

Department of Forensic Science

COPYRIGHT © 20**20**

**MITOCHONDRIAL DNA SECTION
PROCEDURES MANUAL
OF
FORENSIC SCIENCE**

TABLE OF CONTENTS

- 1 Introduction and Sample Requirements**
 - 1.1 Introduction**
 - 1.2 Case Type Requirements**
 - 1.3 Sample Type Requirements**

- 2 Isolation of DNA**
 - 2.1 Equipment**
 - 2.2 Materials**
 - 2.3 Reagents**
 - 2.4 Chelex® Extraction Method for Reference Bloodstains**
 - 2.5 Chelex® Extraction Method for Reference Buccal Swabs**
 - 2.6 Chelex® Isolation of DNA**
 - 2.7 Organic Extraction Method for Loose Hairs**
 - 2.8 Organic Extraction Method for Bone**
 - 2.9 Organic Extraction Method for Bloodstains, Reference Samples, and Alternate Knowns**

- 3 Mitochondrial DNA Amplification**
 - 3.1 Equipment**
 - 3.2 Materials**
 - 3.3 Reagents**
 - 3.4 Amplification of mtDNA for Control Region and Primer Set Sequencing**

- 4 Mitochondrial DNA Amplification Product Evaluation**
 - 4.1 Equipment**
 - 4.2 Materials**
 - 4.3 Reagents**
 - 4.4 Gel and Buffer Preparation**
 - 4.5 Loading and Running the Gel**
 - 4.6 Evaluation of the Gel Results**

- 5 Purification/Sequencing of Mitochondrial DNA**
 - 5.1 Equipment**
 - 5.2 Materials**
 - 5.3 Reagents**
 - 5.4 Enzymatic Clean Up of PCR Amplification Reaction Product**
 - 5.5 Sequencing mtDNA PCR Product**
 - 5.6 Purification of mtDNA Sequencing PCR Product**

- 6 Capillary Electrophoresis of the Sequencing Product**
 - 6.1 Equipment**
 - 6.2 Materials**
 - 6.3 Reagents**
 - 6.4 General Instrument Operation and Setup**
 - 6.5 Data Management**
 - 6.6 Starting the Run**
 - 6.7 Instrument Maintenance Schedule**

- 7 [Sequence Assembly and Analysis with Sequencher™ Software](#)**
- 7.1 Equipment**
 - 7.2 Printing/Displaying Electropherogram Data**
 - 7.3 Creating a Sequencher™ Project**
 - 7.4 Assembling a Contig**
 - 7.5 Editing Assembled Contigs**
 - 7.6 Creating a Variance Table and Report**
 - 7.7 Exporting Sequence Data for CODIS**
- 8 [Interpretation of Mitochondrial DNA Results](#)**
- 8.1 Sequence Confirmation**
 - 8.2 Evaluation of the Reagent Blank**
 - 8.3 Evaluation of the Negative Control**
 - 8.4 Evaluation of the Positive Control**
 - 8.5 Evaluation and Comparison Between Sample Sequences**
 - 8.6 Missing Persons Cases**
- 9 [Population Statistics – Determination of Haplotype Frequency](#)**
- 9.1 Searching the Database**
- 10 [Reporting Mitochondrial DNA Results](#)**
- 10.1 Methods**
 - 10.2 Results and Interpretations**
 - 10.3 Results Table**
 - 10.4 Statistical Table**
 - 10.5 Termination After Examinations Have Begun**
 - 10.6 Request for Known Reference Samples**
- 11 [Quality Assurance](#)**
- 11.1 Case File Documentation**
 - 11.2 General Precautions to Ensure Quality**
 - 11.3 Extraction and Amplification Controls**
 - 11.4 Quality Control of Critical Reagents and Supplies**
 - 11.5 Reagents and Supplies**
 - 11.6 Equipment**
- Appendix A [Hair Collection and Evaluation](#)**
- Appendix B [UV Sterilization](#)**
- Appendix C [Primer Verification](#)**
- Appendix D [Procedure for Drying Down and Resolubilizing Mitochondrial DNA Extracts](#)**
- Appendix E [References](#)**

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 (mitotype) 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.

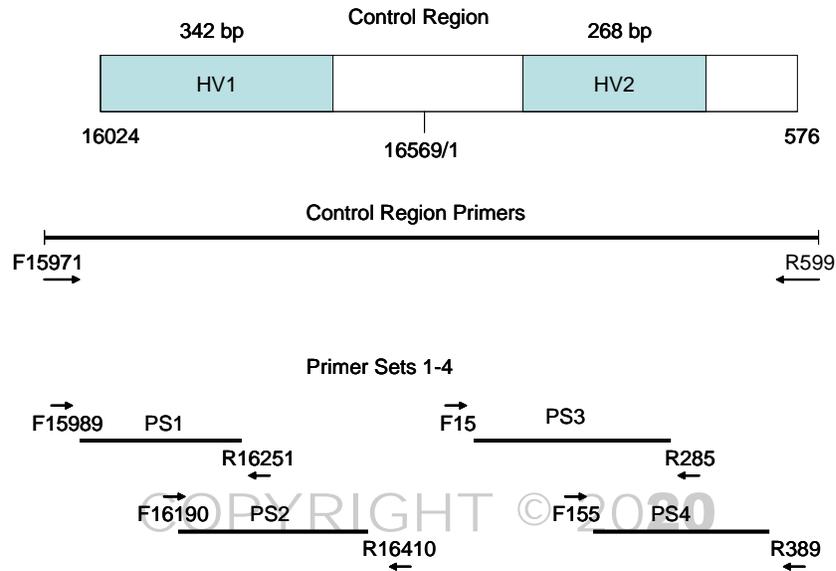


Figure 1 Primer Set Strategy

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, 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

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)

COPYRIGHT © 2020
VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

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.

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.

Evidence Samples

Sample 1
Reagent Blank 1
Sample 2
Reagent Blank 2
Sample 3
Reagent Blank 3
Sample 4
Reagent Blank 4

Reference Samples

Known 1
Known 2
Known 3
Known 4
Reagent Blank

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

COPYRIGHT © 2020

VIRGINIA
DEPARTMENT
OF

FORENSIC SCIENCE

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® YM-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[®] YM-30 concentrators.
- 2.7.25 Add 300 μL TE buffer to each sample reservoir of the Microcon[®] YM-30 concentrators.
- 2.7.26 Transfer the lower aqueous layer to the sample reservoir of the Microcon[®] YM-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[®] YM-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[®] YM-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 μ L of retentate to an appropriately labeled sterile tube.

2.8 Organic Extraction Method for Bone

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% Liqinox, 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 uL 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 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 Bloodstains, Reference Samples, and Alternate Knowns

- 2.9.1 Cut an appropriate 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 μ L extraction buffer
 - 10 μ L 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 μ L 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 μ L 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® YM-30 concentrators.
- 2.9.14 Add 100 μ L TE buffer to each sample reservoir of the Microcon® YM-30 concentrators.
- 2.9.15 Transfer the lower aqueous layer to the sample reservoir of the Microcon® YM-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 μ L TE buffer to the sample reservoir of the Microcon® YM-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 μ L TE buffer wash.
- 2.9.21 Add 60 μ L TE buffer to the filter side of the sample reservoir of the Microcon® YM-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 μ L.
- 2.9.26 Transfer the 100 μ L of retentate to an appropriately labeled sterile tube.

COPYRIGHT © 20**20**

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

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

Control Region (CR) F15971/ R599

Primer pairs 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 5 μ L	5 μ L
2.5 mM dNTP's	(n+1) X 4 μ L	4 μ L
0.625 μ g/ μ l BSA	(n+1) X 2 μ L	2 μ L
Forward primer (10 μ M)	(n+1) X 2 μ L	2 μ L
Reverse primer (10 μ M)	(n+1) X 2 μ L	2 μ L
Taq Gold™(5 units/ μ l)	(n+1) X 1 μ L	1 μ L
ultra-pure water	(n+1) X 24 μ L	24 μ L
Total Volume	(n+1) X 40 μL	40 μL

NOTE: n represents the total number of samples

- 3.4.2 Aliquot 40 μ L 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 μ L, ultra-pure water	Amplification Negative Control
1-10 μ L of sample qs to 10 μ L with ultra-pure water	Sample DNA extracts
10 μ L HL60 (200pg /10 μ L primer sets) 10 μ L HL60 (500pg/10 μ L control region)	Amplification Positive Control
1-10 μ L of the Reagent blank qs to 10 μ L with ultra-pure water	Reagent Blank volume is equal to the maximum sample volume added

NOTE: The total reaction volume is 50 μ L 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 μ L	[~DNA] ng/2 μ 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
- Nuseive® 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 Nuseive® 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 Nuseive® 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 ^a	(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

^aAny 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 ^b	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 ^b	Optimum Sample and Positive Control PCR product concentration should be between 5-20 ng total.

^bWhen 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.

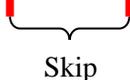
5.5.6 Select and start the following program on the thermal cycler:

- 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 is skipped for reference samples when loading evidence and reference samples onto the same plate for capillary electrophoresis.

	1	2	3	4	5	6	7	8
A	F-Neg	R-Neg			F-Neg	R-Neg		
B	F-S1	R-S1			F-K1	R-K1		
C	F-RB1	R-RB1			F-K2	R-K2		
D	F-S2	R-S2			F-K3	R-K3		
E	F-RB2	R-RB2			F-K4	R-K4		
F	F-S3	R-S3			F-Pos	R-Pos		
G	F-RB3	R-RB3			Blank	Blank		
H	F-Pos	R-Neg			Blank	Blank		



Skip

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 μ L of Hi-Di™ Formamide into any well of the optical plate that contains the dried sequence product.
- 5.6.8 Pipette 10 μ L of Hi-Di™ Formamide into any unused wells of the plate that are in a set of two 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.

COPYRIGHT © 2020

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

6 CAPILLARY ELECTROPHORESIS OF THE SEQUENCING PRODUCT

The Applied Biosystems 3130*xl* Genetic Analyzer is a fluorescent capillary electrophoresis instrument with a 16 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. Sample handling and injection is automated by use of the Autosampler 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 an argon-ion multi-line, single mode laser with primary excitation lines at 488 nm and 514.5 nm to excite fluorescent dyes and a CCD camera records the fluorescence emitted from each of the 16 capillaries. Data analysis including color separation is performed semi-automatically through either the use of Sequence Analysis or Fragment Analysis Software.

Safety Considerations

The Applied Biosystems 3130/3130*xl* Genetic Analyzers use an Argon laser. Under normal operating conditions, the instrument laser is categorized as a Class I laser. The system must be installed and maintained by an Applied Biosystems Technical Representative. 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 is an irritant and may cause skin, eye, and respiratory tract irritation. Gloves are to be worn when handling or cleaning liquid or dried polymer.

6.1 Equipment

- 96-well plate retainer and base
- Applied Biosystems 3130*xl* Genetic Analyzer Data Collection Software
- Applied Biosystems 3130*xl* Genetic Analyzer
- PC Computer
- Pipettes
- Sequencing Analysis Software v5.2 or higher

6.2 Materials

- 50 ml conical tube
- 96-well septum
- Capillary array (36-cm)
- MicroAmp® Optical 96-Well Reaction Plate
- PDP Cleaning kit
- 50 mL conical tube
- Freezer block
- Tips, aerosol-resistant (for pipettes)

6.3 Reagents

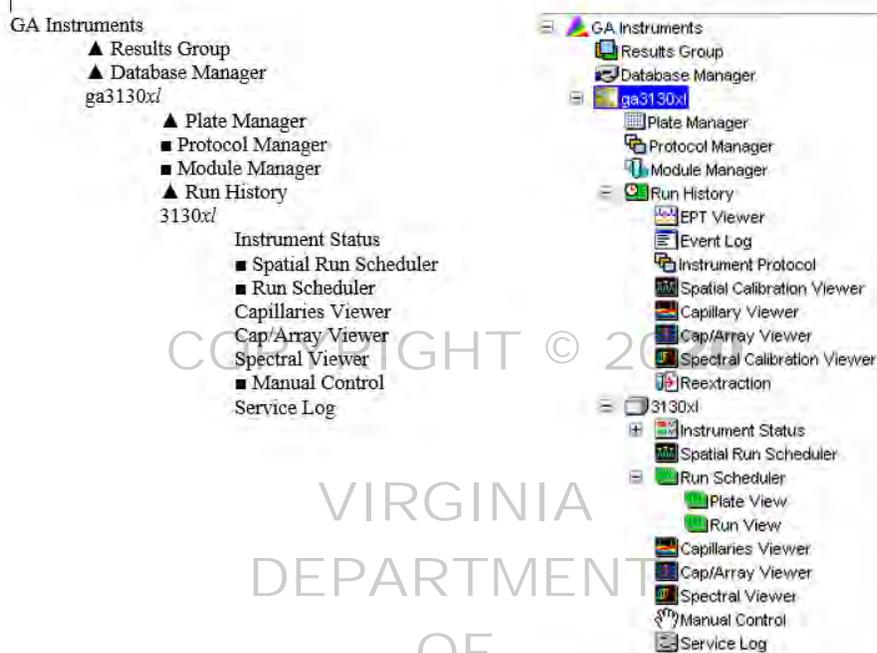
- 10X Genetic Analyzer Buffer with EDTA
- POP-6™ PERFORMANCE OPTIMIZED POLYMER
- Water

6.4 General Instrument Operation and Setup

Ensure that the instrument doors are closed and the green light on the front panel of the instrument is illuminated before opening the 3130 data collection software. The data collection software sets up the instrument for operation, data

collection and management of the database. The software has two basic components Data Management and Instrument Control.

The Tree Pane is used to organize the operations of the instrument. The operations can be categorized into two general groups: Data Management (indicated by ▲) and Instrument Control (indicated by ■).



Wizards are automated instrument processes which allow the user to perform a variety of tasks. Each wizard has been designed with specific instructions to achieve the purpose of the wizard. The wizards are accessed by selecting the desired wizard from the “Wizards” menu and activated when the instrument name is selected in the tree pane.

Available wizards are listed below. See Section 6.8 of this Chapter for the maintenance schedule for the 3130xl.

- Install Array- used to install or replace capillary arrays
- Change Polymer Type- change to a different polymer type (ex. POP-4 to POP-6)
- Replenish Polymer – replace the polymer in the PDP with polymer with the same or different lot number.
- Bubble Remove – remove bubbles (>0.2mm) in the PDP chamber, channels, tubing, as well as the array port
- Water Wash – rinse the PDP chamber, lower polymer block, channels and tubing
- Instrument Shutdown – prepares the instrument for long term storage and removes the capillary array
- Autosampler Calibration – calibrates the positions on the Autosampler
- Update Cap Array Info – correct or update capillary array information

The instrument can also be controlled manually; however, caution should be exercised when controlling the instrument manually as most required user-involved operations have wizards. The most common manual control is to pre-heat the oven to 55°C prior to beginning an electrophoresis run.

6.4.1 Flushing and Filling the Water Trap

6.4.1.1 Fill the supplied 20 mL, all-plastic Luer lock syringe with water and expel any bubbles.

6.4.1.2 Attach the syringe to the forward-facing Luer fitting at the top of the pump block. Hold the fitting with one hand while threading the syringe onto the fitting with the other hand.

6.4.1.3 Open the Luer fitting by grasping the body of the fitting and turning it and the attached syringe approximately one-half turn counterclockwise.

- 6.4.1.4 Open the exit fitting at the top left side of the pump block by turning it approximately one-half turn counterclockwise.
- 6.4.1.5 Hold an empty tube or beaker under the exit fitting to receive approximately 5 mL of waste. Flush the trap by pushing steadily on the syringe plunger.
- 6.4.1.6 Close the fittings in the following order by turning each clockwise until the fittings seal against the block:
- Luer fitting first
Exit fitting second
- 6.4.1.7 Remove the syringe from the Luer fitting. Hold the fitting with one hand while turning the syringe counterclockwise with the other hand.

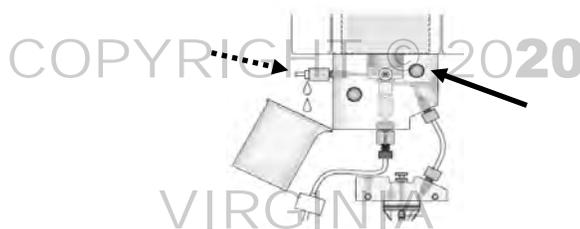
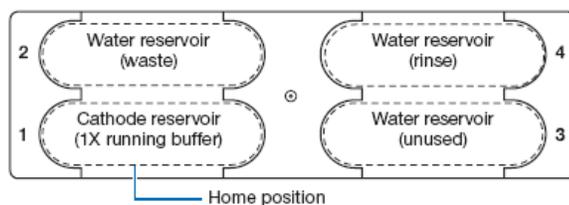


Diagram of the pump block for flushing the water trap.
The solid arrow points to the luer fitting.
The dashed arrow points to the exit fitting.

- 6.4.2 Rinsing and Filling the Buffer and Water Reservoirs
- 6.4.2.1 Ensure the instrument doors are closed.
- 6.4.2.2 Press the TRAY button on the outside of the instrument to bring the Autosampler to the forward position.
- 6.4.2.3 Wait until the Autosampler has stopped moving and then open the instrument doors.
- 6.4.2.4 Remove the anode buffer reservoir by gently pulling down and twisting slightly.
- 6.4.2.5 Remove the cathode buffer reservoir and water reservoirs.
- 6.4.2.6 Discard the solutions into the sink and rinse out the reservoirs with water.
- 6.4.2.7 Dry with a lint-free wipe the cathode and anode reservoirs and then fill the reservoir to the fill line with 1X Genetic Analyzer buffer.
- 6.4.2.8 Fill the water reservoirs to the fill line with water (approximately 17 ml).
- 6.4.2.9 Place a clean septa strip on each reservoir and dry the outside of the reservoirs using a lint-free wipe. Be sure that the septa are dry and fitted flush on the tops of the reservoirs in order to prevent damaging the capillary tips.
- 6.4.2.10 Place the reservoirs into position on the Autosampler as shown below:



6.4.3 Performing a Spatial Calibration

A spatial calibration ensures that the capillary alignment is correct prior to collecting data. It must be performed after each time a capillary array is installed, replaced, or temporarily removed from the detection block, or when the instrument is moved.

6.4.3.1 Select the Spatial Run Scheduler from the 3130x/ menu.

6.4.3.2 In the Spatial Protocol section, select one of the following:

- **3130SpatialNoFill_1** (select if the capillary array contains fresh polymer)
- **3130SpatialFill_1** (select if the capillary array does not contain fresh polymer)

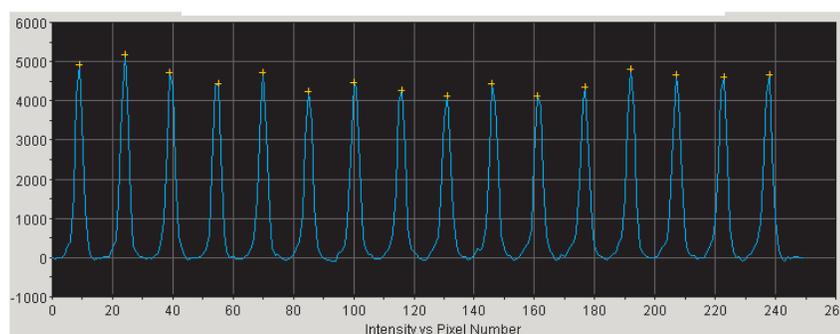
NOTE: It is unnecessary to fill the capillary array each time a spatial calibration is performed.

6.4.3.3 Click Start. The calibration run lasts approximately 2 min (6 min when filling the capillaries as well.)

6.4.3.4 Evaluate the Spatial calibration profile. The following criteria are used to evaluate the data:

Peak Attribute	Criteria
Height	Similar height for all peaks
Orange crosses	One orange crossed marking on the top of every peak. No misplaced crosses.
Shape	Single sharp peak for each capillary. Small shoulders are acceptable.
Spacing	Position values are 13-16 higher than the previous one for every capillary. (Theoretical spacing between capillaries is 15).

Typical Spatial Calibration Profile for 3130x/ system



- 6.4.3.4.1 If the calibration passed and all the peak attributes meet the accepted criteria, click “Accept” to add the calibration data to the database.
- 6.4.3.4.2 If the calibration failed, click reject, and refer to the Applied Biosystems 3130*xl* Genetic Analyzers Maintenance, Troubleshooting and Reference Guide for assistance.

6.4.4 Performing a Spectral Calibration

A spectral calibration must be performed when the capillary array lot number is changed, the laser or CCD camera has been realigned/replaced, or a decrease in spectral separation (pull-up and/or pull down peaks) in the raw or analyzed data is observed.

NOTE: When replacing the capillary array, two to three formamide injections can be run prior to the spectral injection.

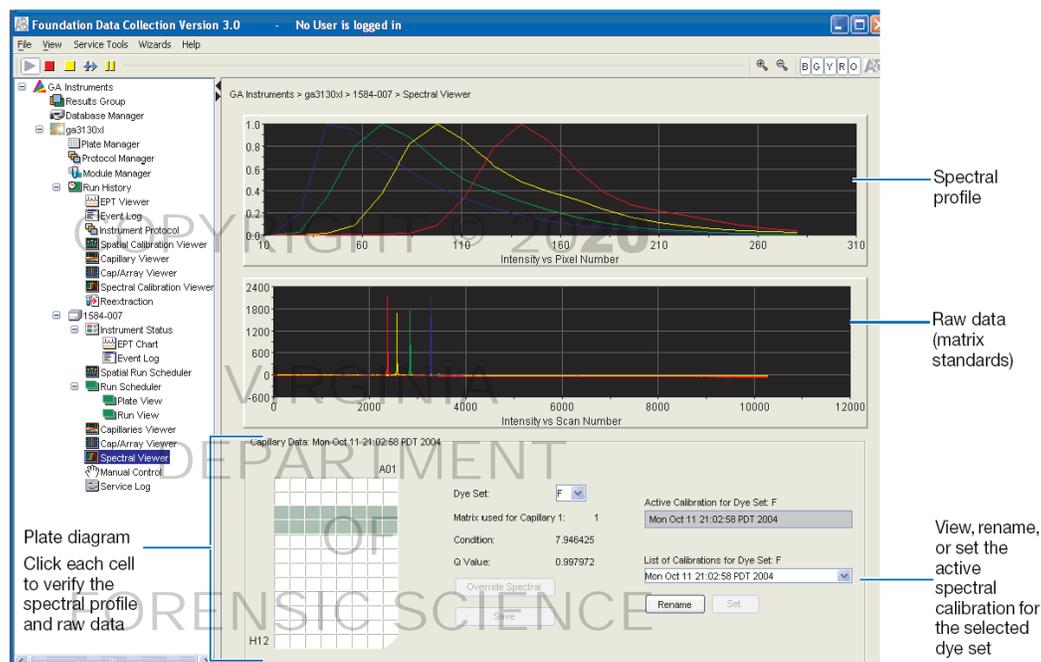
- 6.4.4.1 Add 170µL of Hi-Di™ Formamide to resuspend the Big Dye Terminator v1.1 sequencing standard.
- 6.4.4.2 Thoroughly mix the contents of the sequencing standard tube and spin briefly in a microcentrifuge.
- 6.4.4.3 Dispense 10uL of the sequencing standard into wells A1-H2 of a 96-well reaction plate.
- 6.4.4.4 Cap the wells and denature at 95°C for 2 minutes followed by a brief spin in a microcentrifuge to ensure there are no bubbles in the bottom of the wells.
- 6.4.4.5 Remove caps and assemble the plate assembly as depicted in the diagram for 7.2.3 and load the plate assembly onto the Autosampler.
- 6.4.4.6 Create a new plate for the Spectral Calibration.
 - 6.4.4.6.1 Click on “New” from the Plate Manager on the ga3130*xl* tree pane.
 - 6.4.4.6.2 Enter a name for the plate.
 - 6.4.4.6.3 In the “Application” drop down list, select “Spectral Calibration”.
 - 6.4.4.6.4 Select “96-Well” in the “Plate Type” drop down list.
 - 6.4.4.6.5 Enter the Owner and Operator Names in the corresponding boxes.
 - 6.4.4.6.6 Fill in the sample name column with “BD1.1SizingStandard”.
 - 6.4.4.6.7 Select “Spect36_POP6_1” in the Instrument Protocol 1 drop down box.
 - 6.4.4.6.8 Highlight the entire first row and select “Fill Down Special” from the “Edit” menu.
 - 6.4.4.6.9 Click “OK” to complete and save the plate record.
- 6.4.4.7 Run the Spectral Calibration plate.
 - 6.4.4.7.1 Click on “Plate View” from the Run Scheduler on the ga3130*xl* tree pane.
 - 6.4.4.7.2 Type in the name of the newly created spectral calibration plate and click “Search” or alternatively click “Find All” and find the name of the plate on the list.

6.4.4.7.3 Highlight the plate name and click on the corresponding plate position on the Autosampler indicator to link the plate.

6.4.4.7.4 Click the green button in the toolbar .

6.4.4.7.5 When the Processing Plates dialog box opens, then click “OK”.

6.4.4.8 Evaluate the Spectral Calibration Results.



6.4.4.8.1 Click on “Spectral Viewer” from the ga3130xl tree pane.

6.4.4.8.2 Select the “E-BigDyeV1” from the Dye Set drop-down list.

6.4.4.8.3 Using the plate diagram, select each well on the plate to view the capillary spectral results.

- Passed capillaries appear dark green.
- Failed capillaries appear tan.

6.4.4.8.4 Evaluate the spectral profile and raw data for each capillary.

- Verify the order of the peaks in the spectra profile (blue-green-yellow-red).
- Verify the order of the peaks in the raw data profile (red-yellow-blue-green).
- Verify the peaks in the spectral profile do not contain gross overlap, dips, or irregularities.

6.4.4.8.5 Once all capillaries have passed, ensure the new spectral is active and save it by clicking on “Save”.

6.4.4.8.6 If the capillaries have not passed, the spectral calibration plate will be re-run.

6.4.5 Creating a new Module

Instrument procedures are controlled by the Module Manager. The control of operations such as injection time, oven temperature, voltage during electrophoresis, and data collection interval can be modified.

Modules must be created or modified from the templates provided, but the templates cannot be edited.

- 6.4.5.1 Click on Module Manager in the ga3130xl dropdown list.
- 6.4.5.2 Select the template from the list that most closely resembles the run requirements.
- 6.4.5.3 Click on “New” to open the Run Module Editor Window.
- 6.4.5.4 Fill in the name and select the type of module as well as a template from the dropdown lists.
- 6.4.5.5 Make modifications to the run conditions as necessary to satisfy the run requirements.

Recommended Module Parameters

NOTE: The default 7 second BigDye® Injection Module parameters appear in table. Based on the amount of sequence product and the age of the capillary array, 3 second or 15 second injections may be used instead.

The screenshot shows the 'Run Module Editor' window. The 'Run Module Description' section includes:

- Name: DFS_Rapid36_P6_7second
- Type: REGULAR
- Template: RapidSeq36_POP6
- Description: Standard Injection Module

 The 'Run Module Settings' section contains a table with the following parameters:

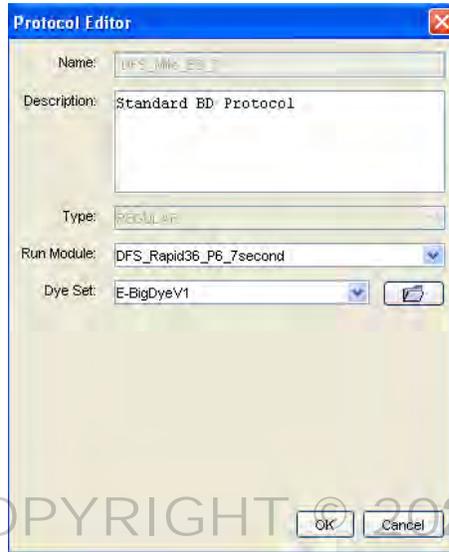
Name	Value	Range
Oven_Temperature	55	18..65 Deg. C
Poly_Fill_Vol	6500	6500..38000 steps
Current_Stability	5.0	0..2000 uAmps
PreRun_Voltage	15.0	0..15 kVolts
Pre_Run_Time	180	1..1000 sec.
Injection_Voltage	3.0	1..15 kVolts
Injection_Time	7	1..600 sec.
Voltage_Number_Of_Steps	40	1..100 nk
Voltage_Step_Interval	15	1..60 sec
Data_Delay_Time	240	1..3600 sec.
Run_Voltage	15.0	0..15 kVolts
Run_Time	2400	300..14000 sec.

- 6.4.5.6 Click “OK” to save the new Module.

6.4.6 Creating a new Protocol

Modules containing the instrument procedures are joined with Auto Analysis procedures and system specific information in the Protocol Manager. A variety of different dye sets for color separation can be used with specific Modules to create different Protocols corresponding with the type of analysis being conducted.

- 6.4.6.1 Click on Protocol Manager in the ga3130xl dropdown list.
- 6.4.6.2 Click on “New” in the upper window to open the Protocol Editor Window.
- 6.4.6.3 Name the Protocol and select the type, a previously created Run Module, and an appropriate Dye Set from the dropdown lists.
 - 6.4.6.3.1 Dye Set “E-BigDyeV1” is used as the default sequencing chemistry.



- 6.4.6.4 Name the Protocol and uncheck the boxes for “Sequence File Parameters”.
- 6.4.6.5 Click on the Base calling tab and select “KB.bcp” as the base caller, as well as a corresponding dye set/primer combination from the dropdown lists.

NOTE: The default Analysis Protocol is “Mitosequencing” and contains the following:

- True/Flat profile (default is True)
- Ending Base & Quality Threshold is not established
- Mixed Base – default is 25% (not selected)
- Clear Range methods – QV default is 4 out of 20 QV < 20

- 6.4.6.6 Click “OK” to save the new protocol.
- 6.4.6.7 Analysis parameters may also be defined, as applicable, in the Sequence Analysis Protocol Editor Window within the Protocol Manager.

6.5 Data Management

NOTE: Some alphanumeric characters are not valid for user names, file names, or sample names. Do not use blank spaces or the following characters: \ / : * ? " < > |

6.5.1 Results Groups

The results of each electrophoresis run are logged and saved in the database. Additionally, the results can be formatted so that information about the sample and the run can be tracked. The data can be saved in formats compatible for analysis in separate analysis programs.

- 6.5.1.1 Create a results group by clicking on “Results Group” in the Tree Pane and click on the “New” button.

OR

Select a previously created results group to modify, click “Duplicate”, and rename the results group.

- 6.5.1.2 Complete the general tab by entering a name for the results group and owner. The comment is not required.

NOTE: For casework, the results group name should consist of the FS Lab number and injection time.

Example: "C08-12345_7sec"

- 6.5.1.3 Select the Analysis tab and click on the Autoanalysis check box.
- 6.5.1.4 Select the Destination tab and choose the location in which the data will be stored by typing in the directory or finding it by using the "Browse" button.
- 6.5.1.5 Select the Naming tab and specify how the sample names will be recorded in the results group under the Sample File Name Format section.
- 6.5.1.5.1 At a minimum the sample name and date of run should be included to differentiate each sample from similar samples on different runs. Casework sample names will include the following:

- FS Lab number
- Item number and type of control
- E followed by the extraction number
- A followed by the amplification number
- Direction of sequencing primer/amplification primer
- Suffix with injection time that matches the results group name injection time

EXAMPLES:

"C08-12345_1a-Hair.E1.A1_F-PS1_7sec_060108"

"C08-12345_1a-HairRB.E1.A1_F-PS1_7sec_060108"

"C08-12345_2-Ref.E1.A1_F-CR3_7sec_060108"

These example names represent the Hair and Reagent Blank for the first extraction and amplification at primer set 1 with the forward sequencing primer and the Reference sample amplified with the control region forward sequencing primer 3.

NOTE: Re-injections with the same injection time are denoted in the full file name with ".2" at the end after the Date of Run.

- 6.5.1.6 The Run Folder Name Format will delineate further where the run information will be saved. Examiners can use the selections within the Run Folder Name Format at their discretion.
- 6.5.1.7 Once the naming is complete, click on "OK" to save the Results Group. This results group will be used during the sample data input in the Plate Manager.

6.5.2 Plate Manager

Sample information is entered into corresponding plate locations along with the individual protocol, results group, and analysis method for each sample loaded into the plate. Each injection group (16 samples corresponding to the 16 capillaries) must have the same instrument protocol, as it represents a single injection. Sample information can be entered manually within the plate manager or imported from an external worksheet. Samples should be organized on the plate in order to maximize efficiency considering the capillaries labeled 1-16 correspond to wells A1-H2, A3-H4, A5-H6, A7-H8, A9-H10, and A11-H12 on the sample plate for each injection. Evidence and reference samples may reside on the same plate, but will be organized such that they are injected separately and that all evidence samples are injected prior to the reference samples. For casework, plate names should include the date of the run.

6.5.2.1 Manual Sample Input

- 6.5.2.1.1 Click on Plate Manager in the ga3130xl dropdown list.
- 6.5.2.1.2 Click “New” at the bottom of the display to open the New Plate Dialog Box.
- 6.5.2.1.3 Complete the information in the New Plate Dialog Box.
 - 6.5.2.1.3.1 For casework, the name of the plate should include the date of the run.
 - 6.5.2.1.3.2 A description is optional.
 - 6.5.2.1.3.3 Select the appropriate sequencing application in the “Application” dropdown list.
 - 6.5.2.1.3.4 Select “96-well” in the “Plate Type” dropdown list.
 - 6.5.2.1.3.5 Type a name or initials for the owner and operator fields.
 - 6.5.2.1.3.6 Click “OK” to open the Sequencing Analysis Plate Editor.
- 6.5.2.1.4 For each sample, complete the following fields.
 - Sample Name – Enter a sample name for each well.
 - Comments (optional) – Enter any comments or notations for the sample.
 - Results Group 1 – Select a Group from the dropdown list or create a new one.
 - Instrument Protocol 1 – Select from the dropdown list.
 - Analysis Protocol 1 – Select from the dropdown list.

NOTE: The priority column automatically populates with a priority of 100. Lowering the number will increase the priority for each set of 16 samples.

To fill in the same sample information for multiple rows more efficiently, highlight the row of interest and select “Edit” and “Fill-Down Special”.

To fill in the same sample information for an entire plate more efficiently, highlight the row of interest and select “Edit” and “Fill Down”.

To add an additional injection of a sample, select “Edit” and “Add Sample Run”. Results Group, Instrument Protocol, and Analysis Protocol columns are added to the right side of the plate record and can be filled in for the sample(s) of interest.

6.5.2.2 Automated Sample Input

- 6.5.2.2.1 The plate data must be created from an outside program such as Microsoft Excel and be in a tab-delimited, .txt, format before it can be imported.
- 6.5.2.2.2 Click on Plate Manager in the ga3130xl dropdown list.

- 6.5.2.2.3 Click “Import” at the bottom of the display to open the “Import Plate” dialog box.
- 6.5.2.2.4 Select the location and file to be imported and click “OK”. The plate will be added to the list of plate records.
- 6.5.2.2.5 Select the plate record and click “Edit” to open the record and ensure the Sample Names, Results Groups, Instrument Protocols, and Analysis Protocols are correct.

6.5.3 Run History

- 6.5.3.1 Accessing and viewing the instrument conditions stored for a previous run
 - 6.5.3.1.1 Click on “Run History” in the ga3130xl dropdown list.
 - 6.5.3.1.2 Select the run of interest from the dropdown list.

6.6 Starting the Run

Two plates may be loaded onto the Autosampler at a time in positions A and B. When loading the Autosampler, ensure the plate is properly positioned in either position A or B. The run scheduler designates which plates are located in which position on the Autosampler. Additionally, each set of injections for each plate is displayed, as well as their priorities. If two plates are loaded into the Autosampler, the first plate linked to its plate map will be the first plate that is injected, independent of whether it is loaded into position A or B (unless the priority column of the plate map has been used to re-prioritize the injections). The plate on the left in the Autosampler is defined as position A and the plate on the right is defined as position B.

- 6.6.1 Ensure the plate(s) have been assembled properly and are placed in their proper location on the Autosampler.
- 6.6.2 Verify the plate information.
- 6.6.3 Click “Run Scheduler” in the tree pane of the Data Collection software to open the Run Scheduler window.
- 6.6.4 Click “Find All” and find the plate map to be linked. Highlight the plate map and the position on the Autosampler picture to link the map to that position.
 - 6.6.4.1 Repeat to indicate the location of a second 96-well plate, if necessary
- 6.6.5 Verify that the active spectral calibration matches the dye set and capillary array length for all scheduled runs.
- 6.6.6 Click the green button  in the toolbar.
- 6.6.7 When the Processing Plates dialog box opens, click “OK”.
 - 6.6.7.1 At this point the software automatically performs a run validation.
 - 6.6.7.1.1 If the validation passed, the run starts, and no further action is needed.
 - 6.6.7.1.2 If the validation fails and the run does not start, check the event log for information.

NOTE: The EPT Chart is a summary of the values of the instrument processes and can be used to assist in troubleshooting.

6.7 Instrument Maintenance Schedule

Day of Use

- Rinse out the respective water reservoirs with water and replace with water to the fill line.
- Prepare 50 mL of 1X running buffer by diluting the 10X stock running buffer, and ensure adequate levels of buffer and water are in the respective reservoirs and the outside of the reservoirs are dry.
- Ensure the plate assemblies are properly assembled and positioned on the deck properly.
- Check for bubbles in the pump block, lower polymer block, interconnect tube, polymer supply tube, and channels. If bubbles are present, remove all bubbles with the Bubble Remove wizard.
- Check the loading-end header to ensure the capillary tips are not crushed or damaged.
- Remove any dried polymer from the capillary tips. Use a lint-free wipe moistened with water.
- Ensure that sufficient volume of polymer is present in the bottle for run.
 - The 3130*xl* uses approximately 50-80 μL of polymer per injection.
- Ensure the pump block and lower polymer block are securely fit on the instrument.
- Ensure the instrument surfaces are clean; always use water to clean.
- Check for leaks around the array knob, interconnecting tube nuts, and check valve.

Weekly

- Flush the water trap.
- Replace the polymer using the Replenish Polymer Wizard.
- Examine the capillary tips and remove dried polymer with a lint free wipe.

Monthly

Run the Water Wash wizard and flush the array port during this wizard.

When Sequence Data is Not of Requisite Quality

- Calibrate the Autosampler when the following symptoms are observed; poor injection results for a small number of capillaries, low signal strength, or no evidence of sample.
- Change the array when the sequence peak morphology and signal strength is no longer of requisite quality to obtain proper sequencing results for multiple capillaries. If a few capillaries demonstrate poor quality results and the remaining capillaries are good, they may be avoided without replacing the entire capillary array.
- Spatial and Spectral Calibrations: A spatial calibration is performed when a new capillary array is installed. A spectral calibration is performed when a new capillary array is installed, the laser or CCD camera has been realigned/replaced or when a decrease in spectral separation is seen in sequence data.

Annually

Have authorized vendor evaluate and perform necessary maintenance and repair.

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
d	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.

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.

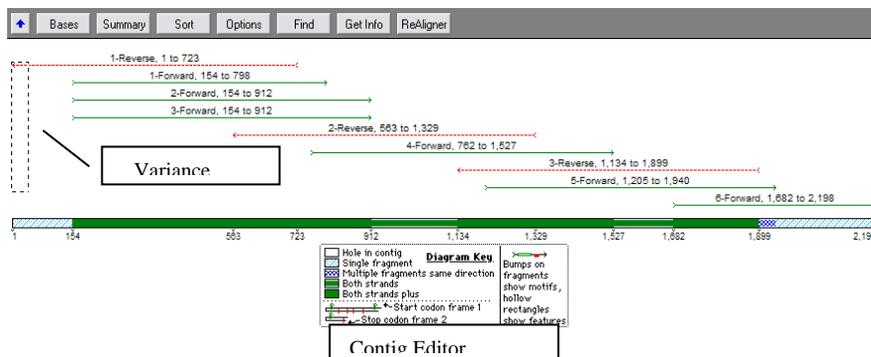
Length heteroplasmy in HVI most commonly arises when there is a substitution of a C for a T at position 16189. The reference type in HVI is C TC₅ TC₄. HVI length heteroplasmy will not be recorded in casework samples. Rather, the sequence reported will be the first 10 cytosines observed beginning at position 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 TC₇ TC₅. A 309.1C insertion in HVII will be annotated C TC₈ TC₅, and the insertions 309.1C, 315.1C will be annotated as C TC₈ TC₆.

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.

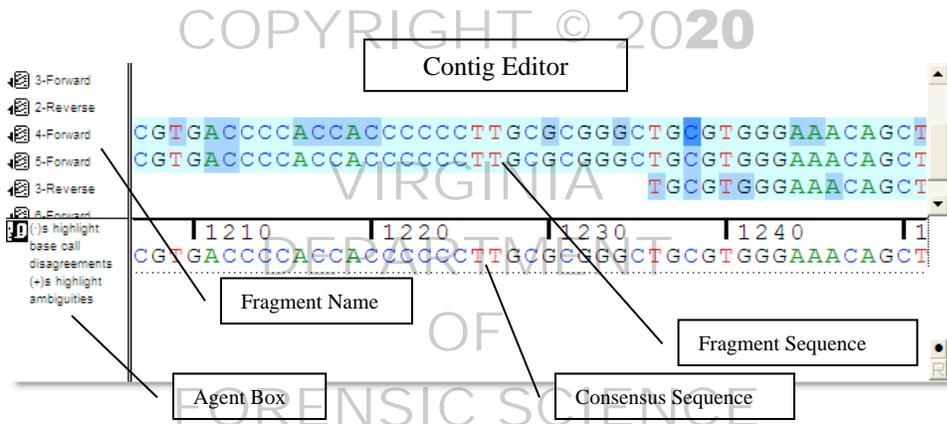
NOTE: Moving the fragments this way may change their alignment to the reference.

- 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.

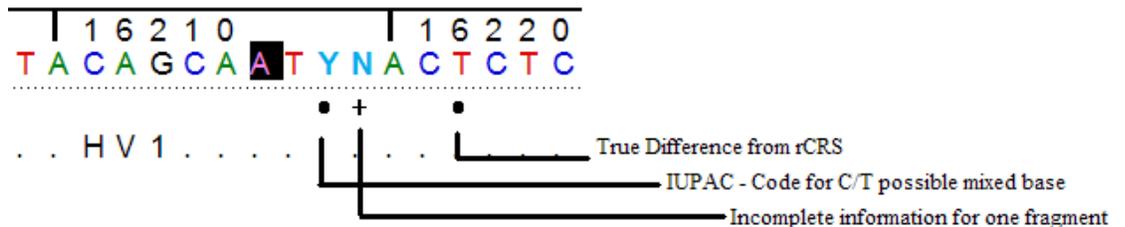


The Agent Box contains descriptive information regarding the sequence and selected areas.

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 C C C T C
T A C A G C A A T C A A C T C T C
T A C A G C A A T C 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.

The screenshot displays the Sequencher software interface with three main windows:

- Variance Table:** A table comparing 10 sequences to a reference sequence. The table has columns for sequence names and positions 16127 through 16474. The reference sequence is CAGCCACCATGAATATGACGGTACCATAAATCCTTGAC. The table shows mismatches between the sequences and the reference.
- Contig Editor:** A window showing the consensus sequence and individual fragment sequences. The consensus sequence is CAGCCACCATGAATATGACGGTACCATAAATCCTTGAC. The fragment sequences are listed on the left, and the consensus sequence is shown on the right.
- Electropherogram Viewer:** A window showing the chromatogram for the selected base. The chromatogram displays peaks for A, C, G, and T. The x-axis represents the sequence position, and the y-axis represents the signal intensity.

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 directly into CODIS.

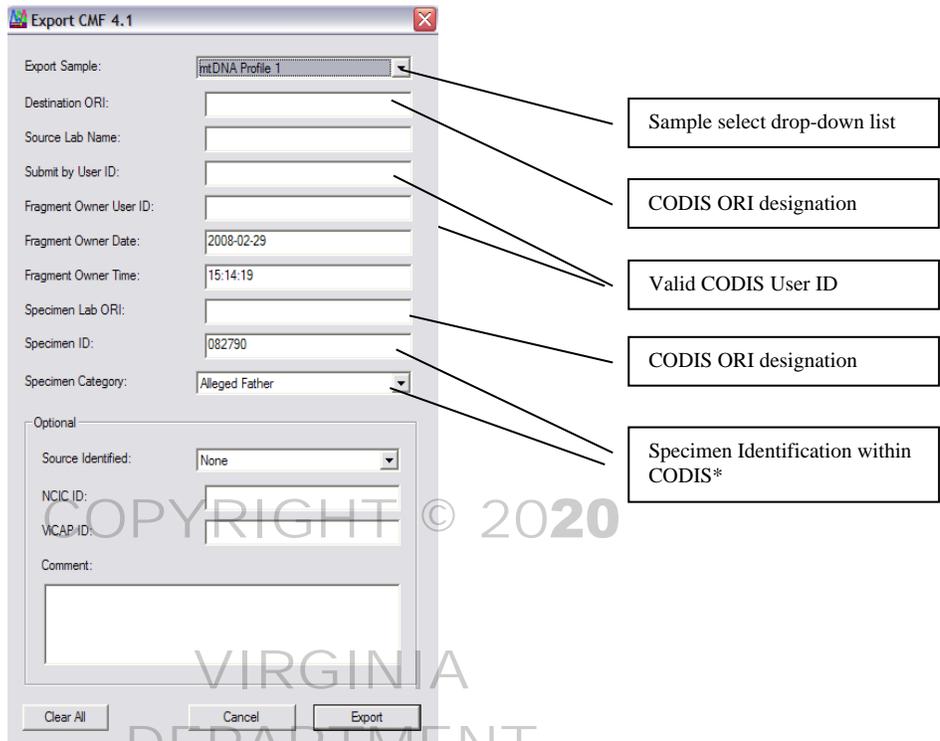
- 7.7.1 Click on “File” and select “Import Sequencher™ Project” to choose the project containing the sequence data.
- 7.7.2 Repeat for the 2nd Sequencher™ project.
- 7.7.3 Ensure at least two contigs for the specimen are highlighted and that “Consensus Inclusively” is selected in the “Contig” menu.
- 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	C	2
⬆	Total	4	2	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.



COPYRIGHT © 2020
VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

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.

COPYRIGHT © 2020

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

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 (SWGDM). 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:

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

α 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.

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 Methods Section (as outlined below) will be placed prior to the Results and Interpretations Section in the Certificate of 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 (np's) are reported as differences from the revised Cambridge Reference Sequence (rCRS).
- A, T, G, C, represent the deoxynucleotides Adenine, Thymine, Guanine, and Cytosine (respectively).

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 obtained 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 obtained from the EVIDENCE/KNOWN.

10.2.2.2 Insufficient length of contiguous sequencing result

The mitochondrial DNA (mtDNA) obtained from EVIDENCE was not sufficient in mtDNA sequence quality to make a meaningful comparison.

10.2.2.3 Apparent mixture of more than one mtDNA sequence

The mitochondrial DNA (mtDNA) sequence obtained 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 obtained 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 obtained 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 obtained 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 obtained 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 obtained 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 heterplasmly

Mitochondrial DNA (mtDNA) sequences were obtained 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 statement:

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

10.3 Results Table

A results table listing the mtDNA sequencing results will be included in all Certificates for which there is a non-elimination.

The mtDNA sequencing results table will be in tabular format, referenced as an appendix, and will include:

- Sample name

- Range of sequencing coverage results
- Nucleotide position(s) of polymorphism(s)
- Difference of sequence with respect to the rCRS
 - Polymorphisms or differences will be reported as defined by IUPAC codes listed in Chapter 7.

Haplotypes from eliminated references are not included in the results table.

10.4 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.

10.5 Termination After Examinations Have Begun

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

10.6 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 buccal, blood, bone or hair sample (depending upon the reagent(s) being quality control checked).

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 The control samples (positive control, negative control, and reagent blank) 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™ Gold DNA Polymerase
- 10X PCR Buffer
- Deoxynucleotide Triphosphates (dNTP's)
- 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 control samples.

11.4.2.3 Evaluate the sequencing results.

11.4.2.3.1 The control 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/μL and 50 pg/μL 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 control 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 μ M and further dilute with ultra-pure water to 10 μ M 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 control 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 Verification 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 control 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 control 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.

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 4315931, 36cm

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 μ 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 μ m filter to sterilize, aliquot, and store at -20°C to -30°C.

DNA MARKER XIV

Roche Diagnostics, Catalog Number 11721933001, 200 μ 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 0.2 μ m 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® YM-30

Fisher Scientific, Catalog number 42410

N-LAURYL SARCOSINE SODIUM SALT

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

PERFORMA™ 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 4363783 or 4352757, 3.5mL and 7.0mL respectively, Store at 4°C

POSITIVE CONTROL DNA (HL-60 cell line)

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

- Dilute to 20 pg/ μ L and 50 pg/ μ L 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 μ M (200 nanomoles/mL).
- Storage consists of small aliquots at 10 μ M (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.

RUNNING BUFFER, 10X

Applied Biosystems, Catalog number 4335613, 500ml. Store at 4°C

- To make the 1X buffer, dilute 5 mL 10X running buffer with 45 mL water.
- Mix thoroughly.

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-ZYME™

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 0.2 μ m 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/cm² 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 3130xl 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.

3130xl AUTOMATED GENETIC ANALYZER:

Spatial and Spectral Calibrations: A spatial calibration is performed when a new capillary array is installed. A spectral calibration is performed when a new capillary array is installed, the laser or CCD camera has been realigned/replaced and when a decrease in spectral separation is seen in sequence data.

Capillary Arrays: New arrays will be installed when sequence data indicates it is necessary.

Annually: Have authorized vendor evaluate and perform necessary maintenance and repair.

Monthly: Perform the “Water Wash” wizard. Flush the array port during the wizard, whether or not bubbles are present in the array port.

Weekly: Perform the “Replenish Polymer” wizard. Flush and fill the Polymer Delivery Pump (PDP) water trap with water to wash out any diluted polymer and clear bubbles. Examine the capillary tips and remove dried polymer with a lint free wipe.

If the 3130xl is taken off line for repair/maintenance, prior to being put back in service, a performance check will be performed. This check will consist of injecting sequencing product from an HL-60 primer set amplification. The resulting sequences must have low background and peaks of high quality. Sequence analysis must yield accurate typing results.

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.

COPYRIGHT © 2020
VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

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

COPYRIGHT © 2020

VIRGINIA
 DEPARTMENT
 OF
 FORENSIC SCIENCE

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.

COPYRIGHT © 2020

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

APPENDIX B – UV STERILIZATIONUV 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/cm² (essentially 1 J/cm²).

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 μ J/cm².
- 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 VERIFICATION

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

REAGENTS, MATERIALS AND EQUIPMENT

BioPhotometer 6131
 Cuvette, UVette® 50-2000µl UV-VIS
 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)

CONCENTRATION DETERMINATION

Primer concentration is verified by UV absorbance measurement.

- Resolubilize the lyophilized primer in TE buffer so that the concentration is 200 µM.
- Vortex the resolubilized primer at least 30 seconds.
- Pulse spin the tube.
- Add 50 µL ultra-pure water to a new UVette and make a blank measurement on the BioPhotometer.
- Pipette out the 50 µL ultra-pure water and discard.
- Add 2.5 µL of the 200 µM primer solution to 47.5 µL of ultra-pure water in a new 0.5 ml tube.
- Vortex tube for at least 30 seconds.
- Pulse spin the tube.
- Add the 50 µL sample into the blank UVette.
- Make absorbance measurement using the Oligo setting on the BioPhotometer.
- Select the conversion button and enter 50 µL for the volume and the number of bases in the primer being quantitated.
- Ensure the concentration is 10 µmoles/L.

NOTE: If the measured primer concentration is not 10 µmoles/L the primer concentration can be adjusted accordingly when making aliquots from the 200µM solution. The aliquot concentration will be re-verified prior to specificity determination. Aliquots will be prepared with ultra-pure water.

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.

COPYRIGHT © 2020

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

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 – REFERENCES

- Anderson, S., *et. al.* (1981) Sequence and organization of the human mitochondrial genome. *Nature*, 290: 457-465.
- Andrews, R. M., *et. al.* (1999) Reanalysis and revision of the Cambridge Reference Sequence for human mitochondrial DNA. *Nature Genetics*, 23: 147.
- Applied Biosystems. ABI Prism® BigDye® Terminator v1.1 Cycle Sequencing Kit Protocol, Rev. A (09/2002).
- Applied Biosystems. ABI Prism® dGTP BigDye® Terminator Ready Reaction Kit Protocol, Rev. D (01/2003).
- Applied Biosystems. 3130/3130xl Genetic Analyzers Getting Started Guide, rev B (11/2004).
- Applied Biosystems. 3130/3130xl Genetic Analyzers Maintenance Troubleshooting and Reference Guide, rev C (09/2006).
- Applied Biosystems. 3130/3130xl Genetic Analyzers Using Data Collection v3.0 User Bulletin, rev A (02/2005).
- Bandelt, H.J. and Parson, W. (2008) Consistent treatment of length variants in the human mtDNA control region: a reappraisal. *International Journal of Legal Medicine*, 122:11-21.
- Baubliene, J., *et. al.* (2003) Evaluation of the DNA extraction method from ancient animal bones. *Ekologija*, 1: 8-11.
- Biochemical Nomenclature and Related Documents*, 2nd edition, Portland Press, 1992. Edited C Liébecq. [ISBN 1-85578-005-4].
- Bio-Rad Laboratories. Chelex® 100 and Chelex® 20 Chelating Ion Exchange Resin, Instruction Manual, rev B (1996).
- Budowle, B., *et. al.* (2003) Forensic and mitochondrial DNA: applications, debates, and foundations. *Annual Review of Genomics and Human Genetics*, 4: 119-141.
- Butler, J. M., (2005) *Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers*, 2nd Ed., Elsevier: Burlington, MA, USA.
- Carracedo, A., *et. al.* (2000) DNA Commission of the International Society for Forensic Genetics: Guidelines for mitochondrial DNA typing. *Forensic Science International*, 110: 79-85.
- Clopper, C.J. and E.S. Pearson, (1934) The use of confidence or fiducial intervals illustrated in the case of the binomial. *Biometrika*, 26: 404-413.
- Comey, C. T., *et. al.* (1994) DNA extraction strategies for amplified fragment length polymorphism analysis. *Journal of Forensic Sciences*, 39: 1254-1269.
- Comey, C. T., *et. al.* (1993) PCR amplification and typing of the HLA DQ α gene in forensic samples. *Journal of Forensic Sciences*, 38: 239-249.
- Edson, S. M., *et. al.* (2004) Naming the dead – Confronting the realities of rapid identification of degraded skeletal remains. *Forensic Science Review*, 16: 63-90.
- Fisher, D. L., *et. al.* (1993) Extraction, evaluation, and amplification of DNA from decalcified and undecalcified United States Civil War bone. *Journal of Forensic Sciences*, 38: 60-68.
- Forensic DNA Evidence Interpretation. CRC Press. 2005. ed Buckleton, J, Triggs, CM and Walsh, SJ.
- Gabriel, M., *et. al.* (2001) Improved mtDNA sequence analysis of forensic remains using a ‘mini-primer set’ amplification strategy. *Journal of Forensic Sciences*, 46: 247-253.

- Gene Codes Corporation. Sequencher™ 4.8 User Manual for Windows (2007).
- Hawes, J., (2006) Evaluation of methods for sequence analysis of highly repetitive DNA templates. *Journal of Biomolecular Techniques*, 17:138-144.
- Hochmeister, M., et al. (1991) Typing of deoxyribonucleic acid (DNA) extracted from compact bone from human remains. *Journal of Forensic Sciences*, 36: 1649-1661.
- Holland, M.M., et al. (1993) Mitochondrial DNA sequence analysis of human skeletal remains: identification of remains from the Vietnam War. *Journal of Forensic Sciences*, 38: 542-553.
- Holland, M., et al. (1999) Mitochondrial DNA sequence analysis - Validation and use for forensic casework. *Forensic Science Review*, 11: 22-50.
- Kreader, C., (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 Gene 32 protein. *Applied Environmental Microbiology*, 6: 1102–1106.
- Levin, B. C., et al. (1999) A human mitochondrial DNA standard reference material for quality control in forensic identification, medical diagnosis, and mutation detection. *Genomics*, 55: 135-146.
- Linch, C., et al. (2001) Human hair histogenesis for the mitochondrial DNA forensic scientist. *Journal of Forensic Sciences*, 46: 844-853.
- Loreille, O. M., et al. (2007) High efficiency DNA extraction from bone by total demineralization. *Forensic Science International: Genetics*, 1: 191-195.
- Millipore Corporation. Amicon® Ultra-4 Centrifugal Filter Devices User Guide, rev A (04/2007).
- Millipore Corporation. Microcon® Centrifugal Filter Devices User Guide, rev M (06/2005).
- Melton, T., (2004) Mitochondrial DNA heteroplasmy, *Forensic Science Review*, 16: 268-286.
- Monson, K., et al. (2002) The mtDNA population database: An integrated software and database resource for forensic comparison. *Forensic Science Communications*, April 2002, Vol.4: Number 2.
<https://archives.fbi.gov/archives/about-us/lab/forensic-science-communications/fsc/april2002/miller1.htm>
- Nelson, K., et al. (2007) Forensic mitochondrial DNA analysis of 116 casework skeletal samples. *Journal of Forensic Sciences*, 52: 557-561.
- Parson, W. and Dur, A. (2007) EMPOP- a forensic mtDNA database. *Forensic Sci Int Genet*, 1(2):88-92.
- Polansky, D., Den Hartog, B.K., Elling, J.W., Fisher, C.L., Kepler, R.B., Budowle, B. (2010) Comparison of Mitotyper Rules and Phylogenetic-based mtDNA Nomenclature Systems. *Journal of Forensic Science*, 55(5):1184-1189.
- Pruvost, M., et al. (2007) Freshly excavated fossil bones are best for amplification of ancient DNA. *Proceedings of the National Academy of Sciences of the USA*, 104: 739-744.
- Salamon, M., et al. (2005) Relatively well preserved DNA is present in the crystal aggregates of fossil bones. *Proceedings of the National Academy of Sciences of the USA*, 102: 13783-13788.
- Scientific Working Group on DNA Analysis Methods (SWGDM). (2013) Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories.
http://swgdam.org/SWGDAM%20mtDNA_ Interpretation_Guidelines_APPROVED_073013.pdf
- Scientific Working Group on DNA Analysis Methods (SWGDM). (2019) Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories.
<https://swgdam.org/publications>

- Scientific Working Group on DNA Analysis Methods (SWGDM). (2014) Guidelines for Missing Persons Casework. <http://swgdam.org/publications>
- Scientific Working Group on DNA Analysis Methods (SWGDM), Guidelines for mitochondrial DNA (mtDNA) nucleotide sequence interpretation. *Forensic Science Communications*, April 2003, Volume 5: Number 2. <https://archives.fbi.gov/archives/about-us/lab/forensic-science-communications/fsc/april2003/swgdammitodna.htm>
- Scientific Working Group on DNA Analysis Methods (SWGDM). (2014) Mitochondrial DNA Nomenclature Examples Document. <https://swgdam.org/publications>
- Tamariz, J., *et. al.* (2006) The application of ultraviolet irradiation to exogenous sources of DNA in plasticware and water for the amplification of low copy number DNA. *Journal of Forensic Sciences*, 51: 790-794.
- Tully, W., *et. al.* (2001) Considerations by the European DNA profiling (EDNAP) group on the working practices, nomenclature and interpretation of mitochondrial DNA profiles. *Forensic Science International*, 124: 83-91.
- Walsh, P. S., *et. al.* (1991) Chelex® 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques*, 10: 506-513.
- Wilson, M. R., *et. al.* (1995) Extraction, PCR amplification and sequencing of mitochondrial DNA from human hair shafts. *Biotechniques*, 18: 662-669.
- Wilson, M., *et. al.* (2002) Further discussion of the consistent treatment of length variants in the human mitochondrial DNA control region. *Forensic Science Communications*, October 2002, Volume 4: Number 4. <https://archives.fbi.gov/archives/about-us/lab/forensic-science-communications/fsc/oct2002/wilson.htm>
- Wilson, M., *et. al.* (2002) Recommendations for consistent treatment of length variants in the human mitochondrial DNA control region. *Forensic Science International*, 129: 35-42.
- Wilson, M. R., *et. al.* (1995) Validation of mitochondrial DNA sequencing for forensic casework analysis. *International Journal of Legal Medicine*, 108: 68-74.