAligner” button.

7.5.6 To display the coverage information once the Contig has been edited completely, click “Overview”.

7.5.6.1 To print the coverage map, select “Print” from the “File” menu.

## 7.6 Creating a Variance Table and Report

While the consensus sequence contains the compiled nucleotide base calls for the fragments in the sample, the differences from the reference sequence are exhibited in the Variance Tables and Report.

The consensus sequence can be reviewed in the review mode OR through the Variance Table with the data supporting each variant displayed.

7.6.1 Ensure that “Consensus Inclusively” is selected in the “Contig” menu.

7.6.2 Select/highlight the contig to be compared, click on “Contig”, and select “Compare Consensus to Reference”.

7.6.2.1 Alternatively, right click on the highlighted contig and select “Compare Consensus to Reference”.

7.6.3 To review the base calls, click “Review”.

7.6.4 To view the variant bases in the Contig Editor and Electropherogram Viewer, click the corresponding base variant in the Variance Table.

7.6.5 Click on “Reports” in the Variance Table Review window.

7.6.6 Select “Variance Detail Report” from the drop down list and click “Open Report”.

7.6.7 Click “save PDF” to retain an electronic copy of the document.

7.6.8 Open the saved .pdf document and select “Print” to print the report.

## 7.7 Exporting Sequence Data for CODIS

The data from two different analysts must be evaluated to ensure the consensus sequences are in agreement prior to exporting data for CODIS.

Two separate Sequencher™ projects created from the same sequence data are required for the sample sequence data to be exported for import to CODIS.

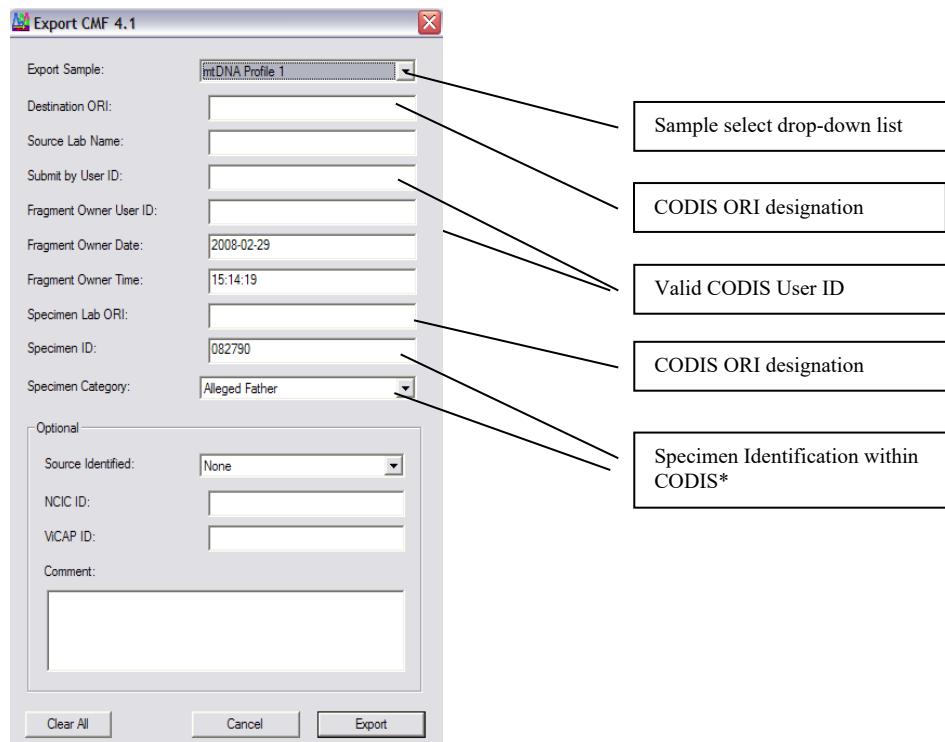
- 7.7.1 Click on “File” and select “Import Sequencher™ Project” to choose the project containing the sequence data.
- 7.7.2 Repeat for the 2<sup>nd</sup> Sequencher™ project.
- 7.7.3 Ensure at least two contigs for the specimen are highlighted and that “Consensus Inclusively” is selected in the “Contig” menu.
- 7.7.4 From the “Contig” menu, click on “Compare Consensus to Reference” to generate the Variance Table to review the two contigs.

Reference	082790	90	Total
16,163	A	N	1
73	A	G	2
183	A	N	1
195	T	C	2
♦	Total	4	6

The sequence and electropherogram data can be viewed or edited by double clicking on the letter listed in the table.

- 7.7.5 Once the two contigs are in agreement, close the Variance Table.
- 7.7.6 With both of the two contigs highlighted, click “Contig”, and select “Validate mtDNA Profiles”.
- 7.7.7 For comparison between two sizings – save the report as a .txt file.
- 7.7.8 Open and print the saved report.
- 7.7.9 Click on “Export CMF” to export the profile for CODIS.
  - 7.7.9.1 Fill in the appropriate blanks in the Export CMF window and click “Export” to create a .xml file that can be uploaded into CODIS.

**NOTE:** Edit the export file in an appropriate application, if necessary, so that the rCRS is in proper alignment for CODIS import.



## 8 INTERPRETATION OF MITOCHONDRIAL DNA RESULTS

### **8.1 Sequence Confirmation**

Sequence confirmation is performed by comparing the independently assembled consensus sequences (comparing the individual Variance Table Reports and Contig Maps) for the negative control, reagent blank(s), positive control, evidence samples, and reference samples.

- Confirmed sequence range for each control or sample is defined as the shortest length of sequence obtained in common from the independent assemblies.

### **8.2 Evaluation of the Reagent Blank**

The reagent blank (RB) monitors for the presence of exogenous DNA from the extraction steps through sequencing and is processed from the DNA extraction procedure through sequencing analysis along with the associated sample(s).

If a reagent blank generates sequence data, the associated sample data may be used under the following conditions:

- The RB does not contain base calls (i.e., the RB contains N base calls only).
- The RB has base calls, but does not align.
- The RB sequence aligns, but the sequence is not concordant with any relevant sample (defined separately below for evidence and reference RBs).
  - If the sequences of the associated sample(s) and the RB are the same within a region, the region in question will not be included in the interpreted and reported sequence for that sample.
    - Alternatively, the sample, along with the RB in question, may be reextracted, reamplified, or resequenced.

#### Relevant samples for evaluation of an evidence reagent blank

When evaluating a reagent blank from within a batch of evidence samples, all evidence samples adjacent to the RB are considered (e.g., Reagent Blank 3, as shown in the Introduction of Chapter 3 of this manual, will be compared to Sample 3 and Sample 4, as necessary). If an evidence RB generates a sequence that is concordant with an associated sample or any adjacent sample within a batch, all evidence samples (whether a single sample or an entire batch) will be reextracted.

#### Relevant samples for evaluation of a reference reagent blank

When evaluating a reagent blank from a batch of reference samples, all associated reference samples are considered such that the reference RB will be compared to all reference samples, as necessary. If a reference reagent blank generates a sequence that is concordant with any one of the associated reference samples, all reference samples will be reextracted.

### **8.3 Evaluation of the Negative Control**

The negative control (NC) monitors for the presence of exogenous DNA in the reagents used from amplification through sequencing.

If the negative control generates a sequence that is in concordance with any associated sample, the results for the associated sample will not be used.

If the negative control generates sequence data, the associated sample data may be used under the following conditions:

- The NC does not contain base calls (i.e., the NC contains N base calls only).
- The NC has base calls, but it is not of requisite quality for comparison purposes or does not align.
- The NC sequence aligns, but the sequence is not concordant with any of the associated samples.
  - If the sequences of associated sample(s) and the NC are the same within a region, the region in question will not be included in the interpreted and reported sequence for the affected sample(s).
    - Alternatively, the affected sample(s), along with the NC, may be reamplified or resequenced.

#### 8.4 Evaluation of the Positive Control

The positive control monitors the success of amplification, sequencing, and sequence analysis.

The positive control must sequence correctly (i.e., the correct polymorphisms are detected at the correct nucleotide positions) in order for sequence data for an associated sample to be interpreted and reported.

Only the ranges in common between the sample and the associated positive control will be considered.

If a positive control fails to amplify or sequence correctly, the associated sample(s) will be reamplified or resequenced.

#### 8.5 Evaluation and Comparisons Between Sample Sequences

No interpretation or sequence comparisons will be made for mixture samples.

Differences between a single source sequence generated from a sample and the rCRS can be compared between evidentiary and reference samples while evaluating the similarities or differences between the mtDNA sequences.

When comparing sequences obtained from samples, only the regions with a common range will be evaluated. For example, if a partial sequence (16024-16365 and 73-284) is obtained for a sample of questioned origin (Q), and a full sequence is obtained for the sample of known origin (K), the comparison will be conducted on positions 16024-16365 and 73-284. In addition, sequence before and after the defined HVI and HVII regions will also be used for comparison purposes, provided this range is common to both samples.

The following interpretations are available for sequence comparisons:

- Elimination

The samples are determined not to have come from the same source if their sequences differ at two or more positions.

- Inconclusive

The determination as to whether or not two samples came from the same source is deemed inconclusive for any of the following situations:

- Their sequences differ at a single position, with no evidence of heteroplasmy.
- Their sequences are the same, but they do not have a common length variant at nucleotide positions 303-309 (HVII).
- Their sequences differ at a single nucleotide position and they don't have a common length variant at nucleotide positions 303-309 (HVII).

**NOTE:** Because of the possibility of undetected heteroplasmy, additional samples may be analyzed when compared evidence and reference sequences differ by a single base. These samples may include blood, buccal swabs, and hair.

- Cannot Eliminate

The samples cannot be eliminated as having come from the same source or from having a maternal relationship if they have the same sequence or demonstrate sequence concordance.

- Sequence concordance is defined as having a common base or common HVII length variant at each position.
  - If one sample has evidence of a C and a T at a given position, and the other has a C, they share the C in common at that position and are concordant.

- Overlap of length variants is considered sequence concordance. However a C and a T are both observed at a given position, and the other has a G at that position, these sequences are not concordant.

## 8.6 Missing Persons Cases

Casework sample haplotypes from Missing Persons cases will be entered into SDIS following appropriate CODIS reviews, as detailed in the FB PM CODIS Operating Policies and Procedures. The haplotypes will be searched and uploaded in accordance with State and National CODIS policies and procedures.

## 9 POPULATION STATISTICS – DETERMINATION OF HAPLOTYPE FREQUENCY

A database search of the HVI and HVII sequence data and determination of a haplotype frequency will be conducted on an evidence sample haplotype when a reference haplotype cannot be eliminated as having come from the same source or from having a maternal relationship.

- If a reference sample haplotype is concordant with multiple evidence sample haplotypes in the same case, a database search and determination of haplotype frequency will only be conducted for the evidence sample with the largest amount of HVI and HVII sequence data.

Determination of haplotype frequency is based upon the counting method and employs the calculation of a 95% upper bound confidence interval.

The database searched is that supplied within the Popstats program contained within the Combined DNA Index System (CODIS) software and is maintained by the Federal Bureau of Investigation (FBI) in collaboration with the Scientific Working Group on DNA (SWGDAM). This database consists of human mitochondrial DNA (mtDNA) nucleotide sequence data from a variety of individuals of European, Asian, and African ancestry generated by forensic DNA typing laboratories and their contractors.

The Popstats program facilitates the search of casework haplotypes against the database to provide frequency estimates.

The database search result report provides the number of database profiles that match the casework profile, as well as a frequency estimate for each population.

The frequency estimates calculated are Clopper-Pearson one-tailed 95% interval upper bound frequency estimates.

- If the searched haplotype is observed in the database, the formula used for calculating the 95% confidence limit is that of an exact confidence interval. The equation seeks a value  $p$  such that:

$$\sum_{k=0}^x p_0^k \binom{n}{k} (1-p_0)^{n-k} = 0.05$$

This corresponds to the margin of error given the size of the database and the corresponding zone of 95% confidence. In 95% of intervals constructed in this way, the true frequency will be higher than this value; thus, with 95% confidence the haplotype frequency will be less frequent than that provided by the upper bound estimate.

- If the searched haplotype is not observed in the database the, formula used is:

$$1 - \alpha^{1/N}$$

$\alpha$  is the confidence coefficient (0.05 for a 95% confidence interval), and N is the number of individuals in the population.

### 9.1 Searching the database

#### 9.1.1 The following settings are selected for searching and visible on the search screen:

- Length Heteroplasmy List – Specifies locations where insertions can be considered
  - 16193, 309, 573
- Maximum Number of Sequence Differences for Match – Specifies number of allowable base differences for CODIS to establish a match
  - 0
- Maximum Number of Sequence Differences for Display – Specifies number of allowable base differences for a match to be displayed
  - 0

- Minimum Number of Overlapping Base Pairs for Search – Specifies the minimum number of overlapped base pairs between target and candidate profiles required before profiles are compared in a frequency search
  - 150
- Minimum Population Group Size for Upper Bound Frequency Estimate – Specifies the smallest sample size for a given population group for which an upper bound frequency will be estimated
  - 100
- List the Match Pairs – Specifies whether the match details will be displayed on the mtDNA Report.
  - Not selected
- Length Heteroplasmy Options – Specify the way in which insertions at length heteroplasmy site are treated.
  - Ignore insertions at the specified heteroplasmy sites – All insertions at the three specified heteroplasmy sites are ignored.

9.1.2 Include only the shortest range of sequence data agreed upon by the two independent analyses/assemblies within HVI and/or HV2.

9.1.3 Search ambiguous bases as “N”.

**NOTE:** This will evaluate any of the four bases as a potential match at that position, thereby preventing that position from having any exclusionary value when compared with other sequences.

9.1.4 Search deletions as “-”.

9.1.5 Include all sequence polymorphisms in the searched haplotype.

**NOTE:** Although all polymorphisms are to be included in the search, length variants at nucleotide positions 16193 (HVI), 309 (HVII), and 573 are ignored during the search and will not provide any additional rarity to a frequency result.

## 10 REPORTING MITOCHONDRIAL DNA RESULTS

The Report wording offered in this manual is meant as a guide that can be applied to all cases. Because not all possible case scenarios can be anticipated or addressed individually, the author of a Report will use his/her judgement in applying the principles behind the wording offered here. The intent is that each author will attempt to conform as much as possible to the wording offered here. Grammatical changes and changes to fit a specific scenario more closely are acceptable and expected; however, unnecessary changes to the wording flow as a whole are not acceptable. Consultation with a Supervisor, Section Supervisor, the Program Manager (Technical Leader), and/or the Assistant Technical Leader is advised, if necessary.

### **10.1 Methods**

The applicable Methods Section(s) (as outlined below) will be placed prior to the Results and Interpretations Section in the Certificate of Analysis.

#### DNA ANALYSIS METHODS:

- Mitochondrial deoxyribonucleic acid (mtDNA) was analyzed using BigDye® Terminator sequencing chemistry.
- Specific primers are utilized for amplifying and sequencing the Control Region (D-Loop) of the human mitochondrion, which includes hyper-variable regions I and II (HVI & HVII).
- mtDNA sequence polymorphisms and nucleotide positions are reported as differences from the revised Cambridge Reference Sequence (rCRS).
- A, T, G, C, represent the deoxynucleotides Adenine, Thymine, Guanine, and Cytosine (respectively).

#### STATISTICAL ANALYSIS METHODS:

- The DNA statistics calculated herein used the CODIS mtDNA Database, which generated a statistic based upon the counting method and applied a 95% upper confidence interval.
- The statistical calculation(s) have been performed in accordance with the Scientific Working Group on DNA Analysis Methods (SWGDAM) 2019 Interpretation Guidelines for Mitochondrial DNA Analysis and Departmental procedures.

### **10.2 Results and Interpretations**

The Results and Interpretations Section will contain the results of all analyses, including comparisons, interpretations, and statistics, as applicable.

#### 10.2.1 Elimination

Different mitochondrial DNA (mtDNA) sequences were developed from EVIDENCE and KNOWN. Therefore, KNOWN INDIVIDUAL is eliminated as the source of the EVIDENCE.

#### 10.2.2 Inconclusive Results

##### 10.2.2.1 Insufficient mtDNA

There was insufficient mitochondrial DNA (mtDNA) present for an mtDNA sequence to be developed from the EVIDENCE/KNOWN.

##### 10.2.2.2 Insufficient length of contiguous sequencing result

The mitochondrial DNA (mtDNA) sequence data developed from EVIDENCE was not of sufficient quality to make a meaningful comparison.

##### 10.2.2.3 Apparent mixture of more than one mtDNA sequence

The mitochondrial DNA (mtDNA) sequence developed from EVIDENCE indicates the presence of a mixture of mtDNA from more than one individual. Mixtures of mtDNA are not interpretable.

- 10.2.2.4 Quality Control (positive control, negative control and/or reagent blank) failure and sample analysis has been exhausted

No reportable mitochondrial DNA (mtDNA) sequence data was developed from EVIDENCE, because a quality control standard was not met.

- 10.2.2.5 The mtDNA sequences differ by only a single nucleotide with no evidence of a common nucleotide.

Mitochondrial DNA (mtDNA) sequences were developed from EVIDENCE and from KNOWN. Due to the one base pair difference observed at position <nucleotide position #>, no conclusion can be reached as to whether KNOWN can be eliminated as the source of the EVIDENCE.

- 10.2.2.6 The mtDNA sequences differ only by the absence of a common length variant at HVII (nucleotide positions 303-309).

Mitochondrial DNA (mtDNA) sequences were developed from EVIDENCE and from KNOWN. Due to the difference of the length of the repeated bases between nucleotide positions 303 to 309, no conclusion can be reached as to whether KNOWN can be eliminated as the source of the EVIDENCE.

#### 10.2.3 Cannot Eliminate

- 10.2.3.1 The mtDNA sequences are concordant

##### Comparison to known reference as the potential source

Mitochondrial DNA (mtDNA) sequences were developed from EVIDENCE and KNOWN. No differences in mtDNA sequence were determined. Therefore, KNOWN INDIVIDUAL and all of their matrilineal relatives cannot be eliminated as a possible source of the EVIDENCE.

##### Comparison to potential relative

Mitochondrial DNA (mtDNA) sequences were developed from EVIDENCE and KNOWN. No differences in mtDNA sequence were determined. This indicates that the EVIDENCE could have originated from a matrilineal relative of KNOWN.

- 10.2.3.2 The mtDNA sequences are concordant with evidence of heteroplasmy

Mitochondrial DNA (mtDNA) sequences were developed from EVIDENCE and KNOWN. No differences in mtDNA sequence were determined, with the exception of position <nucleotide position #>. Due to the common base observed at this position, KNOWN INDIVIDUAL and all of their matrilineal relatives cannot be eliminated as a possible source of the EVIDENCE.

#### 10.2.4 Body Identifications

In addition to the results of the comparisons, as detailed above, all Body Identification Certificates will include the following statement:

Investigators are advised to evaluate all associated case information in addition to this information before declaring the identity of the remains.

### **10.3 Statistical Table**

Statistical results are reported for, at a minimum, the US Caucasian, African American, and US Hispanic populations. In addition, results for any other population in which a match is observed will be included.

Statistical results will be reported in a tabular format, referenced as an appendix, and include:

- Database referenced (CODIS vx.x)
- Number of observations of mtDNA sequence
- Number of individuals in the database
- 95% upper bound frequency estimate
  - This will be reported as a probability by inverting the frequency provided in the search result report and will be truncated to 2 significant figures.

**EXAMPLE:** A frequency estimate provided on the search result report as “0.79” is interpreted as 0.79 percent. Therefore, the reported value would be calculated as follows:

$$1/.0079 = 126.58227 \quad \text{Truncate to 120}$$

Report as: 1 in 120 individuals would be expected to share this haplotype.

### **10.4 Termination After Examinations Have Begun**

The mtDNA analysis was terminated/discontinued at the request of INVESTIGATOR/COMMONWEALTH'S ATTORNEY on DATE.

### **10.5 Request for Known Reference Samples**

Mitochondrial DNA comparisons can be conducted following the submission of two buccal (cheek) swabs (or BLOOD SAMPLE/HAIR SAMPLE) from a suspect (or SUSPECT/VICTIM/ELIMINATION SAMPLE) to the Laboratory.

## 11        QUALITY ASSURANCE

The Mitochondrial DNA Section follows the Forensic Biology Procedures Manual QA Program. Specific requirements pertaining to mtDNA may differ slightly, however, and are addressed in this Chapter as well as throughout this manual.

### **11.1 Case File Documentation**

The following is a list of data printouts that will be maintained in the case file:

- mtDNA Profile Validation Report document for single source samples and positive controls
- Variance Detail Report(s) from examiner Sequencher™ project(s), and as necessary, from the second analyst
- Contig overview map(s) from examiner Sequencher™ project(s), and as necessary, the second analyst
- Trace on a page from examiner Sequencher™ project(s) for all data used in contigs
- Raw Data printouts for the negative control and reagent blank associated with sequence fragments assembled in a contig

### **11.2 General Precautions to Ensure Quality**

Area(s) for examination, photography, and microscopy of evidence must be separated in time or space from the extraction and amplification set-up areas.

The area(s) for sample extraction are physically separated from the post- amplification work area(s), and are separated in time or space from the amplification set-up area.

Laminar flow PCR cabinets equipped with UV light sources are utilized for polymerase chain reaction (PCR) set-up.

The post-amplification work areas are separated physically from the rest of the laboratory. Amplification will be performed in this work area as well as all procedures utilizing the amplified product. All equipment and reagents used in this area are prohibited from entering either the extraction or PCR set-up areas without proper decontamination.

Only one case and only one item of evidence from that case will be opened at the same time by an individual analyst. To the maximum extent possible, evidence samples are processed separately and prior to samples of known origin.

Always use aerosol resistant pipette tips when dispensing solutions that contain DNA.

Extraction reagents (except for ProK, PCIAA, n-butanol, DTT, and 5% Chelex) and tubes used for extraction as well as amplification set-up are exposed to UV light prior to use.

Hoods will be cleaned by wiping down the interior with a 10% bleach solution followed by isopropanol, then UV exposure for a minimum of 15 minutes.

Disposable gloves will be used at all times and will be changed often. Always remove gloves when leaving the post-amplification work area to avoid transfer of amplified DNA into other work areas.

A lab coat will be worn at all times in the laboratory. The lab coat worn in the post-amplification work area is not to be worn in other areas of the laboratory. Disposable lab coats may be utilized as well as disposable arm covers.

Face masks and hair nets will be worn during sample examination, extraction, and amplification.

Lot numbers of the reagents used during all steps of sample processing will be documented.

During analysis, the sample(s) and corresponding controls will be handled in the following order: negative control, sample, reagent blank and positive control.

Both light (L) and heavy (H) strand sequencing reactions are performed to provide confirmatory data. Analysis of evidence samples will be conducted to provide the maximum information with the least consumption of the sample.

General laboratory cleaning will be performed on a schedule determined by the supervisor.

### **11.3 Extraction and Amplification Controls**

At each step of the testing procedure standards and controls must be used to evaluate the effectiveness of the testing process and to assure that the procedure is being properly performed.

#### **11.3.1 Reagent Blanks**

A reagent blank is extracted in conjunction with each sample or batch of samples processed. The reagent blank consists of all reagents used in the test process, excluding the sample. It is processed through the entire extraction, amplification, and sequencing procedures with the sample. The reagent blank is subjected to the same dilution as the corresponding sample. Once the reagent blank has been established as acceptable, it is not necessary to re-amplify the reagent blank when re-amplifying/ re-sequencing associated samples unless a different injection time will be used.

#### **11.3.2 Negative Amplification Control**

A negative amplification control must be used with each amplification. This sample contains all reagents used in setting up the reaction and the appropriate amount of ultra-pure water. No DNA sample is added to this control. This control is processed through all post-amplification procedures.

#### **11.3.3 Positive Amplification Control**

An amplification positive control (HL-60) must be used with each amplification. This control is processed through all post-amplification procedures. The correct sequence results for the mtDNA control region of HL-60 are as follows:

16069T  
16193T  
16278T  
16362C  
73G  
150T  
152C  
263G  
295T  
315.1C  
489C

### **11.4 Quality Control of Critical Reagents and Supplies**

All critical reagents and supplies must be quality control tested for accurate, reliable performance prior to being used in the mtDNA Section on casework samples. The results of all quality control tests will be maintained in a properly labeled file. The following identifies critical reagents/kits/supplies used in mtDNA analysis and describes quality control procedures to ensure their suitability for casework.

If several reagents are being checked together, efforts must be made to isolate the reagent responsible for the failure. This can be accomplished by testing each individual new reagent with existing reagents that have previously been deemed suitable for use in casework.

#### 11.4.1 Extraction Critical Reagents

- Extraction Buffer
- Demineralization Buffer
- Proteinase K
- Dithiothreitol (DTT)
- PCIAA (phenol/chloroform/isoamyl-alcohol 25:24:1 v/v)
- n-Butanol

11.4.1.1 Extract a previously run proficiency sample or in-house sample of known sequence.

11.4.1.2 Amplify the sample, along with the associated controls, in HVI using primer set 2.

11.4.1.3 Sequence and analyze the sample, along with the associated controls.

11.4.1.4 Evaluate the sequencing results.

11.4.1.4.1 All samples must generate the expected result.

- If amplification of the sample is unsuccessful or DNA is detected in the reagent blank during sequencing, the sample will be reamplified.
  - If reamplification fails or DNA is detected in the reagent blank for a second time, the sample will be reextracted.
    - If amplification of the second extract fails or DNA is detected in the reagent blank, the reagent being tested is considered unsuitable for casework.

#### 11.4.2 Amplification Critical Reagents

- AmpliTaq<sup>TM</sup> Gold DNA Polymerase
- 10X PCR Buffer
- Deoxynucleotide Triphosphates (dNTPs)
- Bovine Serum Albumin (BSA)

11.4.2.1 Amplify a positive control (HL-60 or other in-house sample of known sequence) and an associated negative control in HVI using primer set 2.

11.4.2.2 Sequence and analyze the samples.

11.4.2.3 Evaluate the sequencing results.

11.4.2.3.1 The samples must generate the expected result.

- If amplification of the positive control is unsuccessful or DNA is detected in the amplification blank during sequencing, the test will be repeated.
  - If the amplification fails or DNA is detected in the amplification blank a second time, the reagent being tested is considered to be unsuitable for casework.
- Positive Control – HL-60

11.4.2.4 Amplify the 20 pg/ $\mu$ L and 50 pg/ $\mu$ L dilutions prepared from stock lots using control region primers F15971 and R599 and/or primer sets along with a negative control.

11.4.2.5 Sequence and analyze the samples at HVI and HVII.

## 11.4.2.6 Evaluate the sequencing results.

11.4.2.6.1 The positive must yield the expected result, or it is deemed unsuitable for use in casework.

- Amplification/Cycle Sequencing Primers

11.4.2.7 Resolubilize in TE buffer to 200 µM and further dilute with ultra-pure water to 10 µM working concentrations.

11.4.2.8 Amplify a positive control (HL-60 or other in-house sample of known sequence) and an associated negative control.

11.4.2.9 Sequence and analyze the samples.

11.4.2.10 Evaluate the sequencing results.

11.4.2.10.1 The control samples must generate the expected results in order for the primers to be deemed suitable for use in casework.

11.4.2.11 Refer to Appendix C for the Primer Dilution procedure.

Aliquots of working solutions will be stored at -20°C to -30°C.

- ExoSAP-IT®

11.4.2.12 Amplify a positive control (HL-60 or other in-house sample of known sequence) along with a negative control in HVI using primer set 2.

11.4.2.13 Subject the control samples to the ExoSAP-IT® procedure.

11.4.2.14 Sequence and analyze the samples.

11.4.2.15 Evaluate the sequencing results.

11.4.2.15.1 The resulting sequences must have low background and peaks of high quality for the ExoSAP-IT® to be deemed suitable for use in casework.

## 11.4.3 Sequencing Critical Reagents

- Sequencing Kits

11.4.3.1 Amplify a positive control (HL-60 or other in-house sample of known sequence) along with a negative control in either HVI or HVII.

11.4.3.2 Sequence and analyze the samples.

11.4.3.3 Evaluate the sequencing results.

11.4.3.3.1 The resulting sequences must have low background and peaks of high quality as well as the expected result for the region sequenced in order for the sequencing kit to be deemed suitable for use in casework.

## 11.5 Reagents and Supplies

Although the mtDNA Section follows the FB PM QA Program in regard to laboratory prepared reagents and solutions, the following are applicable to the mtDNA Section, specifically.

- The in-house expiration date will not be limited by the chemicals/reagents used for the pH of a reagent.
- Graduated cylinders or pipettes closest in capacity to the volume being measured are used for preparing liquid reagents.
- Type I water that has been autoclaved is used for preparing reagents in the laboratory.
- Formulations may be scaled up or down, as necessary to meet laboratory needs.
- All laboratory prepared reagents and solutions are stored in sterile containers.
- Unless otherwise specified, laboratory prepared reagents and solutions expire 1 year from the QC date.
- Molecular Biology grade reagents will be used where appropriate and available.

The following is an alphabetical list of reagents and consumables. Other reagents and supplies used may be listed in the FB PM QA Program.

Other sources than those listed may be used. If other sources are used, Molecular Biology grade reagents will be used where appropriate and available. Manufacturer's recommended expiration dates are followed unless otherwise specified.

### ACTIVATE™ BLEACH SPRAY

Fisher Scientific, 23-640-502 Deardorff Fitzs No.77051FS for starter kits  
23-640-501 Deardorff Fitzs No.77012FS for bleach cartridge refills. Store at room temperature.

### AGAROSE – NuSieve® 3:1

Lonza, Catalog number 50090, 125g, Store at room temperature.

### AMPLITAQ™ GOLD DNA POLYMERASE with GeneAmp® 10X PCR BUFFER I

Applied BioSystems, Catalog number N8080242 (6 pack) or N8080244 (12 pack). Store at -20°C to -30°C.

### ANODE BUFFER CONTAINER

Applied BioSystems, Catalog number 4393925

### BIGDYE® TERMINATOR v1.1 CYCLE SEQUENCING KIT

Applied Biosystems, Catalog number 4337450, 100 reactions. Store at -20°C to -30°C  
Kit components include:

- Ready Reaction Mix
- pGEM® -3ZF(+) double-stranded DNA Control template
- -21 M13 Control Primer (forward)
- BigDye® Terminator v1.1/3.1 Sequencing Buffer (5X) – this buffer is stored at 4°C

### BIOPHOTOMETER CUVETTES

VWR, Catalog number 62111-412

### BLEACH SOLUTION, 10%

- Bring 50ml household bleach to 500mL with water.
- Make fresh each day of use.
- Store at room temperature.

### BOVINE SERUM ALBUMIN (BSA), 0.625 µg/µL

Ambion, Catalog number AM2616, 50mg, Store at -20°C to -30°C.

- In a 50 mL conical tube, combine 250µL of BSA (50mg/mL) and 19.75mL of ultra-pure water.
- Mix thoroughly.
- Aliquot and store at -20°C to -30°C.

**BROMOPHENOL BLUE**

Sigma-Aldrich, Catalog Number B5525, 10g, Store at room temperature

**1-BUTANOL (n-BUTANOL)**

Fisher Scientific, Catalog number A383-1, 1L, Store at 4°C.

**CAPILLARY ARRAY**

Applied Biosystems, Catalog number 4404687, 36cm

**CATHODE BUFFER CONTAINER**

Applied Biosystems, Catalog number 4408256

**CHELEX® 100 RESIN**

Bio-Rad Laboratories, Catalog Number 142-1253, 50g, Store at room temperature.

- Chelex® is made fresh daily.
  - For a 5% solution, add 0.5g Chelex® to 10mL of ultra-pure water.

**DEMINERALIZATION BUFFER, 0.5M EDTA and 1% lauryl sarcosine**

- Slowly add 186.1g of EDTA to 700mL water.
- Stir vigorously on a magnetic stir plate.
- To dissolve the powder, adjust pH to 8.0 ( $\pm 0.2$ ) by adding approximately 20g of NaOH pellets.
- Check the pH and if small adjustments are needed add NaOH solution, as the pH cannot be accurately controlled with pellets.
- Add 10g of lauryl sarcosine and continue stirring.
- Adjust the final volume to 1 L with water.
- Autoclave, allow to cool, and filter through a 0.2 $\mu$ m filter.
- Store at room temperature.
- UV-irradiate before use.

**dGTP BIGDYE® TERMINATOR v1.0 CYCLE SEQUENCING READY REACTION KIT**

Applied Biosystems, Catalog Number 4307175, 100 Reactions, Store at -20°C to -30°C

Kit components include:

- Terminator Ready Reaction Mix
- pGEM® -3ZF(+) double-stranded DNA Control template
- -21 M13 Control Primer (forward)

**DITHIOTHREITOL (DTT), 1M**

Promega, Catalog number V3151, 5g, Store at -20°C to -30°C.

- Prepare a 1M solution by adding 3.86g of DTT to 15 mL of ultra-pure water.
- Mix thoroughly.
- When DTT is completely dissolved, bring up to a final volume of 25 mL with ultra-pure water.
- Filter through a 0.2 $\mu$ m filter to sterilize, aliquot, and store at -20°C to -30°C.

**DNA MARKER XIV**Roche Diagnostics, Catalog Number 11721933001, 200 $\mu$ L, Store at -20°C to -30°C avoid excessive freeze/thaw cycles.**ETHYLENEDIAMINE TETRAACETIC ACID (EDTA), 0.5M**

Sigma-Aldrich, Catalog number E5134-500G, 500g, Store at room temperature.

- Slowly add 186.1 g EDTA to 800 mL water.
- Stir vigorously on a magnetic stirrer.
- To dissolve the powder, adjust pH to 8.0 ( $\pm 0.2$ ) by adding approximately 20g of NaOH pellets.
- Check the pH and if small adjustments are needed add NaOH solution, as the pH cannot be accurately controlled with pellets.
- Adjust the final volume to 1 L with water.

- Autoclave, allow to cool, and then filter through a 0.2µm filter to sterilize.
- Store at room temperature.

**ETHYL ALCOHOL, 200 PROOF**

Aaper Alcohol and Chemical Co., Catalog number 111000200, 1pt, Store at room temperature.

**ExoSAP-IT®**

Applied Biosystems, Catalog number 78200.200.UL, 100 reactions. Store at -20°C to-30°C.

**ExoSAP-IT® DILUTION BUFFER, 50mM Tris, pH 8.0**

- Dissolve 6.06 g of Tris base in 800 mL water.
- Adjust to pH 8.0 by adding drops of concentrated HCL.
- Adjust to final volume of 1 L with water.
- UV-irradiate before use.

**EXTRACTION BUFFER, 7.6 mM TRIS - 100 mM NaCl - 10mM EDTA - 2% SDS**

- To make a 1 L stock solution, add 5.84g NaCl and 1.21g Tris to 500 mL water.
- Adjust pH to 8.0 with HCl.
- Add 50 mL of 20% SDS and 20 mL of 0.5M EDTA.
- Mix well.
- Bring final volume to 1 L with water.
- Autoclave, allow to cool, then filter through a 0.2µm filter to sterilize.
- Store at room temperature.
- UV-irradiate before use.

**GeneAmp® 10X BUFFER I**

Applied Biosystems, Catalog number N8080189, Store at -20°C to-30°C

**GeneAmp® dNTP BLEND, 10mM**

Applied Biosystems, Catalog number N8080260, Store at -20°C to-30°C

**GLYCEROL**

Invitrogen, Catalog number 15514-011, 500ml, Store at room temperature.

**Hi-Di™ FORMAMIDE**

Applied Biosystems, Catalog number 4311320, 25mL, Store at -20°C to-30°C

**HYDROCHLORIC ACID**

Fisher Scientific, Catalog number MHX060313, 500ml, Store at room temperature.

**LOADING BUFFER, 5X**

- Combine 0.01g bromophenol blue, 5 mL glycerol, 2 mL 0.5M EDTA, 3 mL TE and mix well.
- Divide into 1 mL aliquots and store at -20°C to-30°C.
- Does not expire.

**MICROCON® 30**

Merck Millipore, Catalog number MRCFOR030

**N-LAURYLSARCOSINE SODIUM SALT**

MP Biomedicals, Catalog number 190289, 50g, Store at room temperature.

**PERFORMATM DTR FILTRATION CARTRIDGES**

Edge Biosystems, Catalog Number 42453, Store at 4°C

**PHENOL CHLOROFORM ISOAMYL ALCOHOL (PCIAA), 25:24:1 v/v**

Ambion, Catalog number AM9732, 400ml, Store at 4°C.

**PERFORMANCE OPTIMIZED POLYMER - 6 (POP-6)**  
 Applied Biosystems, Catalog Number 4393717, Store at 4°C

**POSITIVE CONTROL DNA (HL-60 cell line)**

American Type Culture Collection (ATCC), Catalog Number CCL-240D, 10µg, Store at -20°C to-30°C.

- Dilute to 20 pg/µL and 50 pg/µL using ultra-pure water.
- Store the dilutions at -20°C. to-30°C.

**PRIMERS**

Integrated DNA Technologies (IDT) or other suitable vendor. Primers are typically HPLC-purified. Lyophilized primers are stored at -20°C to-30°C.

- Each primer is reconstituted from the lyophilized form using TE to achieve a final concentration of 200 µM (200 nanomoles/mL).
- Storage consists of small aliquots at 10 µM (10 nanomoles/mL) concentration diluted with ultra-pure water stored at -20°C. to-30°C.
- Lyophilized primers expire three years from the day of receipt.
- Typically, the vendor lot# will be used for reconstituted reaction ready aliquots.

**2-PROPANOL**

Fisher Scientific, Catalog number A417-4, 4L, store at room temperature.

**PROTEINASE K, 20mg/ml**

Ambion, Catalog number AM2542, 100mg, Store at -20°C to-30°C

- Add 100mg lyophilized Proteinase K to 5 mL of ultra-pure water.
- When completely reconstituted, divide into aliquots, and store at -20°C to-30°C.

**SODIUM CHLORIDE**

Fisher Scientific, Catalog number S671-500, 500g, Store at room temperature.

**SODIUM DODECYL SULFATE (SDS), 20%**

Sigma-Aldrich, Catalog number L4390-500G, 500g, Store at room temperature.

- Slowly dissolve 200 g of electrophoresis-grade (ultra-pure) SDS in 800 mL water.
- Warming may be required to dissolve solids completely.
- Adjust to a final volume of 1 L with water and mix thoroughly.

**SODIUM HYDROXIDE**

Fisher Scientific, Catalog number S318-500, 500g, Store at room temperature.

**SYBR® SAFE DNA GEL STAIN**

Invitrogen, Catalog number S33102, Store at room temperature.

**TERG-A-ZYMET™**

Fisher Scientific, Catalog number NC9435481, 4lbs, Store at room temperature.

**Tris BASE**

Promega, Catalog number H5131, 500g, Store at room temperature.

**Tris-EDTA BUFFER (TE), pH 7.5**

- Dissolve 0.606 g Tris base in 400 mL water and adjust pH to 7.5 with HCL (add HCL slowly as only a small volume is required).
- Add 0.0185 g of EDTA and readjust pH to 7.5 if necessary.
- Bring final volume to 500 mL.
- Autoclave, allow to cool, then filter through a 0.2µm filter to sterilize.
- Store at room temperature.
- UV-irradiate before use.
- TE will expire three years after preparation date.

**ULTRAPURE™ 10X TAE BUFFER**

Invitrogen, Catalog Number 15558-034, 10L, Store at room temperature.

- Prepare a 1X working solution by diluting 1:10 with water.

**ULTRA-PURE WATER**

- Type I water is filtered with 0.2µm filter, autoclaved, and subjected to UV irradiation.
- 15-50 mL conical tube aliquots will be subjected to 15 J/cm<sup>2</sup> exposure time as determined in Appendix B.
- Ultra-Pure water will not expire.

**WHEATON MICRO TISSUE GRINDERS**

Fisher Scientific, Catalog Number 08-414-15B

200µL EXTRA LONG PIPETTE TIPS  
USA Scientific, Catalog number 1011-0830**11.6 Equipment**

The following is a list of equipment utilized in the mtDNA Section. This list is not meant to be all-inclusive. If parenthetical statement says "e.g.," then equivalent equipment can be used.

- ABI Prism 3500xL Genetic Analyzer
- Autoclave
- Balance
- Biophotometer (e.g., Eppendorf 6131)
- Blender (e.g., Waring Laboratory Blender)
- Compound Light Microscope
- Centrifuges
- Digital Camera w/Microscopy Adapters
- Digital Camera w/UV Transilluminator Adapter (e.g., Fotodyne, Foto/Analyst® Apprentice)
- DC Power Supply
- Electrophoresis Tank
- Flexible Shaft Power Tool (e.g., Foredom Series SR)
- Filter, SYBR Green (e.g., Fotodyne Glass Filter for SYBR® Green Stain)
- Freezers (-20°C,-30°C)
- Heat Block
- Hood - Ductless w/ Particulate Filter (e.g., Purair, Air Science USA)
- Hood - Ductless Fume w/ HEPA filter (e.g., Flow Sciences Inc, model FS2010)
- Hood - Laminar Flow w/ UV light source (e.g., Esco PCR Cabinet)
- Hot Plate Stirrer
- Incubator
- Microcentrifuges
- Microwave Oven
- Nutator
- Orbital Shaker
- Pipettes
- pH Meter
- Refrigerators (4°C)
- Rotary Tool (e.g., Dremel® MultiPro™)
- Ultrasonic cleaner
- Stereomicroscope
- Thermal Cycler (e.g., Applied Biosystems, GeneAmp® System 9700)
- Thermometers, to cover -20°C, 4°C, 50°C, 56°C, 95°C (NIST-traceable)
- Touch Mixers/Vortexes
- UV Crosslinker
- UV Transilluminator

- Vacuum Sample Evaporator Centrifuge (e.g., Savant® ISS110 SpeedVac® Concentrator)

The following is a list of equipment for which the maintenance schedules specific to mtDNA differ (are additional or different) from that listed in the FB PM QA Program.

#### **FREEZERS (-20°C, -30°C):**

Check and record temperatures daily (on work days). If the temperature is greater than -10°C the thermostat will be adjusted to lower the temperature. There will be normal fluctuations in temperatures due to the cycling and use of the freezers. It is expected that freezers will be at or near their set point during periods of non-use. A determination of whether a freezer needs repair is based on a pattern of temperature readings falling outside the limits of the acceptable temperature.

#### **HEAT BLOCKS:**

*Each Use* - Check and record temperature. If the temperature on the thermometer while in the hole of the block designed to hold the thermometer is greater than  $\pm 2^{\circ}\text{C}$  from the specified temperature the thermostat will be adjusted to obtain the correct temperature. If the correct temperature cannot be achieved the heat block will be removed from service until it can be repaired.

#### **HOODS:**

Laminar Flow PCR Cabinets: Annually – Have authorized vendor perform re-certification and any necessary maintenance and repair. UV bulbs are replaced on an annual basis or as needed due to bulb failure. Pre-filters are replaced annually.

Ductless Fume Hood: Annually – Have authorized vendor perform re-certification and any necessary maintenance and repair.

Vented Balance Safety Enclosure: (bone grinding hood): Annually – Have authorized vendor perform re-certification and any necessary maintenance and repair.

#### **INCUBATORS (OVENS):**

*Each Use* - Check and record temperatures. If the temperature is greater than  $\pm 2^{\circ}\text{C}$  from the specified temperature the thermostat will be adjusted to obtain the correct temperature. If the correct temperature cannot be achieved the incubator will be removed from service until it can be repaired.

#### **REFRIGERATORS (4°C):**

Check and record temperatures daily (on work days). The temperature should be between 1-6°C. If not, the thermostat will be adjusted accordingly to bring the temperature into this range. It is expected that refrigerators will be at or near their set points during periods of non-use. A determination of whether a refrigerator needs repair is based on a pattern of temperature readings falling outside the limits of the acceptable temperature range.

#### **ULTRASONIC CLEANER**

Each Day of Use – Ensure the water level is at the correct height.

The ultrasonic cleaner and its basin will be cleaned with a 10% bleach solution followed by a water rinse whenever contamination is thought to have occurred.

## APPENDIX A – HAIR COLLECTION AND EVALUATION

In general all hair examinations should be conducted prior to submission of hair evidence to the Mitochondrial DNA (mtDNA) Section. Hairs previously mounted on microscope slides, typically re-submitted in cold cases, will be forwarded to the Trace Evidence Examiner for de-mounting of the hair(s).

### **REAGENTS, MATERIALS AND EQUIPMENT**

- Bleach, 10% commercial (7 mM sodium hypochlorite solution)
- Cap, Surgical
- Compound microscope
- Digital camera
- Envelopes, glassine
- Forceps
- Gloves
- Isopropanol
- Kimwipes
- Laboratory coat
- Mask, Surgical
- Microscope camera adapters
- Microscope cover slips
- Microscope slides
- Safety glasses
- Stereo microscope
- Ultra-Pure water

### **Special Precautions**

- Disposable gloves, surgical mask, lab coat and surgical cap/hair net will be used.
- Avoid drafts around the examination area.
- Examination work area and forceps will be thoroughly cleaned with fresh 10% bleach. Subsequently, isopropanol or ethanol is used to remove any residual bleach from the surfaces.

### **HAIR COLLECTION**

- If hairs are to be recovered from an item of evidence, use a new, clean piece of paper on the work surface for each item examined.
- Examine the item visually or with the aid of an illuminated magnifier and recover hairs/fibers using one or more of the following methods: forceps, post-it notes, and/or low tack adhesive tape.

**NOTE:** Clear packing tape (high tack tape) can be used to ensure complete recovery of hairs (not fibers), but ONLY for knit articles, such as hats, caps, masks, and scarves. If fibers are probative to the case, high tack tape must not be used.

- As hairs/fibers are being collected, they should be affixed to Post-it type notes and placed in glassine envelopes.
- Place the untested hairs/fibers and remainder of the examined hair(s) in the item packaging when hair evaluation and/or mtDNA analysis is complete.

**NOTE:** If high or low tack tape is used for recovery, do not fold it on itself. It should be placed on clear plastic for return with the evidence. (When a post-it note is used for recovery of hairs/fibers, it can be folded on itself.)

## HAIR EVALUATION

- Hairs may be evaluated with the aid of a stereo microscope and/or by placing a cover slip over the hairs mounted in ultra-pure water on a glass microscope slide, followed by examination with a compound microscope using bright field illumination. When using the stereo microscope, paper/post-it notes or other such material providing varying contrasts with the hairs being examined may be helpful.
- Record the general color (light or dark) and approximate length of the hair and the number of hairs. Distinguish between hairs (those which have roots) and hair fragments (those without roots).
- Hairs which exhibit tissue on the proximal (root end) and/or are in an active growth stage (anagen/catagen) are suitable for nuclear DNA testing and will not be processed for mtDNA.
- Hairs may be grouped based on general appearances (light hairs vs. dark colored hairs) and separated based on this information for screening purposes dependent upon case information.
- Photographic documentation will be made when the hairs or hair fragments will be consumed during mtDNA analysis.

## APPENDIX B – UV STERILIZATION

### UV Lamp Intensity Calibration

Periodically, over the lifespan of the UV bulb the amount of energy delivered will vary. The amount of energy delivered must be estimated so that sufficient irradiation of laboratory equipment will produce a sterilizing effect. This is accomplished by measuring the amount of time the UV equipment takes to deliver 0.9999 J/cm<sup>2</sup> (essentially 1 J/cm<sup>2</sup>).

The energy required to sterilize critical disposables for mtDNA analysis is noted in the table. Based upon the energy output of the UV bulb the required time to obtain the sterilizing effect is calculated and recorded. Ultra-pure water and tube racks are sterilized using the time/energy requirements of conical tubes. Microtissue grinders are sterilized using the time/energy requirements of micro tubes. Blender cups are sterilized using the time/energy requirements of conical tubes. Micro-amp tubes are sterilized using the time/energy requirements of Microcons.

### Materials Required

- Timer
- UV Crosslinker

### Calibration

- Choose 'Energy' mode on the crosslinker and type 9999 into the display and press enter.
- Press start on the crosslinker as you start the timer.
- Measure the length of time (the crosslinker will beep) to deliver 9999 µJ/cm<sup>2</sup>.
- Repeat this measurement for a total of 3 times and enter the times in the table.

The calibration log exists in electronic format with formulas that determine the required times for irradiation based on the calibration (document 212-F101 UV Calibration Worksheets).

## APPENDIX C – PRIMER DILUTION

Primers will be diluted and verified for sequence specificity before being used for casework.

### **REAGENTS, MATERIALS AND EQUIPMENT**

- Pipettes
- Microcentrifuge
- Hood
- Bleach, 10% commercial
- Gloves
- Freezers (-20°C, -30°C)
- Isopropanol
- Kim-wipes
- Laboratory coat
- Racks, tube
- Safety glasses
- Sleeve Protectors
- Water, ultra-pure
- TE Buffer
- Tips, aerosol-resistant (for pipettes)

### **PRIMER DILUTION**

- Resolubilize the lyophilized primer in TE buffer according to manufacturer yield so that the concentration is 200 µM.
- Vortex the resolubilized primer at least 30 seconds.
- Pulse spin the tube.
- Dilute 200 µM stock to 10 µM working concentration with ultra-pure water.
- Freeze aliquots at -20°C.

### **SPECIFICITY DETERMINATION**

Primer specificity is verified by amplifying and sequencing a known mitochondrial sequence (e.g., HL-60).

- Set up amplification using a Negative Control and HL-60 Control DNA.
  - Control Region Amplification
    - 500 pg
  - Primer Set Amplification
    - 200 pg
- Evaluate the amplification products on a product gel
  - Look specifically for a single band, additional banding may indicate non-specific amplification products.
  - Ensure there is sufficient quantity of amplified product for cycle sequencing.
- Cycle sequence the amplified product.
- Primer set primers will also be checked by sequencing Control Region amplification product.
  - Ensure that the sequence matches the HL-60 Control DNA known sequence.
  - Ensure that the sequences do not contain mixtures of DNA sequence.

**APPENDIX D – PROCEDURE FOR DRYING DOWN AND RESOLUBILIZING MITOCHONDRIAL DNA EXTRACTS**  
**REAGENTS, MATERIALS AND EQUIPMENT**

- DNA concentrator/evaporator
- Microcentrifuge
- Pipettes
- Plastic ziploc bags
- Kimwipes
- Ultra-pure water

**PROCEDURE FOR DRYING OF EXTRACTS**

- Open the cap of the microcentrifuge tube and place either the open evidence sample tubes or the reference sample tubes, **BUT NOT BOTH TOGETHER AT THE SAME TIME**, into a DNA concentrator/evaporator until the sample has been dried down. To prevent contamination the open evidence sample tubes are placed into the DNA concentrator/evaporator at a different time or a different DNA concentrator/evaporator than the open reference sample tubes.
- Once the extract has been dried down, close the microcentrifuge cap and store in a plastic ziploc bag or similar storage medium.

**PROCEDURE FOR RESOLUBILIZING EXTRACTS**

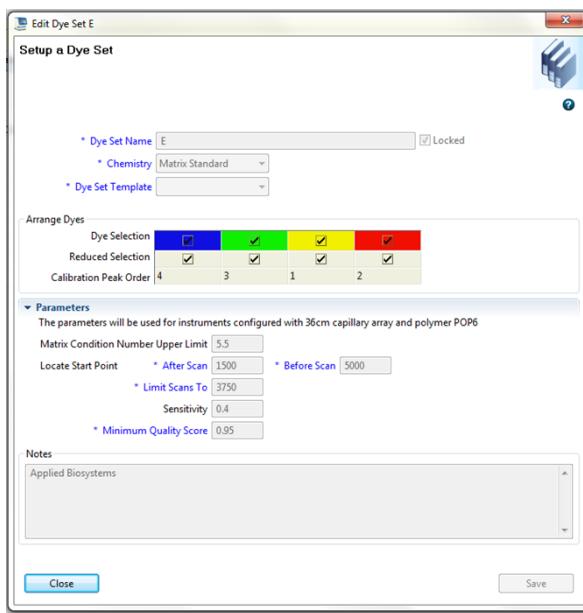
- To resolubilize extracts and reagent blanks, add a volume of ultra-pure water to the microcentrifuge tube that is consistent with the sample volume prior to drying down the extracted DNA. Place the tube in a 56° C incubator or heat block for a minimum of 2 hours, but no more than 3 hours.

## APPENDIX E – INITIAL SETUP OF THE 3500XL AND DATA COLLECTION SOFTWARE

After installation of the 3500 series Data Collection Software for a new 3500xL instrument, a number of customizations must be made. Displayed below are screen captures for settings in use by the 3500xL instruments. These settings may also be exported/imported from an established instrument to a new instrument.

For step by step directions for set up, the user guides (Applied Biosystem's 3500xL Genetic Analyzer User Guide and Applied Biosystems. ABI Prism® BigDye® Terminator v1.1 Cycle Sequencing Kit Protocol) should be consulted.

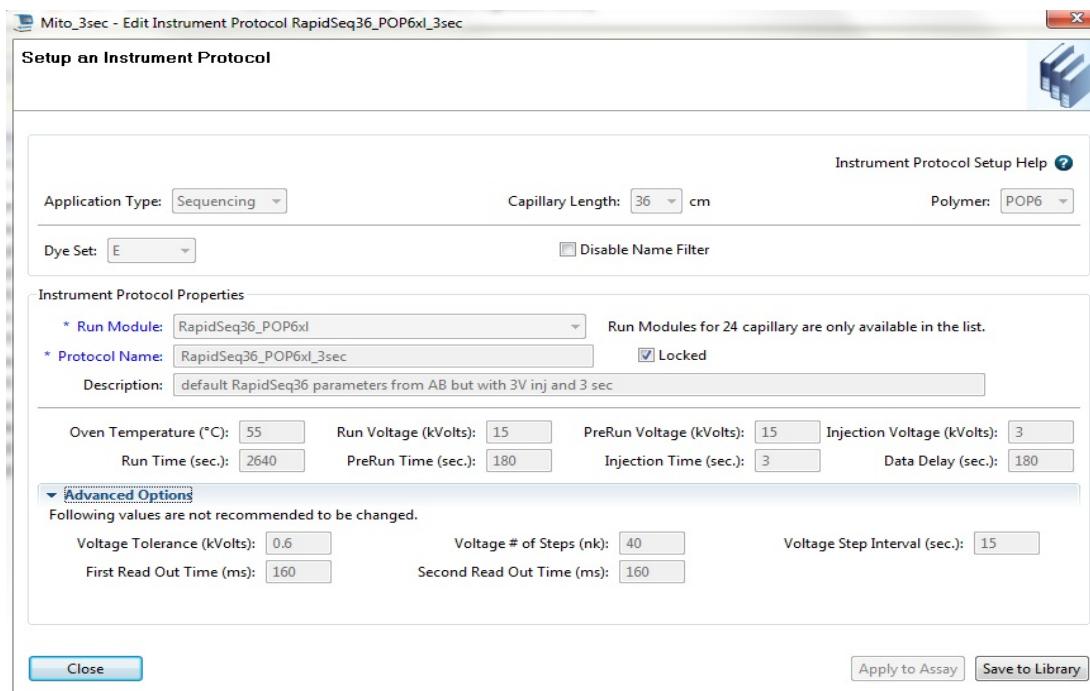
- 1 The figure below shows settings used in creating a dye set for the BigDye® Terminator v1.1 Cycle Sequencing Kit:



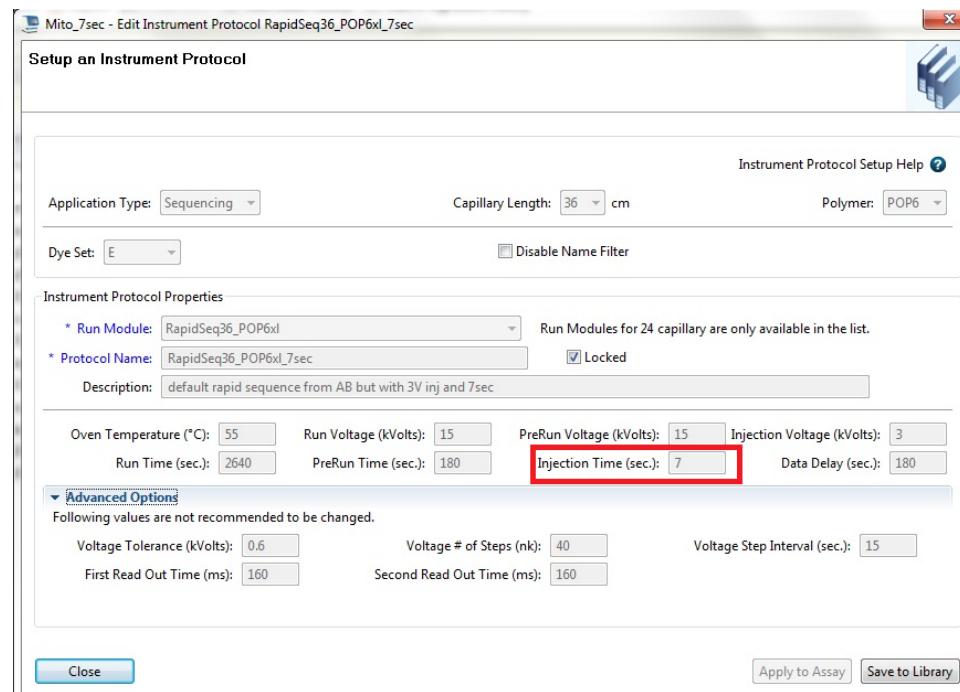
- 2 The figure below shows the Library Resources window of the Data Collection software to access resources to manage settings:

### 3 Creating Instrument Protocols

- 3.1 The figure below shows settings used in creating the “RapidSeq36\_POP6xl\_3sec” Injection Protocol (a 3 second injection protocol).



- 3.2 The figure below shows settings used in creating the “RapidSeq36\_POP6xl\_7sec” Injection Protocol (a 7 second injection protocol, notice all settings are identical except the injection time). Additional injection time change to 7 seconds is used to create the “RapidSeq36\_POP6xl\_15sec” Injection Protocol.



#### 4 Creating Assays

- 4.1 The figures below show the settings used in creating “Mito\_15sec” (15 second assay), which links the basecalling protocol with the instrument protocol. Additional instrument protocols can be added by selecting “Yes” as indicated in the figure.

The image contains two screenshots of the BioNumerics software interface:

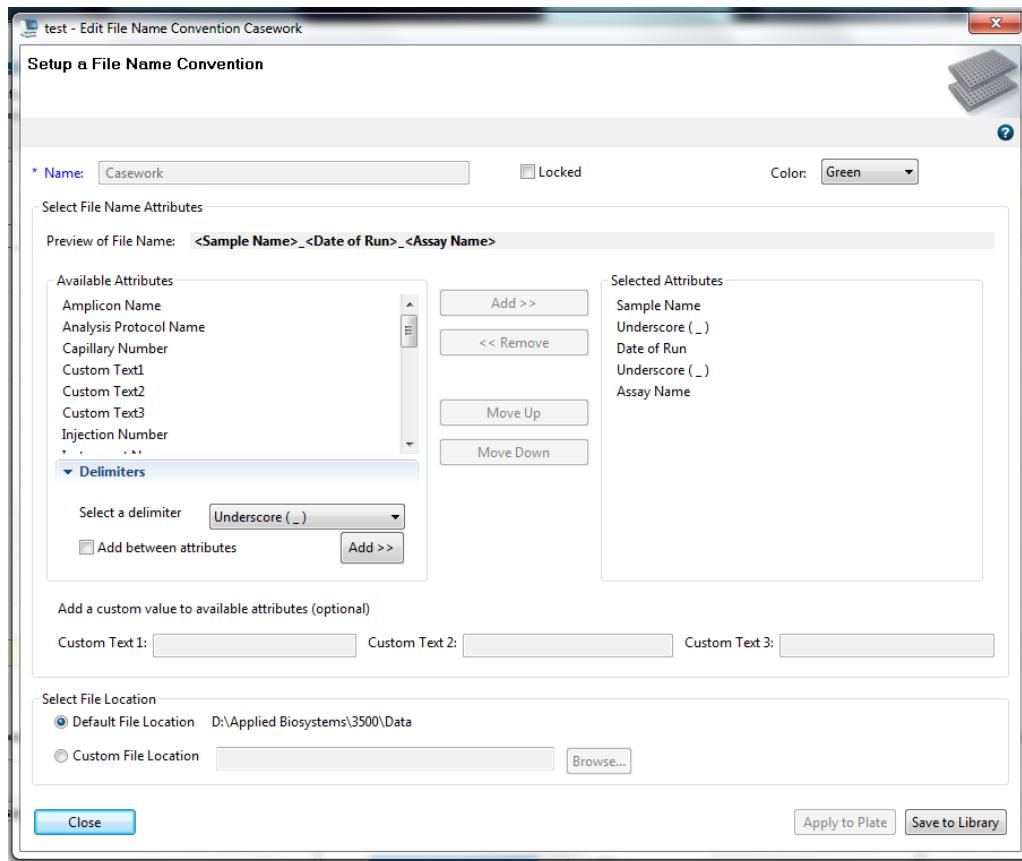
**Screenshot 1: Setup a Basecalling Protocol**

- Protocol Name:** BDTv1.1\_MitoProtocol-POP6
- Description:** (empty)
- Basecaller:** KB 1.4.1
- Analysis Settings Tab:**
  - Mobility File:** KB\_3500\_POP6\_BDTv1
  - Quality Threshold:** Do not assign N's to Basecall
  - Mixed Base Threshold:** Do not use Mixed Base Identification
  - Scaling:** True Profile
  - Clear Range Methods:** Use quality values.
- Buttons:** Close, Apply to Assay, Save to Library

**Screenshot 2: Edit Assay Mito\_15sec**

- Assay Name:** Mito\_15sec
- Locked:**
- Color:** Blue
- Application Type:** Sequencing
- Disable Filters:**
- Protocols Section:**
  - Do you wish to assign multiple instrument protocols to this assay?**  No  Yes (with a red arrow pointing to this radio button)
  - \* Instrument Protocol:** RapidSeq36\_POP6xl\_15sec
  - \* Basecalling Protocol:** BDTv1.1\_MitoProtocol-POP6
- Buttons:** Close, Save

## 4.2 The figure below shows the Casework File Name convention settings:



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