

Department of Forensic Science

COPYRIGHT © 2018

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

TABLE OF CONTENTS

- 1 [Introduction and Sample Requirements](#)
 - 2 [DNA Isolation](#)
 - 3 [Mitochondrial DNA Amplification](#)
 - 4 [Mitochondrial DNA Amplification Product Evaluation](#)
 - 5 [Purification / Sequencing of Mitochondrial DNA](#)
 - 6 [Capillary Electrophoresis & Sequence Analysis](#)
 - 7 [Sequence Analysis with Sequencher™](#)
 - 8 [Interpretation of Mitochondrial DNA Results](#)
 - 9 [Population Statistics – Determination of Haplotype Frequency](#)
 - 10 [Reporting Mitochondrial DNA Results](#)
 - 11 [Quality Assurance](#)
 - 12 [Equipment, Supplies and Reagents](#)
- Appendices
- A [Hair Collection and Evaluation](#)
 - B [Centrifuge Speed Check by Tachometer](#)
 - C [Care and Maintenance of Pipettes](#)
 - D [Thermometer Temperature Calibration](#)
 - E [Thermal Cycler 9700 Temperature Calibration Verification, Temperature Non-Uniformity, and System Performance Diagnostic Tests](#)
 - F [UV Sterilization](#)
 - G [Primer Verification](#)
 - H [Procedure for Drying Down and Resolubilizing Mitochondrial DNA Extracts](#)

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.

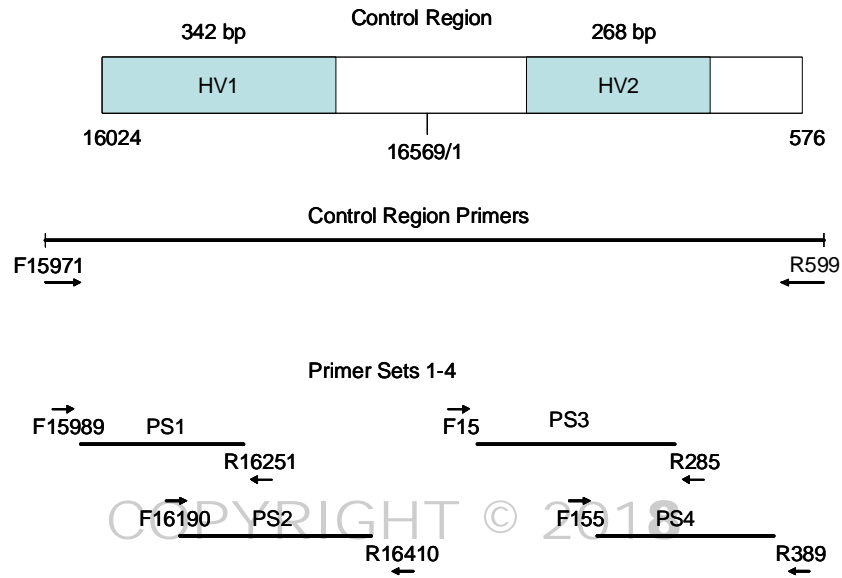


Figure 1 Primer Set Strategy

To the maximum extent possible, evidence samples will be processed completely and consensus sequences determined before reference samples' consensus sequences are determined.

1.2 Case Type Requirements

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

1.2.1 Body identifications

Must submit a reference from the putative maternal relatives and/or alternate known from missing person.

1.2.2 Forensic cases

1.2.2.1 No indication that the sample is from more than one individual (e.g., DNA mixture from mixed body fluids).

1.2.2.2 Evidence samples that have been examined for nuclear DNA and exhibited no result or limited results.

- Actual item of evidence will be re-examined
- Nuclear DNA extracts will not be examined

1.2.2.3 Submission of all references required and includes:

1.2.3 Unidentified human remains

mtDNA analysis can be conducted on bones, hair, and blood/tissue.

1.3 Sample Type Requirements

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

1.3.1 Evidentiary Samples

1.3.1.1 Hairs/hair fragments, including those previously analyzed with nuclear DNA and previously determined to be unsuitable for nuclear testing.

- Any hair examination requests will be conducted prior to submission.
- Nuclear DNA extracts will not be examined.

1.3.1.2 Bones/bone fragments

1.3.1.3 Blood/tissue

1.3.2 Reference Samples

To the maximum extent possible, reference material will be determined to be from a single donor.

1.3.2.1 Buccal swabs

1.3.2.2 Blood sample

1.3.2.3 Hair sample (minimum of 5) may be required for sequence comparison in certain cases

1.3.2.4 Alternate knowns (i.e., toothbrush, razor)

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.

Baubliene, J., *et al.* (2003) Evaluation of the DNA extraction method from ancient animal bones. *Ekologija*, 1: 8-11.

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.

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

Holland, M., *et al.* (1999) Mitochondrial DNA sequence analysis - Validation and use for forensic casework. *Forensic Science Review*, 11: 22-50.

Linch, C., *et al.* (2001) Human hair histogenesis for the mitochondrial DNA forensic scientist. *Journal of Forensic Sciences*, 46: 844-853.

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.

Scientific Working Group on DNA Analysis Methods (SWGDM). (2014) Guidelines for Missing Persons Casework. Available: http://swgdam.org/SWGDAM_Guidelines_for_MP_Casework_APPROVED_01092014.pdf

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.

Wilson, M. R., *et. al.* (1995) Validation of mitochondrial DNA sequencing for forensic casework analysis.
International Journal of Legal Medicine, 108: 68-74.

COPYRIGHT © 2018

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

2 DNA ISOLATION**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, 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 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, a reagent blank is used with each case, alternatively if enough of the reference samples remain for re-analysis a single reagent blank may be used).
- 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

Reagents, Materials And Equipment For General DNA Isolation

Bleach, 10% commercial (7mM sodium hypochlorite solution)
 Boiling Water Bath
 Ethanol
 Forceps
 Freezer, -20°C
 Gloves
 Heat Block
 Hood
 Incubator (56°C)
 Isopropanol

Kim-wipes
 Laboratory coat
 Magnetic Stirring/Hot Plate
 Mask, Surgical
 Microcentrifuge
 Microcentrifuge tubes, screw-top and regular top (1.7 ml to 2.0 ml)
 Pipettes (P-2, P-10, P-20, P-100, P-200, P-1000)
 Racks, tube
 Refrigerator, 4°C
 Safety glasses
 Scalpel
 Scissors
 Sleeve Protectors
 Spin-EASE™ Tubes
 Tips, aerosol-resistant (e.g., for P-10, P-100, P-1000 pipettes)
 Ultraviolet Crosslinker
 Vortex
 Waste containers (general, biohazard, Sharps™)
 Water, ultra-pure
 Weigh Boats

2.1 Chelex® Extractions

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.

Reagents, Materials And Equipment For Chelex® Extractions

Chelex® 100 (Bio-Rad) in ultra-pure water
 Beaker
 Boileezers
 Boiling Water Bath
 Magnetic Stirring/Hot Plate
 Stir Bar

2.1.1 Extraction of DNA from Reference Bloodstains

- Pipette 1.0 ml of ultra-pure water into the appropriately labeled microcentrifuge tubes. A reagent blank must be initiated at this point.
- Add a piece (approximately a 3 mm square) of bloodstained material (including FTA cards) to the tube.
- Vortex at high speed for 10 seconds.
- Incubate at room temperature for a minimum of 15 minutes. (Incubate at 95°C if from FTA card)
- Vortex tubes several times during incubation Centrifuge in microcentrifuge for 3 minutes at approximately 10,000 x g.
- Discard all but 20 - 30 µl of the supernatant. Leave substrate and pelleted material in the tube.

2.1.2 Extraction of DNA from Reference Buccal Swabs

- Using a sterile, disposable scalpel, cut 1/3 of the swab off the applicator stick and place into the lower portion of an appropriately labeled microcentrifuge tube or Spin-EASE™ unit.
- Add 1.0 ml ultra-pure water to the tube. Initiate a reagent blank.
- Vortex at high speed for 10 seconds.
- Incubate at room temperature for 30 minutes.
- Centrifuge in a microcentrifuge for 3-15 minutes at approximately 10,000 x g. Remove and discard top 0.5 ml of supernatant.
- Transfer stain cutting to the basket portion of the Spin-EASE™ tube or similar device. Replace basket in lower portion of the Spin-EASE™ tube.
- Centrifuge for 3 minutes at approximately 10,000 x g. Remove basket, dry and repackage if necessary.
- Alternatively, sterile forceps can be used to remove the cuttings from the liquid in the tube and squeezed to remove the excess fluid from the cuttings. Dry and repackage the cuttings if necessary.
- Remove and discard all but 50 µl of supernatant.

2.1.3 Chelex® Isolation of DNA

- 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.
- Incubate at 56°C for 30 minutes.
- Vortex briefly.
- Incubate in a boiling water bath for 8 minutes.
- Vortex briefly.
- Centrifuge in microcentrifuge for 3 minutes at approximately 10,000 x g.
- Transfer the supernatant from the Chelex® beads to a sterile, appropriately labeled tube and store at -20°C.

2.2 Organic Extractions

Organic extractions are used for extraction of DNA from evidence and reference samples, including alternate knowns.

Reagents, Supplies And Equipment For Organic Extractions

Aluminum Oxide Grinding Stones
Amicon® Ultra-4 Concentrators
Bleach, 10% commercial (7mM sodium hypochlorite solution)
Centrifuge (fixed angle or swinging bucket rotor)
Chisel

Compound microscope
 Demineralization Buffer
 Dremel Tool
 Emery Wheel
 Ethanol, Absolute
 Extraction buffer (10 mM Tris, 100 mM NaCl, 50 mM EDTA, pH 8.0, 0.5% SDS)
 Hammer
 Microcon[®] YM-30 concentrators
 n-Butanol
 Nutator
 Phenol/Chloroform/Isoamyl Alcohol (25:24:1)
 Proteinase K (20 mg/ml)
 Sonicator
 Stereo microscope
 TE Buffer (10 mM Tris, 1mM EDTA, pH 7.5)
 Terg-a-zyme[™]
 Tissue Grinders
 Warring Blender
 Xylene

COPYRIGHT © 2018

2.2.1 Organic Extraction of DNA from Loose Hairs

- Clean micro tissue grinders with 10% bleach, water, and ethanol, in that order. Allow to dry completely before using.
- Irradiate the micro tissue grinders in the UV crosslinker according to Appendix F.
- Carefully remove up to 2 cm of hair shaft material from proximal end of the hair and place in a sterile, appropriately labeled, microcentrifuge 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 regarding collection and evaluation of hairs.)

- Add 1.0 ml of 5% Terg-a-zyme[™] solution and place the tube in the sonicator for approximately 20 minutes.
- Remove the 1.7 ml tube from the sonicator and carefully remove the 5% Terg-a-zyme[™] solution.
- Repeat Terg-a-zyme[™] washes three times.
- Rinse the hair with 1.0 ml of 100% ethanol. Recap tube. Gently agitate several times.
- Remove the 100% ethanol. Rinse the hair with 1.0 ml of ultra-pure water. Recap tube, agitate several times and remove water.
- Prepare reagent blank for each sample as follows: Add 187 μ l of Extraction buffer to micro tissue grinder. Briefly simulate grinding. The reagent blank will be the last specimen processed for the remaining steps.
- Transfer the reagent blank into a labeled 1.7 ml microcentrifuge tube.
- To the same micro tissue grinder add 130 μ l of extraction buffer and place hair(s) in the micro tissue grinder.

- Grind until fragments of hair are no longer visible.
- Transfer the solution into a labeled 1.7 ml microcentrifuge tube.
- Add an additional 57 μ l of extraction buffer to the micro tissue grinder to rinse and transfer it to the 1.7 ml microcentrifuge tube with the sample.
- Add 5 μ l of Proteinase K and 8 μ l of DTT. Vortex and pulse spin.
- Incubate at 56°C for a minimum of 2 hours.
- Add 200 μ l phenol/chloroform/isoamyl alcohol.
- Vortex thoroughly. Centrifuge for 2 minutes at approximately 10,000 x g in a microcentrifuge.
- Transfer upper aqueous layer to a sterile, appropriately labeled microcentrifuge tube. If necessary, repeat extraction with phenol/chloroform/isoamyl alcohol until the interface is clean. Dispose of phenol waste in the appropriate waste container.
- Add 200 μ l n-butanol.
- Vortex thoroughly. Centrifuge 2 minutes at approximately 10,000 x g in a microcentrifuge.
- Remove and discard most of the n-butanol upper layer into the appropriate waste container.
- Label a sufficient number of pre-assembled, irradiated Microcon[®] YM-30 concentrators.
- Add 300 μ l TE buffer to the sample reservoir of the Microcon[®] concentrators.
- Transfer the lower aqueous layer to the sample reservoir of the Microcon[®] concentrators. Avoid pipetting any residual n-butanol.
- Centrifuge column at a maximum of 1,000 x g for 15-30 minutes or until sample has spun through. Discard filtrate.
- Add 300 μ l TE buffer and centrifuge 15-30 minutes at a maximum of 1,000 x g until the buffer has spun through.
- Add 60 μ l of TE buffer to the filter side of each Microcon[®] concentrator.
- Place a retentate cup on the top of each concentrator.
- Briefly vortex the Microcon[®] concentrators with the retentate cups pointing upward.
- Invert each concentrator with its retentate cup and centrifuge in a microcentrifuge at a setting of 10,000 x g for 3 minutes.
- Discard the concentrators. Measure the volume of the retentate with the pipette. Add TE buffer if necessary to bring volume to 100 μ l. Transfer retentate to a sterile, appropriately labeled, microcentrifuge tube.

2.2.2 Organic Extraction of DNA from Bone

- Sand the exposed surfaces of bone with a clean aluminum oxide sanding bit fitted to a rotary tool. Use an Emery Wheel to cut the bone, if necessary. Clean hood appropriately and change the bit, gloves, and disposable sleeves between each specimen.
- Obtain approximately 1.0 g of bone specimen.
- Clean specimen of powdered debris by placing the sanded skeletal fragments into a 50 ml conical tube containing approximately 25 ml of ultra-pure water. Shake the tube back and forth several times. Decant into a waste container. Repeat twice.
- Cover the specimen in the conical tube with 100% ethanol. Shake the tube back and forth several times. Decant into waste container. Repeat twice. Pour fragments into a labeled weigh boat and allow to air dry in the laminar flow hood.

NOTE: Cleaning of the Waring blender cups and lids: Fill cup approximately 1/3 full with 10% Liqinox, attach lid and run blender for 10-20 seconds; remove lid, rinse lid and cup with water, 10% commercial bleach, water, then 95% ethanol. Drain excess ethanol and allow surfaces to dry. Irradiate in UV crosslinker for the same amount of time required for the 50 ml conical tubes.

- Initiate a reagent blank at this time by swabbing the inside surfaces of the Waring blender cup and lid. The reagent blank will be the last specimen processed for the remaining steps.
- Place specimen fragments into a clean Waring Blender cup. Run blender until bone fragments are finely ground. If sample is not completely ground after one minute, shut off blender, tap the container, and repeat.

NOTE: When starting the unit, the bone may become wedged between a blade and the cup wall. If so, shut off power, remove cup and dislodge bone by tapping or rotating the blade spindle from below.

- Pour powder into a clean weigh boat or funnel for transfer to a sterile, appropriately labeled, pre-weighed or tared 15 ml conical tube.
- Determine the weight of the pulverized bone specimen in the conical tube. Approximately 0.2 grams of powdered bone, is needed for extraction.

NOTE: You may stop at this point for the day. Store pulverized bone sample in the -20°C freezer.

- Add 3 ml Demineralization Buffer and 200 µl of Proteinase K to the specimens and the reagent blank. Suspend the bone dust thoroughly in the reagents. Incubate overnight at 56°C on a Nutator.
- Extract with 3 ml phenol/chloroform/isoamyl alcohol.
- Vortex thoroughly. Centrifuge 10 minutes at 10,000 x g using the fixed angle rotor, or at 3270 x g using the swinging bucket rotor centrifuge.
- Transfer upper aqueous layer to a clean labeled tube. Repeat extraction with phenol/chloroform/isoamyl alcohol until the interface is clean. Dispose of phenol waste in the appropriate waste container.
- Extract with 3 ml n-butanol.

- Vortex thoroughly. Centrifuge 10 minutes at 10,000 x g using the fixed angle rotor, or at 3270 x g using the swinging bucket rotor centrifuge. Remove and discard most of the n-butanol upper layer into the appropriate waste container.
- Label a sufficient number of pre-assembled, irradiated, Amicon® Ultra-4 concentrators.
- Transfer the lower aqueous layer to sample reservoir of the corresponding concentrators.

NOTE: Try to avoid pipetting any residual n-butanol.

- Centrifuge approximately 10-30 minutes, at 2000 x g using a swinging bucket rotor. Discard filtrate.
- Add 2 ml of TE Buffer to sample reservoir.
- Centrifuge approximately 10-30 minutes at 2000 x g using a swinging bucket rotor. Discard filtrate. Repeat at least once.
- Pipette extract directly from the sample reservoir and transfer to a sterile, appropriately labeled, microcentrifuge tube. Measure the volume of the retentate with the pipette. Add TE buffer if necessary to bring volume to 100 µl.

NOTE: Take extreme care in not allowing the pipette to touch the sides of the sample reservoir.

- Store samples at 4°C if amplified within 3 weeks or used routinely. Store at -20°C for long-term storage.

NOTE: Extracts may also be used for nuclear short tandem repeat analysis.

2.2.3 Organic Extraction of DNA from Bloodstains, Reference Samples, and Alternate Knowns

- Cut a portion of the stain (3mm² or larger if necessary), swab or sample and place into a sterile, appropriately labeled, microcentrifuge tube. Spin-EASE™ tubes or similar product may be used for this purpose.
- Add 400 µl of extraction buffer to the tube for the sample and reagent blank.
- Add 10 µl of 20 mg/ml proteinase K to each tube. Vortex and pulse spin.
- Incubate at 56°C for a minimum of 2 hours.
- Transfer cutting to the basket portion of the Spin-EASE™ tube or similar device. Replace basket in lower portion of the Spin-EASE™ tube. Centrifuge for 3 minutes at 10,000 x g in a microcentrifuge. Remove basket, dry and repackage if necessary.
- Alternatively, sterile forceps can be used to remove the cuttings from the liquid in the tube and squeezed to remove the excess fluid from the cuttings. Dry and repackage the cuttings if necessary.
- Add 400 µl phenol/chloroform/isoamyl alcohol to extract volume.
- Vortex thoroughly. Centrifuge 2 minutes at 10,000 x g in a microcentrifuge.
- Transfer upper aqueous layer to a sterile, appropriately labeled, microcentrifuge tube.
- Repeat the previous three steps as necessary until the interface is clean.
- Add 400µl n-butanol.

- Vortex thoroughly. Centrifuge 2 minutes at 10,000 x g in a microcentrifuge. Remove and discard most of the n-butanol upper layer into the appropriate waste container.
- Label a sufficient number of pre-assembled, UV irradiated Microcon[®] YM-30 concentrators.
- Add 100 µl TE buffer to the sample reservoir of the Microcon[®] concentrators.
- Transfer the lower aqueous layer to the sample reservoir of the Microcon[®] concentrators. Avoid pipetting any residual n-butanol.
- Centrifuge column at a maximum of 1,000 x g for 15-30 minutes or until sample has spun through. Discard filtrate.
- Add 400 µl TE buffer and centrifuge 15-30 minutes at a maximum of 1,000 x g or until the buffer has spun through. Discard filtrate and repeat 400 µl TE buffer wash.
- Add 60 µl of TE buffer to the filter side of each Microcon[®] concentrator and place a retentate cup on the top of each concentrator.
- Briefly vortex the Microcon[®] concentrators with the retentate cups pointing upward.
- Invert each concentrator with its retentate cup and centrifuge in a microcentrifuge at a setting of 10,000 x g for 3 minutes.
- Discard the concentrators. Measure the volume of the retentate with the pipette. Add TE buffer if necessary to bring volume to 100 µl. Transfer retentate to a sterile, appropriately labeled, microcentrifuge tube.

When the mtDNA analysis is complete, the remaining evidence samples, dried extracted DNA, and associated reagent blanks will be returned to the submitting agency. (Refer to Appendix I for the procedure.) If the biological sample is consumed during the analysis then the dried extracted DNA sample and associated reagent blanks, and when possible the cutting, will be returned to the submitting agency. Amplified DNA product and sequencing product will not be retained or returned to the submitting agency.

DNA extracts from reference samples and their associated reagent blanks do not need to be dried and returned to the submitting agency unless the sample was consumed, a majority of the sample was used to obtain a result the first time, or the sample is degraded and the analyst believes the best chance of obtaining a result in possible future retesting is from the extract.

REFERENCES

Bio-Rad Laboratories. Chelex[®] 100 and Chelex[®] 20 Chelating Ion Exchange Resin, Instruction Manual, rev B (1996).

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.

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.

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.

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)

Nelson, K., *et. al.* (2007) Forensic mitochondrial DNA analysis of 116 casework skeletal samples. *Journal of Forensic Sciences*, 52: 557-561

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.

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

3 MITOCHONDRIAL DNA AMPLIFICATION

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

Special Precautions

- Dedicated lab coats are worn in the mtDNA isolation laboratory, replace at least weekly.
- Personnel entering the mtDNA isolation laboratory will step onto adhesive floor mats, top layer changed when no longer effective.
- The 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.

Reagents, Materials And Equipment

Bleach, 10% commercial (7 mM sodium hypochlorite solution)
 Bovine Serum Albumin (BSA) (0.625 µg/µl), DNA grade
 Deoxynucleotide triphosphate (dNTP) mix, 2.5 mM of each dNTP (dATP, dCTP, dGTP, dTTP)
 Freezer, -20°C
 Gloves
 Kim-wipes
 Laboratory coat
 Laminar flow hood
 Mask (surgical or dust)
 Microcentrifuge
 PCR buffer, 10X (100mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM MgCl₂)
 Pipettes (P-2, P-10, P-20, P-100, P-200, P-1000)
 Positive control DNA (HL-60 or other in-house equivalent)
 Primers, 10 µM (see full listing below)
 Racks, tube
 Refrigerator, 4°C
 Safety glasses
 Sleeves, disposable
 Taq Gold™ DNA Polymerase
 Thermal cycler GeneAmp® PCR Systems 9700
 Tips, aerosol-resistant (e.g., for P-10, P-100, P-1000 pipettes)
 Tubes, microcentrifuge (1.7 ml to 2.0 ml)
 Tubes, thin-walled PCR (0.2 ml)
 Ultraviolet crosslinker
 Waste containers (general, biohazard, Sharps™)
 Water, ultra-pure

General Guidelines

- Amplify evidence 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	

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

Control Region (CR)	F15971/ R599
---------------------	--------------

Primer pairs for samples with low levels of DNA 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.

3.1 Amplification of mtDNA for Control Region and Primer Set Sequencing

- Prepare the master mix in a UV sterile 1.7-2.0 ml tube as follows:

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

n represents the total number of samples.

- Aliquot 40 µl of master mix into UV sterile, pre-labeled thin walled PCR tubes corresponding to the appropriate samples or controls.
- Pipette the following sample and control volumes (as appropriate) to the corresponding PCR tubes one at a time:

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

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

- Vortex the PCR tubes briefly and pulse spin prior to placing the tubes in the thermal cycler.
- 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

REFERENCES

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.

Kreader, C., (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 Gene 32 protein. *Applied Environmental Microbiology*, 6: 1102–1106.

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.

REAGENTS, MATERIALS AND EQUIPMENT

10X TAE buffer
 5X Loading buffer
 Balance
 DC power supply
 DNA MW Marker XIV
 Erlenmeyer or flat-bottom boiling flask
 Fotodyne FOTO/Analyst® Apprentice-UV system (digital camera/gel hood)
 Gel beds
 Gel lane combs
 Gel tank, cover and electrodes
 Gloves
 Graduated Cylinder
 Laboratory coat
 Microtiter plate
 Microwave oven
 Nuseive® 3:1 agarose
 Orbital platform shaker
 Pipettes (P-10, P-20)
 Racks, tube

Safety glasses
SYBR[®] Safe DNA gel stain
Tips, aerosol-resistant (P-10, P-20 pipettes)
Water
Weigh boat
Ziploc bag

4.1 Gel and Buffer Preparation

- Make up day of use at least 600 ml 1X TAE buffer by making a 1:10 dilution of the stock 10X TAE solution to 1X TAE buffer (e.g., 60 ml of stock 10X TAE into 540 ml water for a total of 600ml).
- Each 3% (w/v) gel contains 1.5g Nuseive[®] 3:1 agarose in 50 ml 1X TAE buffer. Calculate the weight and volume required for a maximum of 4 gels.
- 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.
- Add the appropriate amount of Nuseive[®] agarose to the flask slowly while swirling the buffer to avoid clumping of the agarose.
- Place the weigh boat upside down on the top of the flask to use as a lid and heat in the microwave oven. Suggested time for 50 ml gel is 1 minute at 50% power, remove and swirl. Repeat 1 minute at 50% power. Heating time will depend upon the volume and number of flasks, as well as the characteristics of the microwave oven.
- Place on an orbital shaker, and rotate for 5 minutes at 90 rpm. If necessary, return the flask to the microwave and heat for 30-45 seconds to completely dissolve the agarose.
- Pulse spin the SYBR[®] Safe DNA gel stain 10,000X concentrate in microcentrifuge.
- Stop the orbital shaker and add 5 µl of the concentrate per 50 ml of agarose from the stock solution. Restart the orbital shaker.
- Assemble the gel bed(s) in either the casting tray or by placing the gel bed sideways in the electrophoresis tank.
- When the flask is cool enough to handle (approximately 5-10 minutes at room temperature), the agarose can be poured into prepared gel beds.
- Ensure the gel bed is level and pour molten agarose into center of gel bed. Remove any bubbles with the tip of a pipette. Place the comb(s) in position. Allow the gel to solidify for approximately 15 minutes at room temperature. Once the comb(s) are removed, the gel is ready to be used.
- If the gel will not be used the same day of preparation, the gel may be stored. 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.

4.2 Loading and Running the Gel

- Ensure that the gel tank is situated so that the red (positive) electrode is farthest from the loading wells. 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 1mm.
- Prepare the DNA MW Marker XIV by adding 2 µl of 5X loading buffer for each (1:2) standard to be run.

- 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.
- Add 4 µl of the amplified sample to the appropriate well containing 5X loading buffer. Store the remainder of the sample at 4°C.
- Begin each origin with a DNA MW Marker XIV standard, then load the entire amount of sample from the microtiter plate into the designated well of the gel. Continue until all of the samples have been loaded.
- Place the cover on the gel tank. Plug the red (positive) electrode into the positive plug of the power supply. Plug the black (negative) electrode into the negative plug of the power supply.
- Turn on the power supply and set the voltage to 150 volts and run the gel for a minimum of 45 minutes, or until the loading buffer moves approximately 4 cm.
- 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.

- Turn on the UV transilluminator and while using the macro setting on the camera, zoom into the gel to take the photo.
- Turn the UV transilluminator off and dispose of the gel.
- Once the photo is taken, it can be transferred to the computer using the proper cable and photo software.
- The product gel will be used to define and quantify contamination in the analytical procedure. Visible product in the Negative Control and / or the Reagent Blank lanes will necessitate reamplification or reextraction of samples depending on where the contamination is seen.
- Evaluate the quality and quantity of amplified product by comparing the samples and controls to the DNA Molecular Weight Marker XIV.
 - Primer Set amplification products should display a single band between the 200bp and 300bp marker bands.
 - Control Region amplification products should display a single band between the 1000bp and 1500bp marker bands.
 - The concentration of amplified DNA determined from the product gel will be used to determine the volume of input DNA required for Linear Array Hybridization and cycle sequencing.
 - 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.

REAGENTS, MATERIALS AND EQUIPMENT

BigDye® Dilution Buffer
 BigDye® Terminator 1.1 Ready Reaction Mix
 Bleach, 10% commercial (7 mM sodium hypochlorite solution)
 Centrifuge, 96 well micro-plate
 dGTP BigDye® Terminator Ready Reaction Mix
 ExoSAP-IT®
 ExoSAP-IT® Dilution Buffer
 Freezer, -20°C
 Gloves
 Hi-Di™ Formamide
 Kim-wipes
 Laboratory coat
 MicroAMP™ clear adhesive film
 Microcentrifuge
 Optical plate, 96 well
 Performa® DTR Gel Filtration cartridge
 Pipettes (P-10, P-20, P-100, P-200)
 Racks, tube
 Refrigerator, 4°C
 Safety glasses
 Sequencing Primers (10µM – see list below)
 Speedvac concentrator, Savant ISS110
 Thermal cycler GeneAmp® PCR Systems 9700
 Tips, aerosol-resistant (P-10, P-20, P-100, P-200 pipettes)
 Tubes, microcentrifuge (1.7 ml to 2.0 ml)
 Waste containers (general, biohazard, Sharps™)
 Water, ultra-pure

5.1 Enzymatic Cleanup of PCR Amplification Reaction Components.

- Pulse spin PCR product prior to opening tubes.
- Prepare a master mix of ExoSAP-IT® and ExoSAP-IT® dilution buffer as outlined:

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

- Add 20 μl of the master mix to each sample.
- Mix the PCR tubes and pulse spin prior to placing the tubes in the thermal cycler.
- Select the following program from the 9700 thermal cycler:
 - 37°C for 30 min
 - 85°C for 15 min
 - 4°C soak

5.2 Sequencing mtDNA PCR Product

- 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

^a 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'

- Add 7 μl of master mix to a labeled PCR tube.
- Use the product gel DNA estimates to determine the amount of DNA to be sequenced from the samples and controls use the following table as a guide:

NOTE: Performa[®] DTR Gel Filtration cartridge and BigDye[®] XTerminator[™] procedures are equally effective purification processes, the XTerminator may provide some additional ease of handling larger numbers of samples.

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.

^b When sequencing reference samples the maximum volume used for sequencing is 7 μl when sequencing all eight primers at once

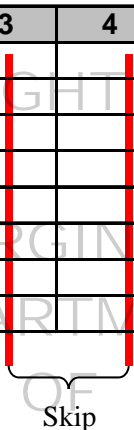
- Add the samples and the controls to the corresponding PCR tubes one a time.
- The total reaction volume is 20 μl per PCR tube.

- Vortex the PCR tubes briefly and pulse spin prior to placing the tubes in the thermal cycler.
- Start the appropriate PCR program listed below:
 - 96°C for 1min
 - 25 cycles of 96°C for 10sec, 50°C for 5sec, 60°C for 4min
 - 4°C soak

5.3 Purification of mtDNA Sequencing PCR Product

When handling samples, ensure they remain in the proper order outlined in General Guidelines in Chapter 3 Mitochondrial DNA Amplification. Ensure a column is skipped for Reference samples when loading Evidence and Reference samples onto the same plate for XTerminator purification and/or 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

5.3.1 Edge Column procedure using individual gel filtration cartridges

- Spin the Performa® DTR Gel Filtration cartridge for 1 minute at 750x g.
- 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.
- Close the cap and spin at 750x g for 2 minutes.
- Remove and discard the cartridge from each tube. Transfer the sample(s) to a 96 well optical plate.
- Spin the plate in a speedvac centrifuge until dry (30-60 minutes).
- Add 10 µl of Hi-Di™ Formamide to the wells of the optical plate that contain the dried sequence product.
- Place a 96 well septa on the plate and vortex to help re-suspend the samples.
- Pulse spin the plate.

5.3.2 Edge Column procedure using 96-well gel filtration plate

- Assemble the Performa® DTR Gel Filtration Plate onto a 96-well microtiter plate waste receptacle and secure edges with tape. Spin for 3 minutes at 850x g with ramp up and ramp down setting level 5 on the Eppendorf 5804 centrifuge.

- Transfer filtration plate onto to 96-well optical plate (ensure to keep the filtration plate level during the transfer). Add the samples to the packed column ensuring that the fluid is placed in the center of the gel.
- Cover with adhesive and spin at 850x g for 5 minutes with ramp up and ramp down setting level 5 on the Eppendorf 5804 centrifuge.
- Remove and discard the filtration plate.

NOTE: The liquid volume in unused well locations on the optical plate may be manually removed before drying.

- Spin the plate in a speedvac centrifuge until dry (30-60 minutes).
- Add 10 µl of Hi-Di™ Formamide to the wells of the optical plate that contain the dried sequence product.
- Place a 96 well septa on the plate and vortex to help re-suspend the samples.
- Pulse spin the plate.

REFERENCES

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).

Hawes, J., (2006) Evaluation of methods for sequence analysis of highly repetitive DNA templates. *Journal of Biomolecular Techniques*, 17:138-144

6 CAPILLARY ELECTROPHORESIS & SEQUENCE ANALYSIS

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.

REAGENTS, MATERIALS AND EQUIPMENT

10X Genetic Analyzer Buffer with EDTA
 50 ml conical tube
 96-well plate retainer and base
 96-well septum
 Applied Biosystems 3130/3130*xl* genetic analyzer Data Collection software
 Applied Biosystems 3130/3130*xl* genetic analyzer
 Capillary array (at least 36-cm)
 MicroAmp® Optical 96-Well Reaction Plate
 PC Computer
 PDP Cleaning kit
 POP-6™ PERFORMANCE OPTIMIZED POLYMER
 Sequencing Analysis Software v5.2 or higher
 Water

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.

Special Precautions

- Performance Optimized Polymer is an irritant, always wear gloves when handling or cleaning liquid or dried polymer. It may cause skin, eye, and respiratory tract irritation.

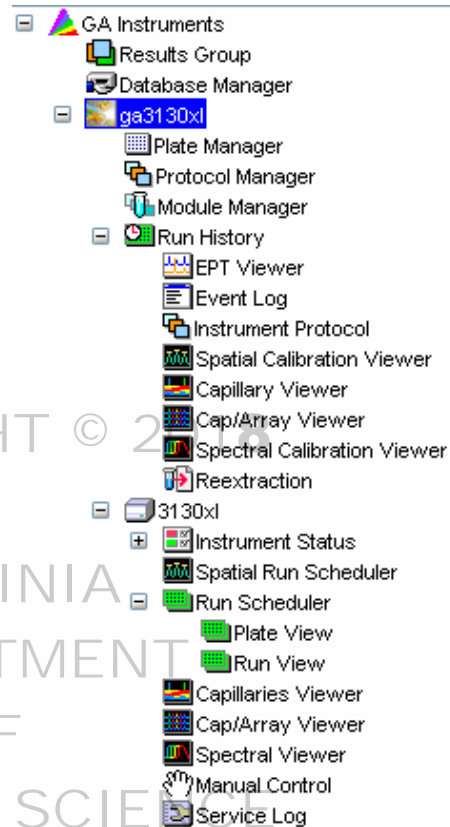
INSTRUMENT OPERATION

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 ■)

GA Instruments

- ▲ Results Group
- ▲ Database Manager
- ga3130xl
 - ▲ Plate Manager
 - Protocol Manager
 - Module Manager
 - ▲ Run History
 - 3130xl
 - Instrument Status
 - Spatial Run Scheduler
 - Run Scheduler
 - Capillaries Viewer
 - Cap/Array Viewer
 - Spectral Viewer
 - Manual Control
 - Service Log



6.1 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: \ / : * ? " < > |

6.1.1 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.
- 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 and click “Duplicate” and rename the results group. Casework should use the Case number and the injection time as the Results Group name.
 - Example: “C08-12345_7sec”
- Complete the general tab by entering a name for the results group and owner, the comment is not required.
- Select the Analysis tab and click on the Autoanalysis check box.
- Select the Destination tab and choose the directory which the data will be stored by typing in the directory or finding it by using the “Browse” button.

- Select the Naming tab and specify how the samples names will be recorded in the results group under the Sample File Name Format section. At a minimum the Sample Name, and Date of Run should be included to differentiate each sample from similar samples on different runs. Casework samples will keep the following naming convention:
 - Sample Names include the following:
 - Case 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 Full File Name:

“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.

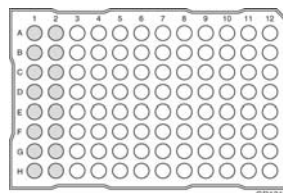
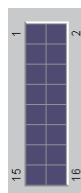
NOTE: Re-injections with the same injection time are denoted in the full file name with “.2” at the end after the Date of Run.

- 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.
- 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.

6.1.2 Plate Manager

Sample information is entered into corresponding plate locations along with the individual protocol, results group, and analysis method for each sample loaded on 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 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.

Capillaries 1-16 equal single injection,
(left diagram)



- Click on the Plate Manager from the ga3130xl dropdown list.

Manual Sample Input

- Click the “New” button at the bottom of the display to open the New Plate Dialog box.
- Complete the information in the New Plate Dialog: Type a name and description (optional) for the plate.
- Select your sequencing application in the “Application” drop-down list.
- Select 96-well in the “Plate Type” drop-down list.
- Type a name for the owner and operator.
- Click “OK” to open the Sequencing Analysis Plate Editor.

For each sample, fill in the following:

- Sample Name, enter a sample name then click the next cell. The Priority column will fill in automatically. Lowering the number (100 is default) for each set of 16 samples increases the priority.
- Comments column, enter any comments or notations for the sample (optional)
- Results Group 1/ Instrument Protocol 1 columns select a group from the drop-down list or create a new one.

Well	Sample Name	Comment	Priority	Results Group 1	Instrument Protocol 1	Analysis Protocol 1
A01						
B01						
C01						
D01						
E01						
F01						

Complete the plate record based on the samples loaded in your plate:

For single runs that use the same samples and protocols

- Highlight the entire row, then select “Edit”, “Fill-Down Special”, the software automatically fills in the appropriate well numbers.

For entire plates that use the same samples and protocols

- Highlight the entire row, then select “Edit”, “Fill Down” to fill down the entire plate editor.
- To do more than one injection of the same sample, select “Edit”, “Add Sample Run”. Results Group, Instrument Protocol and Analysis Protocol columns are added to the right end of the plate record. Complete the columns for the additional runs.
- When finished with the plate, click “OK”.

Automated Sample Input

- The plate data must be created from and outside program such as Microsoft Excel and be in a tab-delimited .txt format before it can be imported.

- Click on the “Import” button on the bottom of the display to open the “Import Plate” dialog box.
- Select the location and file containing the plate data and click on “OK”. The plate will be added to the list of plate records.
- Check the plate record by selecting the plate and clicking “Edit” to ensure the plate samples, results groups, protocols, and analysis methods are correct.

6.1.3 Run History

- Previous electrophoresis runs are stored in the database and are accessed by run and plate name. This allows the user to view the instrument conditions during the run. For casework, plate names should include the case number and the date of the run.
- To view different runs, click on “Run History” from the Tree Pane and select the run from the drop down list. Each parameter available for that run is listed in the Tree Pane just as it was listed in the original run. Re-extraction of the sample data can be selected from the bottom of the Tree Pane.

6.2 Instrument Control

6.2.1 Module Manager

Instrument procedures are controlled by the module manager. The control of operations such as injection time, oven temperature, voltage during electrophoresis, data collection interval and z-axis (not adjustable by user) can be modified. Modules must be created or modified from the templates provided, but the templates cannot be edited.

- To create a new module select the template from the list that most closely resembles the run requirements.
- Click on “New” and a Run Module Editor window appears.

Recommended Module Parameters

The image displays two screenshots of the Run Module Editor dialog box. The left screenshot shows a module named 'DFS_Rapid36_P6_7second' with a description of 'Standard Injection Module' and an injection time of 7 seconds. The right screenshot shows a module named 'BDx-RapidSeq36_POP6_3sec' with a description of 'Standard XTerminator Injection Module' and an injection time of 3 seconds. Both screenshots show a table of parameters with their values and ranges.

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.

- Fill in the name and select the type of module as well as a template from the drop-down list. Make modifications to the run conditions as necessary to satisfy the run requirements.
- Click on “OK” to save the new module.

NOTE: BigDye® XTerminator™ modules (labeled “BDx_”) are specific for the BigDye Purification procedure.

- The default 7 second BigDye® Injection Module parameters appear in table (left side). Based on the amount of sequence product and the age of capillary, 3 second and 15 second injections may be utilized.
- The default 3 second BigDye® XTerminator™ Module injection parameters appear in table (right side). Based on the amount of sequence product and the age of capillary, 1 second and 7 second injections may be utilized.

6.2.2 Protocol Manager

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.

- Select the Protocol Manager from the Tree Pane.

Instrument Parameters

- Click on the “New” button in the upper window and a Protocol Editor window appears.

Recommended Instrument Protocol Parameters

The screenshot shows a 'Protocol Editor' dialog box with the following fields and values:

- Name: UFS_Mic_EB1
- Description: Standard BD Protocol
- Type: REGULAR
- Run Module: DFS_Rapid36_P6_7second
- Dye Set: E-BigDyeV1

Buttons: OK, Cancel

- Name the protocol and select the type of protocol run module and dye set from the drop down lists to correspond with the desired protocol. (Dye set E-BigDyeV1 is used for default sequencing chemistry.)
- Default BigDye® Instrument Protocol parameters appear in table (above).

Analysis Parameters

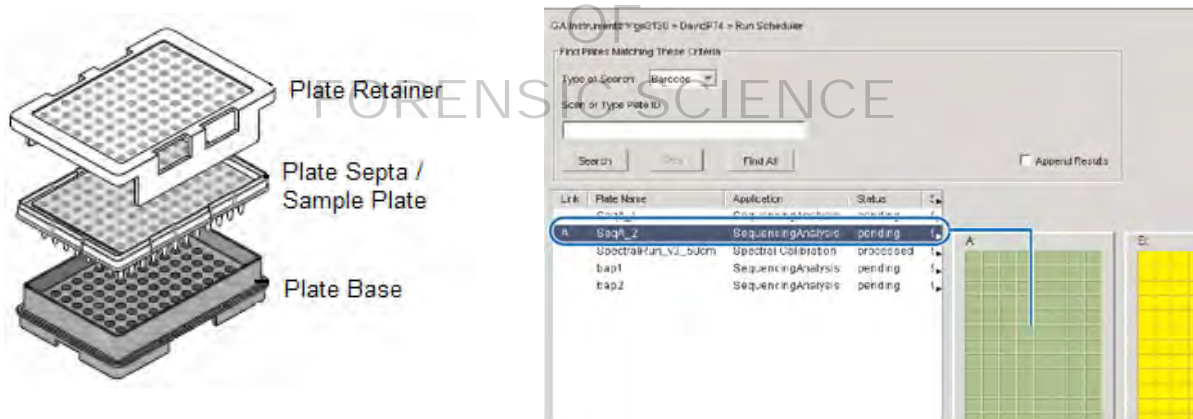
- Click on the “New” button in the lower window and a Sequence Analysis Protocol Editor window appears.
- Name the protocol and uncheck the boxes for “Sequence File Parameters”.
- Click on the Basecalling tab and select “KB.bcp” as the base caller as well as a corresponding dye set/primer combination from their drop down boxes.
- Click “OK” to save the new protocol.

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

6.2.3 Run Scheduler

Two plates can be loaded onto the Autosampler at a time in positions A or B. The run scheduler designates which plates are located in what position on the Autosampler. Additionally, each set of injections for each plate is also displayed as well as their priorities with relation to one another is displayed. When loading the Autosampler, ensure the plate assembly is properly positioned.



6.3 Instrument Setup And Maintenance

6.3.1 Maintenance Schedule

Day of Use

- Rinse out with water, the respective water reservoirs and replace with water to the fill line.
- Prepare 50ml 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.

- 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.
- Restart the computer and instrument.

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 and when a decrease in spectral separation is seen in sequence data.

Annually

- Have authorized vendor evaluate and perform necessary maintenance and repair.

6.3.2 Maintenance Procedures

Wizards

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, activated when the instrument name is selected in the tree pane.

- 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

Flushing and Filling the Water Trap

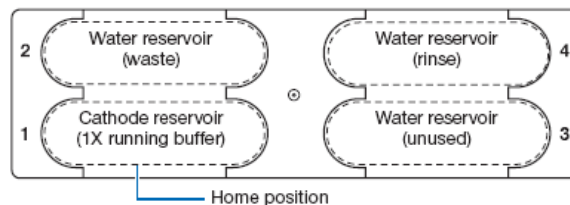
The water trap in the Polymer Delivery Pump creates a water barrier between the pump chamber seal and the actual pump. This barrier is flushed in order to prevent the build up of polymer around the pump.

- Fill the supplied 20 mL, all-plastic Luer lock syringe with water and expel any bubbles.
- 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.
- Open the Luer fitting by grasping the body of the fitting and turning it and the attached syringe approximately one-half turn counterclockwise.
- Open the exit fitting at the top left side of the pump block by turning it approximately one-half turn counterclockwise.



Rinsing and Filling Buffer and Water Reservoirs

- Ensure the instrument doors are closed
- Press the TRAY button on the outside of the instrument to bring the Autosampler to the forward position.
- Wait until the autosampler has stopped moving and then open the instrument doors.
- Remove the anode buffer reservoir by gently pulling down and twisting slightly.
- Remove the cathode buffer reservoir and water reservoirs.
- Discard the solutions into the sink and rinse out the reservoirs with water.
- 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.
- Fill the water reservoirs to the fill line with water (approximately 17 ml).
- 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.
- Place the reservoirs into position on the autosampler as shown below:



Spatial Calibration

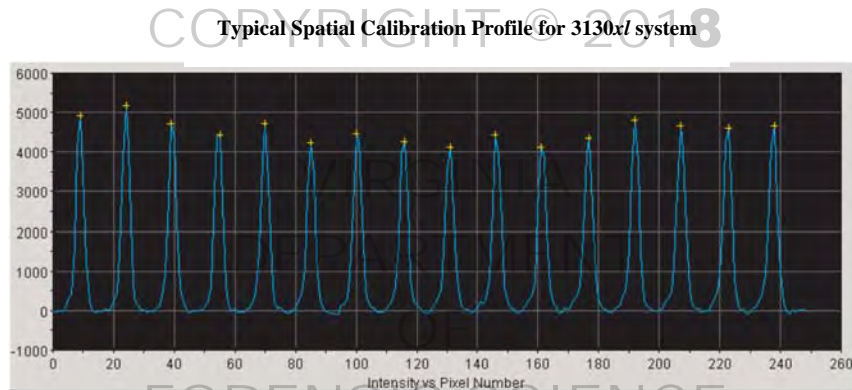
A spatial calibration ensures that the capillary alignment is correct prior to collecting data. It must be

performed after each time a capillary array is installed, replaced, temporarily removed from the detection block, or when the instrument is moved.

- Select the Spatial Run Scheduler from the 3130*x*/ drop down menu.
- In the Spatial Protocol section, select one of the following:
 - If the capillary array contains fresh polymer, select **3130SpatialNoFill_1**.

NOTE: You do not need to fill the capillary array each time you perform a spatial calibration.

- Select **3130SpatialFill_1** if the capillary array does not contain fresh polymer.
- Click Start.



- 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).


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

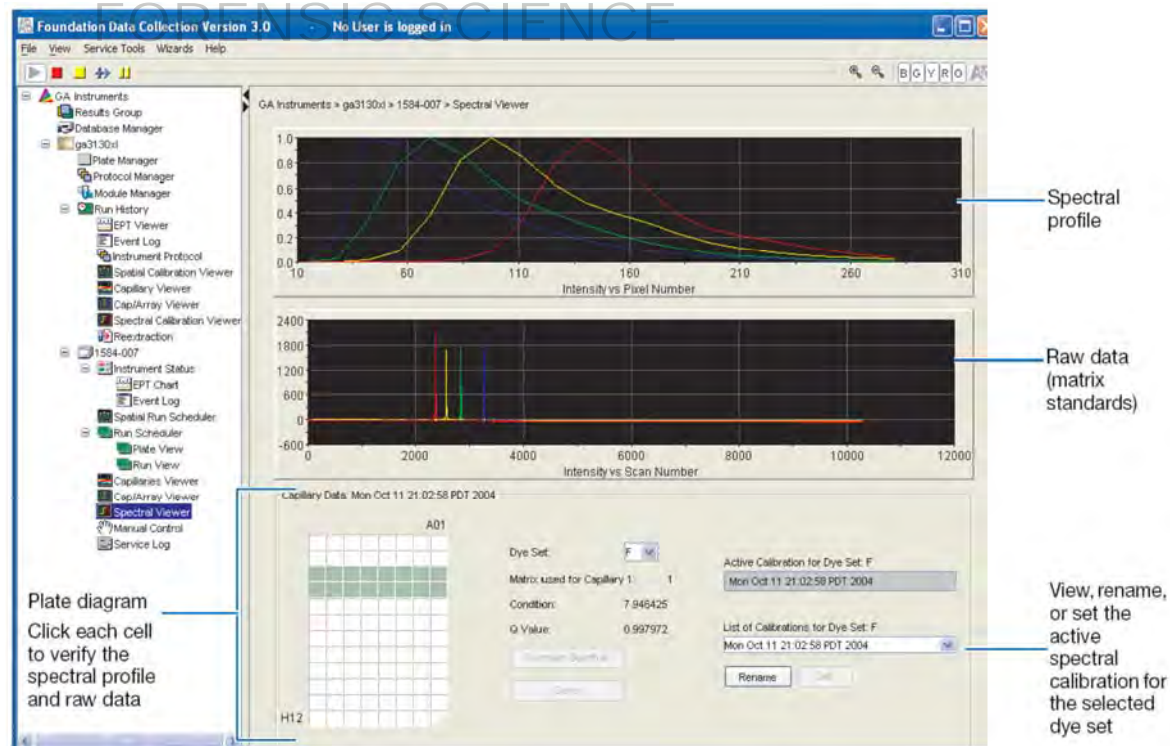
Spectral Calibration

A spectral calibration must be performed whenever 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.

- Add 170µL of Hi-Di formamide to resuspend the Big Dye Terminator v1.1 sequencing standard.
- Thoroughly mix the contents of the sequencing standard tube and spin briefly in a microcentrifuge.

- Dispense 10ul of the sequencing standard into wells A1-H2 of a 96-well reaction plate.
- 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.
- Remove caps and assemble the plate assembly as depicted in the diagram for 7.2.3 and load the plate assembly onto the autosampler.
- Create a new plate for the spectral calibration
 - Click on “New” from the Plate Manager on the ga3130xl tree pane
 - Enter a name for the plate
 - In the “Application” drop down list, select “Spectral Calibration”.
 - Select “96-Well” in the “Plate Type” drop down list.
 - Enter the Owner and Operator Names in the corresponding boxes.
 - Fill in the sample name column with “BD1.1SizingStandard”.
 - Select “Spect36_POP6_1” in the Instrument Protocol 1 drop down box.
 - Highlight the entire first row and select “Fill Down Special” from the “Edit” menu.
 - Click “OK” to complete and save the plate record.
- Run the spectral calibration plate
 - Click on “Plate View” from the Run Scheduler on the ga3130xl tree pane.
 - 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.
 - Highlight the plate name and click on the corresponding plate position on the autosampler indicator to link the plate.
 - Click the green button in the toolbar .
 - The Processing Plates dialog box opens, then click “OK”.



- Evaluate the spectral calibration run
 - Click on “Spectral Viewer” from the ga3130xl tree pane.

- Select the “E-BigDyeV1” from the Dye Set drop-down list.
- 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.
- 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.
- Once all capillaries have passed, ensure the new spectral is active and save it by clicking on “Save”.
- If the capillaries have not passed, the spectral calibration plate will be re-run.

6.3.3 Manual Control

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.

GA Instruments > ga3130xl > 3130xl > Manual Control

Manual Control


Send Defined Command For: Oven

Command Name	Value	Range
Turn On/Off oven	On	

Comments:
Turns main oven on and off.

Send Command

6.4 Starting the Run

- Ensure that the plate(s) have been assembled properly and are placed in their proper location on the Autosampler.
- Verify the plate information and link the plate to its location.
- The linked plate on the run scheduler will appear green.
- Verify that the active spectral calibration matches your dye set & capillary array length for all scheduled runs.
- Click the green button in the toolbar .
- The Processing Plates dialog box opens, then click “OK”.

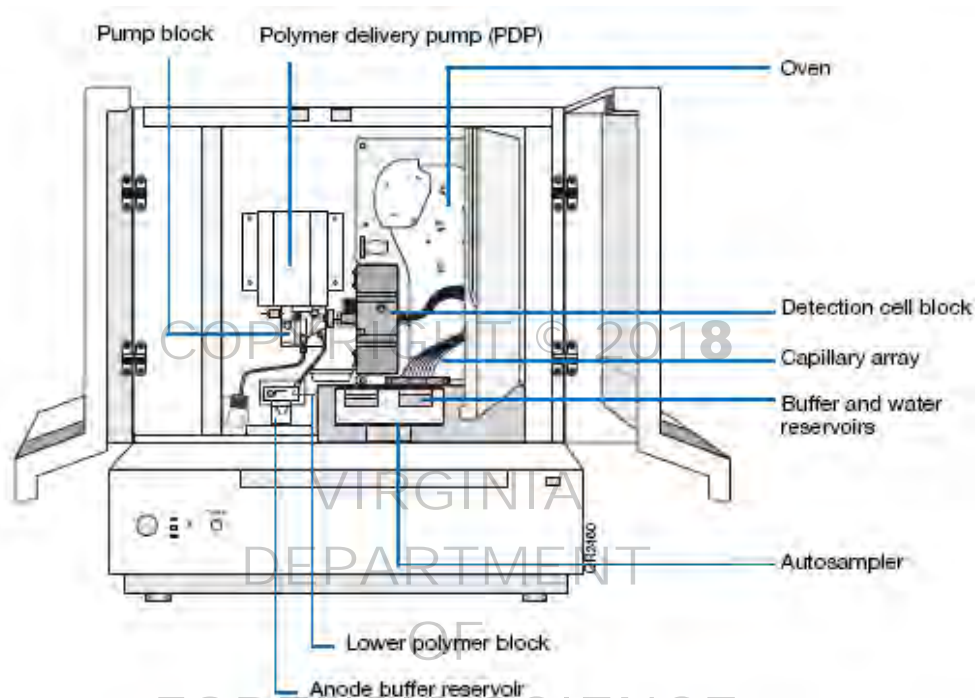
6.4.1 The software automatically performs a run validation:

- If the validation passes, the run starts.

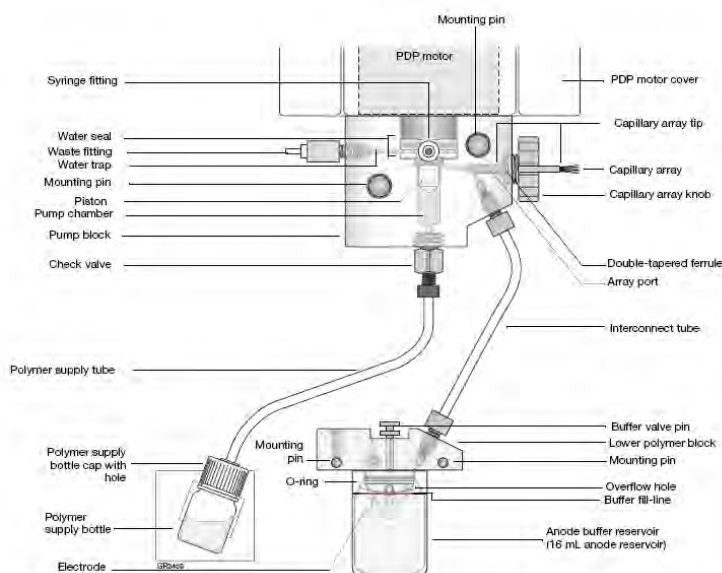
- If any of the validation tests fail, the run does not start. Check the event log for information.

NOTE: The EPT Chart is a summary of the values of the instrument processes over the time of the run.

Instrument Overview Schematic



Polymer Delivery Pump Schematic



REFERENCES

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).

COPYRIGHT © 2018

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

7 SEQUENCE ANALYSIS WITH SEQUENCHER™

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 (contig) consensus sequence. The consensus sequence can then be compared to the Revised Cambridge Reference Sequence (rCRS) to report the differences to the reference. These differences can also be compared between evidentiary and reference samples in evaluating the similarities or differences between the mtDNA sequences.

MATERIALS

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

Data Process

Each sample goes from sequence fragment to contig by assembling the sequences with respect to the rCRS. The Sequencher™ software only allows for one project to be edited at a time. Therefore, the samples will be processed in a specific order keeping evidence samples in separate project(s) from the reference sample project(s). To the maximum extent possible, evidence samples will go completely through this process before being compared to reference samples. Accordingly, all evidence sample contigs will be re-assembled and edited in the same order by a second examiner or forensic lab specialist. Evidence contigs will then be evaluated for concordance between the two scientists. Once the evidence has been completed, this will be repeated for the reference samples.

7.1 Printing/Displaying Electropherogram data

- Open the Sequence Scanner software.
- Click on the “File” menu and select “Import Traces”.
 - Click on the desired trace file or folder of trace files and click on the “Add Selected Traces” button. The traces which will be added will appear in the right side window and can be cleared individually or in groups.
 - To clear or change the traces which will be imported, select them and click on “Clear”.
 - Click on the “OK” button to import all the traces listed in the traces to add window.
- From the “Trace Manager” tab the imported traces can be selected to be viewed or printed.
 - To view the traces, double click the trace listed in the details window.
 - Opening multiple traces will show each displayed in a different tab.
 - To print the trace, ensure that it is highlighted in the details window and select “Print” from the “File” menu.

Additional information can be viewed in the Reports section.

- Click on “Reports” tab to view the reports, there are three very useful reports for sequencing.
 - 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.

- The reports can be printed or exported to a data file.
- Select the desired report and click “Print” from the “File” menu to print the report.
- 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.

7.2 Creating a Sequencher™ Project

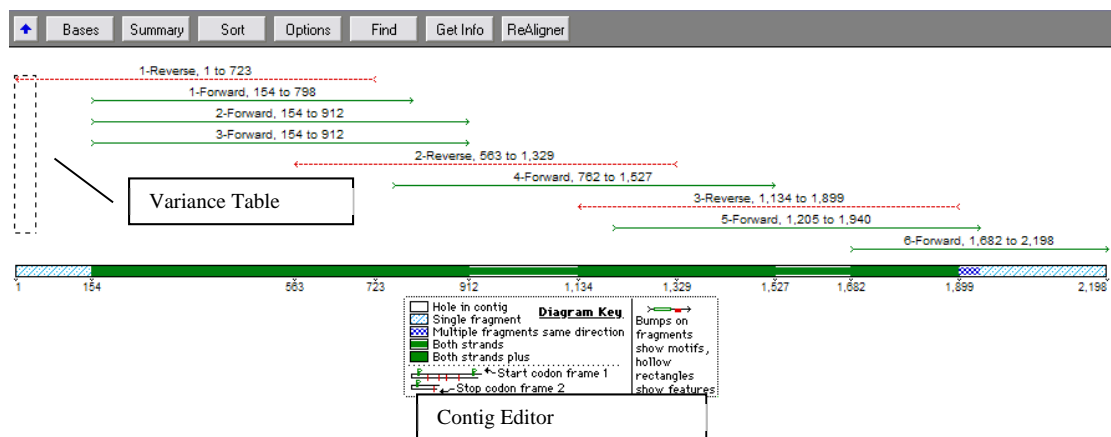
- Open the Sequencher™ software.
- If an untitled project window doesn't already appear with the rCRS DNA reference, create a new one.
- Click on the File menu and select “New Project from template” and choose “rCRS” as the template.
- Add DNA sequence fragments to the project by selecting “Import” from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

7.3 Assembling a Contig

- Select the reference and sequence fragments to include in the contig by using the control key and clicking on each fragment.
- With the mouse over the selected fragments, right click and choose Assemble Contig from the menu.
- Select “Assemble to Reference” or “Assemble to Reference by Name” if you have the “Assemble by Name” option active.
- Assure the contig is named with at least the case number and item number. To change the name, Right click on the newly assembled contig name text and select “Rename Contig”. This is the name that will appear as the title in subsequent printouts of the data associated with this contig.

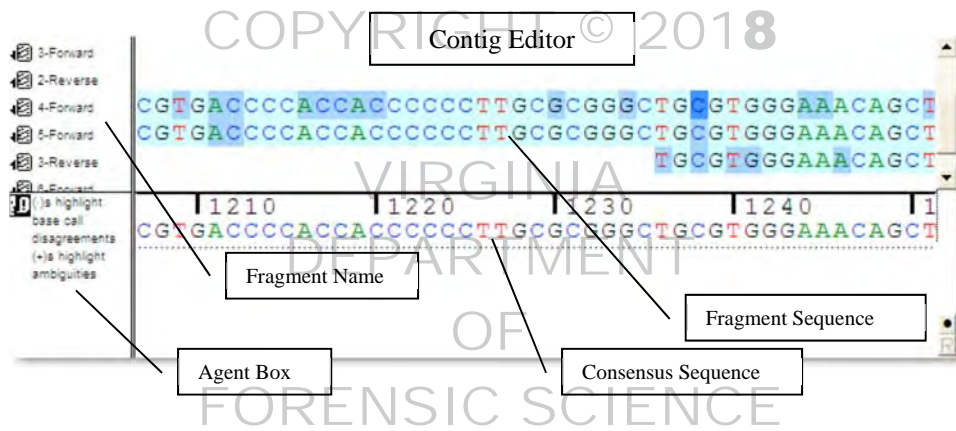
7.4 Editing Assembled Electropherograms

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



- The fragments can be sorted and organized within the overview display.

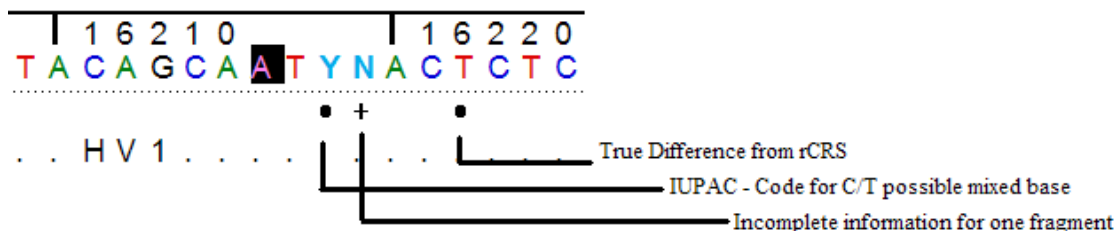
- Click on the “Sort” button on the contig editor to select the sort method of choice and the fragments will be sorted based on that criterion.
- Fragments can also be sorted by using the grab function by pressing the control key and using the mouse to click and drag the fragments. Moving the fragments this way may change their alignment to the reference.
- Clicking on the fragment name while pressing the control key will also grab the fragment for sorting within the fragment name box.
- The bases may be edited by moving the Selection Marquee to choose the region to edit and click on the “Bases” button at the top of the contig editor. Clicking the “Overview” button will return to the previous view of all the fragments.
- The contig editor 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 listed in Chapter 9.3.

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

- Select a base on either the consensus sequence or a fragment sequence to navigate the contig by clicking on the “select” menu and choosing the options listed. Once the option has been selected, use the spacebar to repeat the selection.

NOTE: Edited bases will be reflected as lowercase letters

- Click on “Next ambiguous base” to find the next base designated “N”.
- Click on “Next contig disagree” to find the next base which disagrees with the reference sequence.
- Click on “Next edited base” to find the next base which has been edited previously.
- Click on “Next low confidence base” to find the next base which has a low quality score.
- The electropherogram can be viewed for the selected base(s) by clicking on the “Show Electropherogram” button in the contig editor.

The screenshot displays three windows from the Sequencher software:

- Compare Bases to Reference Sequence:** Shows a table comparing 10 sequences to a reference sequence. The table has columns for position (15884-15971) and quality scores (4-6). A callout box labeled "Variance Table" points to this window.
- Contig Editor:** Shows a sequence alignment with a highlighted region. A callout box labeled "Contig Editor" points to this window.
- Chromatograms from GWB:** Shows an electropherogram with peaks corresponding to the bases. A callout box labeled "Electropherogram Viewer" points to this window.

- The electropherogram may be scrolled through by using the scroll bars or by using the arrow key.
- Peaks within the electropherogram corresponding to the bases by type can be viewed by toggling the bases on or off using the **A**, **C**, **G**, and **T** buttons.
- Bases can also be edited by selecting them in the electropherogram view window by clicking on the base and typing a base letter. Edited bases will appear in lower case.
- Bases can be added or subtracted by selecting the base or an adjacent base and using the options in the “Sequencing” menu.
- Click “Delete Bases” and “fill void from right” or “fill void from left” to delete bases. Depending on which way the void is filled will affect the remaining alignment for that fragment.
- Click “Insert Gap or Move Bases” to insert a colon “:” and move the bases by clicking “left” or “right”. Depending on which way the gap is inserted will affect the remaining alignment for that fragment.

NOTE: Use the “Delete Bases” function to remove the sequencing primer bases which remain on its complimentary fragment. (e.g., remove the forward primer bases which are sequenced on a reverse sequence fragment)

- Click on “Call secondary peaks” to make additional edits where there may be mixed bases. The default value is 15%.
- The sequence fragments alignment to the reference sequence can be optimized by clicking the “ReAligner” button on the contig editor.
- Once the Contig has been edited completely, the coverage map can be printed. Select “Overview” to display the coverage information. Select “Print” from the “File” menu and print the coverage information.

7.5 Creating a Variance Table and Report

- The consensus sequence contains the compiled nucleotide base calls for the fragments in the sample and the differences from the reference sequence are exhibited in the variance tables and reports.
- The consensus sequence can be reviewed in the review mode and through the variance table, the data supporting each variant can be displayed.
- Ensure that “Consensus Inclusively” is selected in the “Contig” menu. Select/highlight the contig to be compared and click on the “Contig” menu and select “Compare Consensus to Reference”. Alternatively, right click on the highlighted contig and select “Compare Consensus to Reference”.
- Click on the “Review” button to review the base calls. Click the base variant in the variance table to view the corresponding base in the contig editor and electropherogram viewer to review the variant bases.
- Click on the “Reports” button in the review variant table window.
- Select “Variance Detail Report” from the drop down list and click “Open Report”.
- Click the “save PDF” to retain an electronic copy of the document.
- Open the saved .pdf document and select “Print” to print the report.

7.6 Exporting Sequence Data to CODIS and Comparing Sequence Data Between Two Scientists

- Sample data can be exported directly into CODIS when there have been two separate Sequencher™ projects made from sequence data.
- The data from two different analysts must be evaluated to ensure the consensus sequences are in agreement prior to exporting data into CODIS.
- Click on the “File” menu and select “Import Sequencher Project” and choose the project containing the sequence data. Repeat for the 2nd Sequencher project.
- Ensure at least two contigs for the specimen are highlighted and that “Consensus Inclusively” is selected in the “Contig” menu.
- 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	<u>N</u>		1
73	A	<u>G</u>	<u>G</u>	2
183	A	<u>N</u>		1
195	T	<u>C</u>	<u>C</u>	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.

- Once the two contigs are in agreement close the variance table and while the two contigs are highlighted, click “Validate mtDNA Profiles” from the “Contig” menu.
- For comparison between two sizings – save the report as a .txt file.
- Open the saved report and “print”.
- Click on “Export CMF” to export the profile to CODIS. Fill in the appropriate blanks in the Export CMF window and click “Export” to create a text file that can be uploaded into CODIS.

The screenshot shows the 'Export CMF 4.1' dialog box with the following fields and callouts:

- Export Sample:** mtDNA Profile 1 (Callout: Sample select drop-down list)
- Destination ORI:** (Callout: CODIS ORI designation)
- Source Lab Name:** (Text input field)
- Submit by User ID:** (Text input field)
- Fragment Owner User ID:** (Text input field)
- Fragment Owner Date:** 2008-02-29 (Callout: Valid CODIS User ID)
- Fragment Owner Time:** 15:14:19 (Callout: Valid CODIS User ID)
- Specimen Lab ORI:** (Text input field)
- Specimen ID:** 082790 (Callout: CODIS ORI designation)
- Specimen Category:** Alleged Father (Callout: Specimen Identification within CODIS*)
- Optional:**
 - Source Identified:** None (Callout: Specimen Identification within CODIS*)
 - NCIC ID:** (Text input field)
 - VCAP ID:** (Text input field)
 - Comment:** (Text area)

Buttons at the bottom: Clear All, Cancel, Export.

REFERENCES

Gene Codes Corporation. Sequencher™ 4.8 User Manual for Windows (2007)

8 INTERPRETATION OF MITOCHONDRIAL DNA RESULTS

8.1 Quality Assurance of Interpretation

In order to prevent unintentional bias by an Examiner during analysis and interpretation, the Mitochondrial DNA (mtDNA) Section will process samples/data in the following manner:

- To the maximum extent possible, evidence samples will be handled separately from reference samples during the analytical process.
- To the maximum extent possible, evidence samples in a case will be processed prior to the reference samples.
- Independent assembly of sequence data by a second Examiner or Forensic Lab Specialist will be performed on all samples.
- To the maximum extent possible, the interpretation of evidence sequence data will be completed prior to the interpretation of sequence data for reference samples.

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 are performed to provide confirmatory data. Two forward strands (L) or two reverse strands (H) from separate primer set amplifications can also be used to confirm data. Two forward strands (L) or two reverse strands (H) from the same control region amplification can also be used to confirm data if the data is generated from a different primer. Data that has not been confirmed (e.g., single stranded data) is not used for interpretation.

8.1.1 Sequence Confirmation

All sample sequences will be confirmed by a second qualified Examiner or Forensic Lab Specialist. Confirmation involves independently assembling sequences for the NC, RB, + control, evidentiary and known samples from the analysis data.

- Separate Sequencher™ printouts are generated and initialed by each Examiner or Forensic Lab Specialist.
- Confirmed sequence range is defined as the shortest length of sequence obtained by the two sizings.
- When a sample is analyzed for QC purposes, analysis by only one Examiner or Forensic Lab Specialist is required.

8.1.2 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 procedure along with the evidentiary sample(s).

If product is observed in the RB lane on a product gel, the corresponding sample must be reextracted.

If no product is observed in the RB lane on a product gel, but generates a sequence that is in concordance with the sample, the results of the sample amplification reaction are not used for comparison purposes. The evidence sample sequence can be used under the following conditions:

- The RB does not contain base calls (e.g., N base calls only).
- The RB has base calls but does not align.
- The RB sequence aligns, but the sequence is not in concordance with the evidence sample.
- The RB sequence aligns and the samples were processed in a batch. (Refer to chapter 3 regarding batching samples.)

Batched RB for **evidence samples** includes adjacent samples in the batch. For example aligned sequence obtained from sample RB 3 will be compared to sample 3 and sample 4 following the guidelines in 8.2.2.

Batched RB for **reference samples** apply to all samples in the batch. For example aligned sequence obtained from the reference RB will be compared to all known samples in the batch following the guidelines in 8.2.2.

If an aligned mtDNA sequence is obtained from the reagent blank, an attempt is made to determine the source of this DNA.

8.1.3 Negative Control

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

If product is observed in the NC lane on a product gel, the corresponding samples must be reamplified.

If no product is observed in the NC lane on a product gel, but generates a sequence that is in concordance with the evidence sample, the results of the sample amplification reaction are not used for comparison purposes. The evidence sample sequence can be used under the following conditions:

- The NC does not contain base calls (e.g., N base calls only).
- The NC has base calls but is not of requisite quality for comparison purposes or does not align.
- The NC sequence aligns, but the sequence is not in concordance with the samples.
- The NC sequence aligns and the samples were processed in a batch. The NC will be compared against all samples in a batch following the guidelines in 8.2.2.

If an aligned mtDNA sequence is obtained from the negative control, an attempt is made to determine the source of this DNA.

8.1.4 Positive Control

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

- The record of the particular positive control used and its lot number is maintained for each case.
- Only the ranges in common between the sample and the corresponding positive control will be considered.

The mtDNA analysis of a particular sample extract is repeated, going back to the appropriate step, if the positive control fails to amplify and/or sequence correctly.

8.2 Cycle Sequencing Interpretation Guidelines

Mitochondrial DNA sequencing results are reported from a consensus sequence derived from multiple sequence results within the Control Region. This consensus sequence is compared to the rCRS and differences (polymorphisms) from this sequence are noted on the Sequencher™ printouts. They are noted by stating the nucleotide position followed by the code for the polymorphic base (for example, 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 table 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

8.2.1 Sequence Profile Reporting

- Profiles are characterized so that known patterns of polymorphisms (a.k.a. phylogenetic alignments) are maintained (e.g., maintain deletions at positions 249, 290 and/or 291 when present).
- Maintain the least number of differences from the rCRS unless it 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.
- Maintain the AC repeat motif in HVIII region from np 515-525.
- Prefer substitutions to insertions/deletions (indels).
- Prefer transitions to transversions unless this conflicts with the first bullet in 9.3.1.
- Place indels contiguously when possible.
- Place indels on the 3' end of the light strand.

- Insertions are listed to the right of a particular nucleotide position to minimize the number of differences from the rCRS. Insertions are described by first noting the site immediately 3' to the insertion followed by a point and a "1" (for the first insertion), a "2" (if there is a second insertion), and so on, and then by the nucleotide that is inserted (for example, 315.1C).

8.2.2 Sequence Comparisons

- Evaluation of Control Sequences

The positive control run with the sample must type correctly in order to report the sequence for that sample (e.g., the correct polymorphisms are detected at the correct nucleotide positions).

After the RB and NC are analyzed, a determination is made as to whether any analyzable sequence is present. If there is not, continue to the section "Comparison Among Samples". If there is, the analyzable sequence obtained from the controls must first be compared to any sequence obtained from forensic samples associated with those controls. To determine whether any contamination seen in the RB or NC is not the source of the DNA from the sample, the sequence of the sample and the corresponding RB or NC must differ by at least one base within each region. If the sequence of the RB or NC within a region is the same as that of the sample, the sample may be re-extracted, re-amplified, or re-sequenced; otherwise, the region in question will not be included in the reported sequence for that sample.

- Evaluation of 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 C₅TC₄. 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 C₇TC₅. A 309.1C insertion in HVII will be annotated C₈TC₅, and the insertions 309.1C, 315.1C will be annotated as C₈TC₆.

- Comparison Among Samples

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.

8.3 Interpretation of Sequence Comparisons

Samples exhibiting three or more areas of apparent heteroplasmy within the control region may be considered as mixtures. No interpretation or sequence comparisons will be made for mixture samples.

The following interpretations are available for sequence comparisons:

8.3.1 Exclusion:

If the samples differ at two or more positions, they can be excluded as coming from the same source.

8.3.2 Inconclusive:

If the samples differ at a single position, with no evidence of heteroplasmy the comparison are reported as inconclusive. Samples that have the same sequence, but do not have a common length variant at nucleotide positions 303-309 (HVII), will also be reported as inconclusive. Additionally, samples differing at a single nucleotide position and not having a common length variant at nucleotide positions 303-309 (HVII) will be reported as inconclusive. Because of the possibility of undetected heteroplasmy additional samples may be analyzed when Q and K sequences differ by a single base. These samples may include blood, buccal swabs, and hair. Hairs from a known hair standard may be combined and processed as a single known sample.

8.3.3 Cannot Exclude:

If samples have the same sequence, or demonstrate sequence concordance, they cannot be excluded as coming from the same source or from having a maternal relationship. Sequence concordance is defined as having a common base, or common HVII length variant at each position. For example, 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. Similarly, overlap of length variants is considered sequence concordance. However, if 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.

8.4 Missing Persons Cases

Casework samples from Missing Persons cases will be entered into the SDIS following appropriate CODIS reviews outlined in the Forensic Biology CODIS Operating Policies and Procedures Manual. The profiles will be handled in accordance with State and National CODIS policies and procedures.

REFERENCES

- Biochemical Nomenclature and Related Documents*, 2nd edition, Portland Press, 1992. Edited C Liébecq. [ISBN 1-85578-005-4]
- 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.
- 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.
- Melton, T., (2004) Mitochondrial DNA heteroplasmy, *Forensic Science Review*, 16: 268-286.
- 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.
- Scientific Working Group on DNA Analysis Methods (SWGDM), Guidelines for mitochondrial DNA (mtDNA) nucleotide sequence interpretation. *Forensic Science Communications*, 5: Issue 2.
Available: <http://www.fbi.gov/hq/lab/fsc/backissu/april2003/swgdammitodna.htm>

Scientific Working Group on DNA Analysis Methods (SWGAM). (2013) Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories.

Available: http://swgdam.org/SWGDAM%20mtDNA_Interpretation_Guidelines_APPROVED_073013.pdf

Scientific Working Group on DNA Analysis Methods (SWGAM). Mitochondrial DNA Nomenclature Examples Document. Available: http://swgdam.org/SWGDAM_Mitochondrial_DNA_Nomenclature_Examples_FINAL-080713.pdf

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.

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., *et. al.* (2002) Further discussion of the consistent treatment of length variants in the human mitochondrial DNA control region. *Forensic Science Communications*, 4: Issue 4.

<http://www.fbi.gov/hq/lab/fsc/backissu/oct2002/wilson.htm>

COPYRIGHT © 2018

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

9 POPULATION STATISTICS – DETERMINATION OF HAPLOTYPE FREQUENCY

The Federal Bureau of Investigation (FBI) Laboratory, in collaboration with the Scientific Working Group on DNA Analysis Methods (SWGDM), maintains a database consisting of human mitochondrial DNA (mtDNA) nucleotide sequence data from a variety of individuals of European, Asian, and African ancestry. The SWGDM database consists of haplotypes generated by forensic DNA typing laboratories and their contractors.

- 9.1. The Popstats program, which is supplied by the FBI and contained within the Combined DNA Index System software (CODIS), is used to search casework haplotypes against the database to provide frequency estimates. The range of the sequence data to be searched consists of the shortest range of sequence agreed upon by the Examiners reviewing the data or the full HVI (16024-16365) and HVII (73-340) regions, whichever one is the smallest. Ambiguous base positions in both database samples and casework samples are searched as an N (meaning the search includes all four bases at that position) and do not have any exclusionary value when compared with other sequences. Deletions are searched as a "-". Those database samples whose range(s) are fully included within the casework sample range are used in the database search result. The database search result provides the number of database profiles that match the casework profile, as well as the number of profiles in the database that differ by up to five positions.
- 9.2. All sequence polymorphisms in the sample searched are entered; however, all length variants at nucleotide positions 16193 (HVI) and 309 (HVII) are ignored in database searches of relevant concordant sequences. Hence, length variability in these regions will not add any additional rarity to a database profile search. The Examiner confirms that profiles listed as the same are in fact the same as the casework profile being searched.
- 9.3. A confidence interval can be calculated from the results of a database search in order to estimate the population frequency of a profile. An upper 95% confidence interval value (upper bound) maybe presented as a percentage of individuals within each population who are estimated to be included as potential contributors of a questioned profile. The upper bound estimate is dependent on the size of the database, and these estimates are therefore likely to change as the database size changes.

9.3.1. The following formulae are used to calculate the upper bound estimate:

- 9.3.1.1. If the haplotype has been 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.

- 9.3.1.2. If the haplotype has not been observed in the database

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

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

The results of these upper bound frequency estimate calculations for the three major population groups in the United States (African-American, US Caucasian, and US Hispanic) are included in the report. In addition, the report will contain the search results and upper bound frequency estimate of any other population where a matching type is found.

- 9.3.1.3. The number of individuals in which the haplotype would be expected to be observed will be truncated to 2 digits.

Example: 1 in 5,278 individuals would be expected to share this haplotype
1 in 5.2 thousand individuals would be expected to share this haplotype

REFERENCES

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.

Forensic DNA Evidence Interpretation. CRC Press. 2005. ed Buckleton, J, Triggs, CM and Walsh, SJ.

Monson, K., *et. al.* (2002) The mtDNA population database: An integrated software and database resource for forensic comparison. *Forensic Science Communications*, 4: Issue 2.
<http://www.fbi.gov/hq/lab/fsc/backissu/april2002/miller1.htm>

Scientific Working Group on DNA Analysis Methods (SWGAM). (2013) Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories.
Available: http://swgdam.org/SWGDAM%20mtDNA_Interpretation_Guidelines_APPROVED_073013.pdf

COPYRIGHT © 2018
VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

10. REPORTING MITOCHONDRIAL DNA RESULTS

10.1. Methods

The METHODS SECTION (as outlined below) will be placed prior to the RESULTS 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

The RESULTS SECTION will contain a description of the evidence, the results of all analyses including comparisons, conclusions and statistics as appropriate. This will follow the METHODS section in the Certificate of Analysis. The following are examples of typical wording that can be used. Determination of exact wording is made on a case to case basis.

10.2.1. Exclusion:

- "Different mitochondrial DNA (mtDNA) sequences were obtained from EVIDENCE and KNOWN. Therefore, KNOWN INDIVIDUAL can be excluded as the source of the EVIDENCE."

10.2.2. Inconclusive:

Insufficient mtDNA:

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

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."

There is an 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. Because mixtures of mtDNA are not interpretable, no conclusions can be reached."

The quality controls (positive control, negative control, reagent blank) were not met and the sample analysis has been exhausted:

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

The mtDNA sequences differ only by a single nucleotide and no evidence of a common nucleotide is present:

- “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 could be reached as to whether KNOWN can be excluded as the source of the EVIDENCE.”

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 KNOWN. Due to the difference of the length of the repeated bases between nucleotide positions 303 to 309, no conclusion could be reached as to whether KNOWN can be excluded as the source of the EVIDENCE.”

10.2.3. Cannot Exclude:

Cases in which the mtDNA sequences are concordant, cannot exclude known sample:

- “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.”

Cases in which mtDNA sequences are concordant for the purposes of identifying remains:

- “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.”

Cases in which the DNA sequences are concordant with evidence of heteroplasmy:

- “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 excluded as a possible source of the EVIDENCE.”

10.2.4. Body Identifications:

All Certificates will be issued with the following statement:

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

10.2.5. Results Table

All cases which the known or evidence samples cannot be excluded will have a table listing the mtDNA sequencing results.

The mtDNA sequencing results will be included in a tabular format and referenced in an appendix, including:

- Sample name
- Range of sequencing coverage results
- Nucleotide position of polymorphism
- Difference of sequence with respect to the rCRS
- Polymorphisms or differences will be reported as defined by IUPAC codes listed in chapter 9.

10.2.6. Statistical Table

Statistical calculations will be conducted and reported for evidence samples that cannot be excluded from a reference. When multiple samples are concordant with a reference, the sample which has the largest amount of HV1 and HV2 sequence data will be reported. The type of statistical calculation reported by DFS for mtDNA typing results is based on the counting method. The 95% upper bound confidence frequency estimate for at least the Caucasian, African American, and Hispanic populations will be made. See section 10.4.1 of chapter 10 addressing statistical calculations.

Statistical calculations will be reported in a tabular format including:

- Database referenced
- Number of observations of mtDNA sequence
- Number of individuals in the database
- Upper bound frequency estimate (for 95% confidence)

10.2.7. Request for Known Samples:

When a known sample has not been submitted and/or a haplotype has been obtained in the case for which the source is unknown, a request will be made using the following statement:

- “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.”

10.2.8. Termination During Examinations:

If DNA analysis has begun in the case, and a request for terminating the case has been received, this will be reported as follows:

- “The mtDNA analysis was discontinued at the request of INVESTIGATOR/COMMONWEALTH’S ATTORNEY on DATE.”

11 QUALITY ASSURANCE

The quality system of the Mitochondrial DNA (mtDNA) Section is directed by the Department of Forensic Science Quality Manual, hereinafter referenced as the Quality Manual.

11.1 Goals And Objectives

To support criminal justice agencies in the Commonwealth by performing mtDNA analysis of selected biological materials associated with criminal investigations.

To ensure the quality, integrity, and accuracy of the analytical data through the implementation of a detailed quality control program.

Establish and monitor quality requirements for reagents, supplies, equipment, and analytical procedures.

Ensure that the entire mtDNA analytical procedure is operating within the established performance criteria and that the quality and validity of the analytical data is maintained.

Ensure that problems are noted and that the appropriate corrective action is taken and documented in a Memorandum of Record or case file documentation for the affected case file (s) and, if necessary, for the Quality Control records.

11.2 Organization and Management Structure

The organization and management of the Department of Forensic Science is addressed in the Quality Manual, Section 3, Management and Authority.

The management structure for the Department is set forth in the Department Organizational Chart.

11.3 Personnel Qualifications and Training

The procedure for the qualification and training of personnel is addressed in the Quality Manual, Section 19, Personnel and Training.

The policy that addresses attending meetings and seminars is addressed in the Quality Manual, Section 19.8, Continuing Education and Training.

Each laboratory will maintain the training record for each assigned person.

All analysts must receive 8 hours of continuing education on a yearly basis. The technical leader shall approve the continuing education program which will be documented on the Department's Report of Training / Conference Attendance Form. Records of attendance will be maintained by the laboratory.

A review of scientific literature will be conducted through the use of a reading list. The technical leader will approve the articles on the list. Documentation of the completion of the required reading shall be maintained by the section supervisor of the mtDNA or nuclear DNA Section, or their designee(s).

11.4 Facilities

The Facilities of the Department are addressed in the Quality Manual, Section 20, Facilities, Security and Safety.

11.5 Evidence Control

The procedure for the assignment of laboratory numbers to submission of physical evidence, and item [sub-item] numbers to evidence contained in the submission of physical evidence is addressed in the Quality Manual, Section 15, Records and Case Files.

The procedure for the handling of physical evidence is addressed in the Quality Manual, Section 14, Evidence Handling. DNA extracts and amplified product are defined as work product.

11.6 Validation

The validation of new DNA technologies/methodologies by the mtDNA Section is addressed in the Quality Manual, Section 5, Technical Procedures and Manuals and in Section VI, Chapter 4 of the Forensic Biology Section Procedures manual. A summary of each validation study is maintained with the data. The summary and data are maintained by the Biology Program Manager as directed by Section 5 of the Quality Manual, Technical Procedures and Manuals.

11.7 Analytical Procedures

The DNA analytical procedures used by the mtDNA Section are set forth in the Section Procedures Manual. Establishment of these procedures is guided by the Quality Manual, Section 4, Quality System Documents, and Section 5, Technical Procedures and Manuals.

Estimation of the uncertainty of measurement: no critical measurements are taken during the analytical process in the Section; therefore, no estimation of uncertainty of measurement is necessary.

11.8 Calibration And Maintenance

Procedures for the calibration and maintenance of laboratory equipment are set forth in Chapter 12, Quality Assurance. Establishment of these procedures is guided by the Quality Manual, Section 5, Technical Procedures and Manuals, Section 8, Supplies and Services, and Section 21, Equipment.

11.9 Proficiency Testing

The proficiency testing program for the Department is referenced in Section 17. 5 of the Quality Manual, Proficiency Testing, as well as Section 10 of the Quality Manual, Nonconformities and Corrective Actions.

11.10 Corrective Action

The procedure for corrective action is set forth in the Quality Manual, Section 10, Nonconformities and Corrective Actions.

11.11 Reports

The procedure for generating a Certificate of Analysis is addressed in the Quality Manual, Section 16, Reporting Test Results.

The procedure for the preparation, storage and disposition of case file records is addressed in the Quality Manual, Section 15, Records and Case Files.

The procedure for the expungement of records is addressed in the Quality Manual, Section 15.11, Expungement of Records.

11.12 Review

The procedure for administrative and technical review of case files is addressed in the Quality Manual, Section 17, Monitoring Results.

The procedure for the monitoring of the testimony of casework examiners is addressed in the Quality Manual, Section 17.4, Testimony Monitoring.

The administrative review also includes a review of the chain of custody and the disposition of the evidence. The chain of custody review includes the written chain of custody on the RFLE and the electronic chain information maintained for the item/container.

On occasion, there can be initial disparities between the conclusions drawn by qualified examiners in the technical review process. The first step towards achieving a unified scientific conclusion should be a discussion between the examiner and technical reviewer. In this discussion, each party should provide details supporting the basis of their conclusions. If consensus cannot be achieved, the supervisor should be consulted. The Biology Program Manager can be contacted for further technical discussions between the examiner and technical reviewer/supervisor.

11.13 Safety

The policies and procedures for safety in the laboratory are addressed in the Department Safety Manual and Section 20 of the Quality Manual, Facilities, Security and Safety.

11.14 Audits

The procedure for audits of the Department is addressed in the Quality Manual, Section 12, Audits.

A FBI Quality Assurance Audit of the Section is conducted by an external agency at a minimum of every other year.

The official record and response to prior audits is maintained by the Department Quality Assurance Coordinator in the Department's headquarters laboratory.

11.15 Outsourcing

Outsourcing is addressed in the Quality Manual, Section 7, Subcontracting of tests.

Technical specifications of the outsourcing agreement are approved by the technical leader prior to the outsourcing agreement being awarded.

A technical review of the vendors data is conducted prior to entering sample haplotypes in SDIS. The following elements are included in the technical review:

- A review of haplotypes to ensure they are supported by the analytical data

- Associated controls

- A review of the final report if provided by the vendor

- Verification of the haplotypes, eligibility and correct specimen categories for entry to CODIS

On-site visits are conducted prior to the beginning of casework analysis. Annual on-site visits are conducted as required. The on-site visit will be documented and maintained by the laboratory.

The on-site visit will assess the following elements:

- Evaluate training and credentials of new personnel involved in the project

- Evaluate any new validation which will be implemented on contract samples

- Review procedures

- Inspect facilities

11.16 Document Retention

The document retention policy of the Virginia Department of Forensic Science is addressed in the Quality Manual and is summarized below:

Proficiency tests are maintained for at least six years

Corrective actions are maintained for at least six years

Audit records are maintained for at least six years

Training records are permanent records

Continuing education is a permanent record

Case files are retained for 50 years

Court testimony monitoring is maintained for at least six years

11.17 Case File Documentation

Technical records that are generated electronically and printed are considered complete after the document is printed and the print session is closed. Changes to these records after this time must be tracked and made in accordance with the Department QM.

Technical records that are generated electronically and saved for printing upon completion are considered complete when the last analytical procedure documented on that record (e.g., worksheet) has been completed. Changes to these records after this time must be tracked and made in accordance with the Department QM.

Note: For example – in the Excel workbooks used for DNA analysis, each individual worksheet will be considered complete once the last procedure documented on that worksheet has been completed.

Examination documentation that is saved electronically and not printed (e.g., additional injections not used for conclusions) is considered complete when saved to a disk (or other suitable media) which is then initialed, dated and uniquely identified by the examiner prior to the technical review. Changes to this documentation after it is complete will need to be regenerated electronically and saved to the original disk or to a new disk which is then initialed, dated and uniquely identified by the examiner. The original and/or subsequent disks are saved as part of the case record.

11.18 Quality Control for Forensic Mitochondrial DNA Analysis

The purpose of the procedures in this portion of the chapter is to ensure that the parameters of the testing process are routinely monitored in the manner necessary to maintain the success and reliability of the testing procedures. Upon the completion of the quality control testing of the equipment or critical reagents the supervisor or designee will review, initial and date the quality control documentation prior to being used to conduct casework analyses.

Controls utilized during each phase of the testing procedure are designed to signal potential problems in the analysis. If acceptable results are obtained on these controls, it is reasonable to assume that the results for other samples analyzed using the same equipment, reagents, and supplies will be accurate and acceptable. If the controls indicate a problem with the analysis, it may be possible to determine the source of the problem and correct for it. Depending on the nature of the problem the sample may need to be re-extracted, re-amplified, re-sequenced or re-injected.

Where the samples are irreplaceable and/or limited in amount (as in most casework), it is highly desirable to minimize the need for repeat analysis due to failure of equipment, materials or reagents. To that end, quality control (QC) procedures should focus as much as possible on preventing problems before they occur rather than on dealing with them after they happen.

11.18.1 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.

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 and DTT) 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 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.

11.18.2 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. Refer Chapter 9 for the interpretation guidelines of the following controls.

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 (according to section 9.1.2), it is not necessary to re-amplify the reagent blank when re-amplifying/ re-sequencing samples unless a different injection time is used.

Negative Amplification Control

An amplification blank 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 the post- amplification procedures.

Positive Amplification Control

An amplification positive control (HL-60) must be used with each amplification. This control is processed through the 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

11.18.3 INSTRUMENTS AND EQUIPMENT QUALITY CONTROL

Instruments and Equipment Inventory

The section will maintain an equipment inventory.

Operating Manuals

Warranty information and operating manuals will be filed in the laboratory and will be readily available to all operators of instruments and equipment.

Calibration/Maintenance/Repair Records

Anytime an instrument or piece of equipment requires calibration, service or maintenance, that fact will be documented on form 210-F600, "Equipment Calibration/Maintenance/Repair Form" or comparable log form. Additionally, instruments/equipment on routine service contracts will have routine service calls documented. These forms will be maintained in a properly labeled file.

Instrument/Equipment Calibration and Maintenance

Routine maintenance and/or calibration will be performed on each instrument/piece of equipment listed, based upon the following schedule. Records will be maintained. Instruments/pieces of equipment from this list which are considered critical are:

Analytical Balance
 Automated Genetic Analyzer
 Thermal Cycler Temperature Verification Unit
 Thermal Cycler
 NIST Traceable Thermometer
 Pipettes (including multi-channel)

NOTE: If an instrument/equipment is out of calibration with the manufacturer's or the mtDNA Unit Section specifications, the instrument/equipment will immediately be taken off line for repair and the maintenance/repair will be documented on the "Equipment Calibration/Maintenance/Repair Form". If an instrument/equipment is out of calibration and cannot be repaired it will be taken off line and the attempted repair will be documented on the "Equipment Calibration/Maintenance/Repair Form". Subsequently, the instrument/equipment will be removed from the section's inventory and will be documented.

ANALYTICAL BALANCES:

Annually - Serviced and calibrated by outside vendor.

If the balance is taken off line for repair/maintenance, prior to being put back in service, the annual calibration will be conducted or the calibration check will be conducted as a performance check.

Calibration check - the weights used to verify the calibration of the analytical balance should include at least 3 data points covering the range for which the analytical balance is used.

PASS/FAIL RANGES FOR EACH DATA POINT (WEIGHT)

0.1 g \pm 0.005 g
 1.0 g \pm 0.01 g
 10 g \pm 0.1 g
 50 g \pm 0.1 g
 100 g \pm 0.5 g
 400 g \pm 1.0 g

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. Restart the instrument and computer. In addition to the specified tasks listed above, the hard drive of the computer operating the instrument will be defragmented annually.

If a 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.

CENTRIFUGES:

Annually - Using a photoelectric tachometer, check and record the speed of the rotor. (Refer to Appendix B for the procedure.)

If a centrifuge is only used to pulse spin samples it is not necessary to check the centrifuge rotor speed using a tachometer. If a centrifuge is taken off line for repair/maintenance, prior to being put back in service, the annual tachometer check will be conducted.

FREEZERS (-20°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) Have authorized vendor perform re-certification and any necessary maintenance and repair. HEPA filters in this hood are replaced when indicated by the hood alarm system.

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.

pH METER:

Each Day of Use - Check and record the calibration at two set points. Check solution level in probe and replace if necessary.

PIPETTES:

Annually - Have an authorized vendor clean, calibrate and perform necessary maintenance and repair. Retain the calibration certificate generated by the vendor.

In the interim, pipettes can be partially disassembled and cleaned with 10% bleach and isopropanol. (Refer to Appendix C for the procedure.) Once the pipette has been reassembled conduct a quick calibration check using the graduated Rainin Pipette Tips discussed in Appendix C.

If a pipette appears to be out of calibration between normally scheduled performance/calibration checks, the pipette will be sent to an authorized vendor for repair. Retain the calibration certificate generated by the vendor.

MULTI-CHANNEL PIPETTES:

Annually - Have an authorized vendor clean, calibrate and perform necessary maintenance and repair. Retain the calibration certificate generated by the vendor.

If a multi-channel pipette appears to be out of calibration between normally scheduled performance/calibration checks, the pipette will be sent to an authorized vendor for repair. Retain the calibration certificate generated by the vendor.

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.

THERMAL CYCLER VERIFICATION UNIT:

Annually - Send the thermal cyler verification unit to an authorized vendor for calibration.

If the thermal cyler verification unit appears to be out of calibration between normally scheduled performance/calibration checks, the unit will be sent to an authorized vendor for repair. Retain the calibration certificate generated by the vendor.

THERMAL CYCLERS:

Quarterly - Perform and record the results of the temperature calibration, temperature non-uniformity and the system performance diagnostic tests. If any of the tests fail, contact an authorized vendor. Record the results of the diagnostics on the appropriate worksheet provided in Document 210-F601. (Refer to Appendix E for the procedure.)

If a thermal cyler is taken off line for repair/maintenance, prior to being put back in service, the quarterly diagnostic tests will be conducted.

THERMOMETERS:

Thermometers that are purchased as NIST Traceable will be considered as calibrated until their expiration date. No annual calibration check is required.

Thermometers which have exceeded their NIST Traceable expiration date and those that are not NIST Traceable will be subjected to the following:

Annually - Check calibration of each thermometer in use against a NIST or NIST traceable thermometer. (Refer to Appendix D for procedure.) If the temperature is greater than $\pm 1^{\circ}\text{C}$ from the temperature of intended use (e.g., 37°C , 56°C , or 95°C) the thermometer will not be used to monitor temperatures at that range. If the thermometer is greater than $\pm 1^{\circ}\text{C}$ for more than one temperature range the thermometer will be removed from service and documented.

A calibration check will be performed on all new thermometers which are not NIST Traceable against a NIST or NIST traceable thermometer prior to putting into service.

TYPE 1 WATER SYSTEM:

As Needed - Change filters/cartridges, sanitize, and/or have an authorized vendor clean and check unit.

UV CROSSLINKER:

Quarterly – Determine the amount of time required to irradiate laboratory tube racks, disposables and ultra-pure water in order to achieve a sterilizing effect. (Refer to Appendix F for the procedure.) All UV bulbs in a crosslinker will be replaced when the amount of time required to deliver 1 joule/cm² exceeds six minutes, or as necessary for individual bulbs due to outage.

WATER BATH:

Each Day of Use - Ensure the water level is at the correct height. If the temperature is greater than $\pm 1^{\circ}\text{C}$ from the specified temperature the thermostat will be adjusted to obtain the correct temperature. Record the final temperature. If the correct temperature cannot be achieved the water bath will be removed from service until it can be repaired.

Water baths will be cleaned on a quarterly basis or whenever contamination is thought to have occurred. Water baths will be cleaned by draining the basin and rinsing with a 10% bleach solution followed by a water rinse.

11.18.4 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.

Extraction Critical Reagents

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

A buccal, blood, bone or hair sample (depending on reagent(s) being quality controlled) will be extracted. The sample along with associated controls will be amplified in HVI using primer set 2. The sample must yield product comparable to the positive control. Control samples (HL-60, Negative amplification Control and Reagent Blank) must be sequenced and generate expected results. If the amplification of the sample is unsuccessful or DNA is detected in the reagent blank during sequencing, the sample will be re-amplified. If the amplification fails or if DNA is detected in the reagent blank for a second time, the sample will be re-extracted. If the amplification of the second extraction fails or DNA is detected in the reagent blank, the reagent being tested is considered to be unsuitable 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.

Amplification Critical Reagents

AmpliTaq™ Gold DNA Polymerase, 10X PCR Buffer, Deoxynucleotide Triphosphates (dNTP's), Bovine Serum Albumin (BSA)

Positive Control DNA (HL-60 or other in-house sample of known sequence) and associated negative control will be amplified in HVI using primer set 2 and sequenced. 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 the reagent being tested is considered to be unsuitable 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.

Positive Control – HL-60

New stock lots of HL-60, or the 20 pg/μl and 50 pg/μl dilutions prepared from a previously tested stock lot, will be amplified using control region primers F15971 and R599 and sequenced at HVI and HVII along with a negative amplification control. Sequence analysis must yield accurate typing results. Any additional dilutions will be created from the 20 or 50 pg/μl dilutions and will be considered valid for use.

Amplification/Cycle Sequencing Primers

Amplification/Sequencing primers will be resolubilized in TE buffer and diluted with ultra-pure water to 10μM working concentrations. Positive Control DNA (HL-60 or other in-house sample of known sequence) and associated negative control will be amplified and sequenced. Sequence analysis must yield accurate typing results for the areas being tested. Aliquots of working solutions will be stored at -20°C. (Refer to Appendix G for the procedure).

ExoSAP-IT®

Positive Control DNA (HL-60 or other in-house sample of known sequence) and associated negative control will be amplified in HVI using primer set 2. Resulting product will be subjected to the ExoSAP-IT® procedure and sequenced. The resulting sequences must have low background and peaks of high quality.

Sequencing Critical Reagents

Sequencing Kits

New lots of sequencing kits are tested by sequencing either HVI or HVII of positive control DNA (HL-60 or other in-house sample of known sequence) and an amplification negative control. The resulting sequences must have low background and peaks of high quality. The correct sequence must be obtained for the region tested.

Standard Reference Material (SRM) Kit 2392-I

The SRM kit is intended to provide quality control when performing the polymerase chain reaction (PCR) and sequencing of human mtDNA for forensic identifications, medical diagnosis, or mutation detection. The SRM material will provide the mtDNA Section with the ability to ensure the analysis procedure is functioning properly and can be calibrated to the national standard provided by the National Institute of Standards and Technology (NIST). The SRM (HL-60) is amplified and sequenced according to Section protocols. This check is performed annually.

11.18.5 REAGENTS, CHEMICALS AND SUPPLIES QUALITY CONTROL

Upon receipt of all reagents, chemicals and supplies the date of receipt, date opened, date of verification (if appropriate) and initials of the person performing the verification and/or opening the reagent/standard will be marked on all incoming reagents/chemicals/supplies.

NOTE: Reagents and supplies which have passed the expiration date will not be used on casework samples. However, these reagents/supplies may be used on non-critical samples (i.e., training samples, research samples, etc.) and will be appropriately labeled as such.

Safety Data Sheets (SDSs)

SDSs for chemicals used in the analysis of DNA can be found in the binder maintained in the Forensic Biology Section. SDSs for chemicals unique to the analysis of mtDNA can be found at the manufacturer's website or from a variety of online resources (e.g., <http://hazard.com/msds/>)

Laboratory Prepared Reagents and Solutions

All laboratory prepared reagents and solutions will be made with great care and using good laboratory practices. A log will be maintained for each laboratory prepared reagent/solution, including dilutions of laboratory concentrates. Each reagent/solution prepared will have the following information recorded in the log book:

- chemicals used
- commercial sources of chemicals
- lot numbers of chemicals
- date prepared
- initials of individual preparing reagent/solution
- expiration date
- other pertinent data, such as pH
- laboratory lot number

The lot number assigned to laboratory prepared reagents/solutions will consist of the date prepared (two digit numerical representation of month, day and year) and the initials of the preparer.

Example: Lot number 071411BCJ represents a reagent/solution prepared on July 14, 2011 by Brad C. Jenkins.

Exceptions to this lot number generation scheme are noted in Chapter 13, Equipment, Supplies and Reagents.

Labeling Requirements

All laboratory prepared reagents/solutions will be clearly labeled (as specified in Chapter 13 of this Manual).

Commercial reagents/chemicals will be labeled with the identity of the reagent and the expiration date. For commercial reagents without a supplied expiration date, a date of 3 years from the date of receipt will be used.

Storage and Disposal

All chemicals must be stored, used, and disposed of in a manner conforming to established safety requirements for that chemical.

A listing of commercial sources for reagents, chemicals, and supplies is found in the Mitochondrial DNA Section Procedures Manual, Chapter 13

REFERENCES

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.

Scientific Working Group on DNA Analysis Methods (SWGDM), Guidelines for mitochondrial DNA (mtDNA) nucleotide sequence interpretation. *Forensic Science Communications*, 5: Issue 2.
<http://www.fbi.gov/hq/lab/fsc/backissu/april2003/swgdammitodna.htm>

Scientific Working Group on DNA Analysis Methods (SWGDM). (2013) Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories.
Available: http://swgdam.org/SWGDAM%20mtDNA_Interpretation_Guidelines_APPROVED_073013.pdf

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.

COPYRIGHT © 2018

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

12 EQUIPMENT, SUPPLIES AND REAGENTS

12.1 The following are general instructions applicable to the preparation of all in-house reagents used in the Mitochondrial DNA (mtDNA) protocol:

Use graduated cylinders or pipettes closest in capacity to the volume being measured for preparing liquid reagents.

Type I water that has been autoclaved is used for preparing reagents in house.

Formulations may be scaled up or down as necessary to meet laboratory needs.

Store all in house reagents in sterile containers. Label all reagents with name of reagent, date prepared, lot number, and expiration date. (1 year from QC date if not otherwise specified)

12.2 SUPPLIES AND REAGENTS

12.2.1 The reagents and consumables below are listed in alphabetical order. Other sources for reagents and consumables may be used. If other sources are used, molecular biology grade reagents will be used where appropriate and available. Follow manufacturer's recommended expiration date.

12.2.2 For each reagent and consumable listed the company and catalog number is included.

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.

BIGDYE® TERMINATOR v1.1 CYCLE SEQUENCING KIT

Applied Biosystems, Catalog number 4337450, 100 reactions. Store at -20°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

BIGDYE® XTERMINATOR™ PURIFICATION KIT

Applied Biosystems, Catalog number 4376487, 20ml, Store at 4°C.

Kit components include:

- XTerminator™ Solution
- SAM™ Solution

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), 0.625µg/µl
Ambion, Catalog number AM2616, 50mg, Store at -20°C.

In a 50ml conical tube, combine 250µl of BSA (50mg/ml) and 19.75ml of ultra-pure water. Mix thoroughly. Aliquot and store at -20°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. (Smaller quantities can also be made).

DEMINEALIZATION 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, continue stirring. Adjust the final volume to 1 L with water. Autoclave, allow to cool and filter through a 0.2µm 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

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.

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

DNA MARKER XIV
Roche Diagnostics, Catalog Number 11721933001, 200µL, Store at -20°C upon receipt, 4°C when in use.

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 then filter through a 0.2µm filter to sterilize. Store at room temperature.

ETHYL ALCOHOL, 200 PROOF

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

ExoSAP-IT®

GE Healthcare, Catalog number US78201, 500 reactions. Store at -20°C.

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

Dissolve 6.06g of Tris base in 800ml water. Adjust to pH 8.0 by adding drops of concentrated HCL. Adjust to final volume of 1L 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

GeneAmp® dNTP BLEND, 10mM

Applied Biosystems, Catalog number N8080260, Store at -20°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

HYDROCHLORIC ACID

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

LOADING BUFFER, 5X

Combine 0.01g bromophenol blue, 5ml glycerol, 2ml 0.5M EDTA, 3ml TE and mix well. Divide into 1 mL aliquots and store at -20 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, 10µg, Store at -20°C.

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

PRIMERS

Trilink Biotechnologies or other suitable vendor, Primers are typically HPLC-purified. Lyophilized primers are stored at -20°C.

Each primer is reconstituted from the lyophilized form using TE to achieve a final concentration of 100 µM (100 nanomoles/ml). Storage consist of small aliquots at 10 µM (10 nanomoles/ml) concentration diluted with ultra-pure water stored at -20°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

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

SODIUM PHOSPHATE MONOBASIC (NaH₂PO₄ • H₂O), 99% min.

VWR, Catalog number JT4011-1, 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 0.606g Tris base in 400ml water and adjust pH to 7.5 with HCL (add HCL slowly as

only a small volume is required). Add 0.0185g of EDTA and readjust pH to 7.5 if necessary. Bring final volume to 500ml. Autoclave, allow to cool then filter through a 0.2µm filter to sterilize. Store at room temperature. UV-irradiate before use. TE will expire three years after preparation date.

ULTRAPURE™ 10X TAE BUFFER

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

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

ULTRA-PURE WATER

Type I water is filtered with 0.2µm filter, autoclaved, and subjected to UV irradiation. 15-50ml conical tube aliquots will be subjected to 15 J/cm² exposure time as determined in Appendix F. 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

250µL WIDE ORIFICE PIPETTE TIPS

Rainin, Catalog number HR-250WS

12.3 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., Freedom Series SR)

Filter, SYBR Green (e.g., Fotodyne Glass Filter for SYBR® Green Stain)

Freezers (-20°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 (various sizes, to cover 1 μ L to 1000 μ L and 1ml-50ml)

pH Meter

Refrigerators (4°C)

Rotary Tool (e.g., Dremel® MultiPro™)

Scanner, Flatbed

Sonicator

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

Ultrasonic Water Bath

UV Crosslinker

UV Transilluminator

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

Water Baths, Rotating (e.g., Bellco Hot Shaker)

COPYRIGHT © 2018
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

Special Precautions

- Disposable gloves, surgical mask, lab coat and surgical cap 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 © 2018

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

APPENDIX B – CENTRIFUGE SPEED CHECK BY TACHOMETER

Upon receipt the calibration of each centrifuge will be checked by verifying the speed of the rotor. Once a year thereafter or following adjustments or repairs, the speed of the rotor will be checked.

The speed or number of rotations per minute (RPM) will be checked using a photoelectric tachometer. Use a high contrast label to mark a visibly accessible spot on the rotor. Follow the manufacturer's instructions provided with the tachometer to take the RPM readings. The speed of the centrifuge will be measured with the rotor installed and the cover closed. If the rotor speed is found to vary more than ± 1000 RPMs from the set speed of 10,000 RPMs, an authorized vendor will be called to recalibrate the speed of the centrifuge.

COPYRIGHT © 2018

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

APPENDIX C – CARE AND MAINTENANCE OF PIPETTES**1. Cleaning and Checking the Sealing System**

Remove the tip ejector arm by pulling downward. Unscrew the shaft coupling slowly while holding the body and shaft firmly. The internal piston is spring loaded and will release when disengaged. Remove the white shaft from the piston assembly.

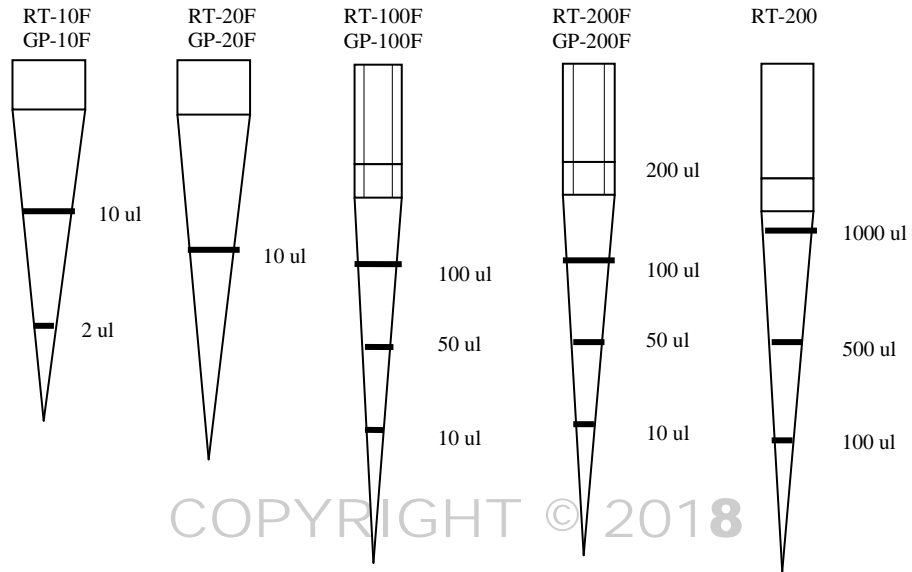
- a. Shaft: Inspect the tip sealing end of the shaft for scratches, wear marks and splits.
- b. Piston: Expose the piston surface. If the stainless steel surface is highly-polished, the piston is in good condition and is not damaged. If the piston is highly stained or dirty, clean with isopropyl alcohol and a Kimwipe. Allow to air dry before reassembly. If the piston surface is scratched, heavily stained, corroded, or bent, the piston must be replaced.
- c. Polyethylene Seal: Slide the white polyethylene seal up and down the piston. If the seal has resistance throughout the full length of the piston, it remains in good condition. If the resistance is spotty or non-existent, replace the seal. Seal replacement will not effect the pipette calibration.

2. Routine In-House Verification of the Pipette Calibration

Once the pipette has been reassembled after cleaning, conduct a quick calibration check using graduated Rainin Pipette RT tips. Verify the pipetting volumes of the pipette as specified in the Pipette Verification Table below. The pipette tips are guaranteed to be accurate $\pm 3\%$ at the specified reference lines. Should the pipette be visibly incorrect according to the reference line, the pipette will be sent to the manufacturer for repair.

Pipette Verification Table

Pipette	Tip RT-10F		Tip RT-10F	Tip RT-20F	Tip RT-200F			Tip RT-200		
	2 μ L	10 μ L								
P-10	2 μ L	10 μ L								
P-20			2 μ L	10 μ L						
P-100					50 μ L	100 μ L				
P-200					50 μ L	100 μ L	200 μ L			
P-1000								100 μ L	500 μ L	1000 μ L



VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

APPENDIX D – THERMOMETER TEMPERATURE CALIBRATION

Thermometers will be verified upon receipt for accuracy at the temperature of intended use. If available, a thermometer certified by the National Institute of Standards and Technology (NIST) should be used for comparison. However, a thermometer that is traceable to a NIST thermometer may also be used.

1. Tag each thermometer with an identifying number.
2. Place all of the thermometers in a vessel containing water at a temperature approximately 4°C below the temperature of intended use. For the temperature range 1-6°C, add salt and ice to the water to bring the temperature below 0°C.
3. Record the temperature shown on each thermometer.
4. Next warm water to approximately 4°C above the temperature of intended use.
5. Place thermometers into the warmed water and record the temperature of each thermometer.
6. All thermometers should be within $\pm 1^\circ\text{C}$ of the NIST thermometer or a thermometer traceable to a NIST thermometer. Any thermometer that varies more than $\pm 1^\circ\text{C}$ in the range of intended use will not be used.

NOTE: If a thermometer is not acceptable at a specified temperature range (e.g., 37°C, 56°C, or 95°C) this does not preclude the thermometer from being used at another temperature providing it has passed the quality control test.

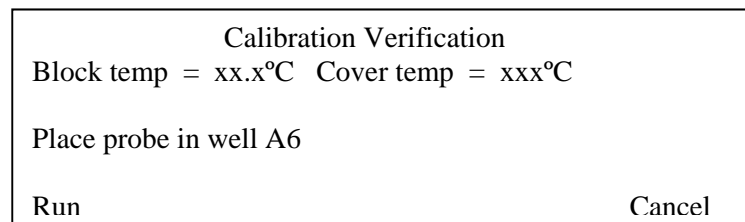
COPYRIGHT © 2018
VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

APPENDIX E – THERMAL CYCLER 9700 TEMPERATURE CALIBRATION, TEMPERATURE NON-UNIFORMITY, AND SYSTEM PERFORMANCE DIAGNOSTIC TESTS

1. DNA THERMAL CYCLER 9700 TEMPERATURE CALIBRATION VERIFICATION TEST

1.1. Verifying the Temperature Calibration

- 1.1.1. If the sample block heated cover is in the forward position, lift the lever, then slide the heated cover back.
- 1.1.2. Place the 9700 Temperature Verification Frame on the sample block.
- 1.1.3. Coat well A6 with mineral oil using a cotton swab.
- 1.1.4. Place the 0.2 mL probe assembly into well A6. Carefully thread the probe wire through the channel in the 9700 temperature verification frame to prevent damage to the probe and lead wires.
- 1.1.5. Make sure the probe is connected to the digital thermometer and is seated properly. Close the cover carefully by sliding the heated cover forward, and pulling the lever down.
- 1.1.6. Turn on the digital thermometer and follow the instructions in the Temperature Verification Kit on how to operate the digital thermometer, Model 4500.
- 1.1.7. Turn on the GeneAmp PCR System 9700. The main menu appears:
 - 1.1.7.1. Select Utilities (F4)
 - 1.1.7.2. Select Diagnostics (F1)
 - 1.1.7.3. Select TmpVer (F3)
- 1.1.8. The Temperature Verification screen appears, select Temp (F1). This automatically configures the system 9700 for the Calibration Verification Test. The following screen appears:



F1 F2 F3 F4 F5

- 1.1.9. Use the digital thermometer to take temperature readings of the sample well connected to the 0.2 mL Probe Assembly. You will take a reading at two different setpoint temperatures.

- 1.1.10. Select Run (F1) to start the Calibration Verification Test. The following screen appears with the setpoint values displayed.

Calibration Verification	
Block temp = xx.x°C	Cover temp = xxx°C
Setpoint is 85°C	
Cover must be within 10°C of 85°C	
Cancel	

F1 F2 F3 F4 F5

- * The cover must be within 1°C of 105°C. It may take several minutes for the system to ramp up.

- 1.1.11. The Calibration Verification screen counts down the time until the setpoint is reached. When the “Stabilization at setpoint” value decrements to zero, read the digital thermometer.

- 1.1.12. Using the numeric keys, type the value displayed on the digital thermometer in the “Enter actual block temperature” field displayed on the screen.

- * The digital thermometer displays a four-digit value; round this off to three digits before typing it in the Calibration Verification screen.

- 1.1.13. Press Enter on the keypad. The system automatically begins the second reading (45°C setpoint). The Calibration Verification screen appears with the setpoint value displayed.

Calibration Verification	
Block temp = xx.x°C	Cover temp = xxx°C
Setpoint is 45°C	
Cover must be within 30°C of 45°C	
Cancel	

F1 F2 F3 F4 F5

- * The cover must be within 1°C of 105°C.

- 1.1.14. Repeat steps 1.1.11 and 1.1.12, then press Enter from the keypad.

- 1.1.15. The system 9700 evaluates the calibration of the sample block temperature for the setpoint values that were entered and displays the results. A summary screen appears at the conclusion of the test.

Calibration Verification	
Actual temperature at 85°C	xx.x°C
Actual temperature at 45°C	xx.x°C
Accept	Cancel

F1 F2 F3 F4 F5

- 1.1.16. Record the results on the Calibration Verification Test Data Sheet (Document 210-F601), then select Accept (F1).
- 1.1.17. If the sample block module is properly calibrated the Calibration Verification screen will display “Calibration is Good”.
- 1.1.18. If the sample block module does not pass the Calibration Verification Test the screen will display “Instrument may require service. Contact Applied Biosystems Technical Support”
 - 1.1.18.1. If the test fails, repeat the procedure to make sure the digital thermometer was not misread or that errors were not made entering data.
 - 1.1.18.2. If the test fails again, contact Applied Biosystems Technical Support.
- 1.1.19. End the test by selecting Exit (F5).

2. TEMPERATURE NON-UNIFORMITY TEST

- 2.1. Verify the temperature uniformity of the system 9700 with a 0.2 mL sample block module

Perform the procedure as described in the following steps.

- 2.1.1. If the sample block heated cover is in the forward position, lift the lever, then slide the heated cover back.
- 2.1.2. Place the 9700 Temperature Verification Frame on the sample block.
- 2.1.3. Coat wells A1, A12, C4, C9, F4, F9, H1, and H12 with mineral oil using a cotton swab.
- 2.1.4. Place the 0.2mL probe assembly into well A1. Carefully thread the probe wire through the channel in the 9700 temperature verification frame to prevent damage to the probe and lead wires.
- 2.1.5. The system 9700 evaluates the calibration of the sample block temperature for the setpoint values that were entered and displays the results. A summary screen appears at the conclusion of the test.

Calibration Verification	
Actual temperature at 85°C	xx.x°C
Actual temperature at 45°C	xx.x°C
Accept	Cancel

F1 F2 F3 F4 F5

- 2.1.6. Record the results on the Calibration Verification Test Data Sheet (Document 210-F601), then select Accept (F1).
- 2.1.7. If the sample block module is properly calibrated the Calibration Verification screen will display “Calibration is Good”.
- 2.1.8. If the sample block module does not pass the Calibration Verification Test the screen will display “Instrument may require service. Contact Applied Biosystems Technical Support”.

Appendix E – Thermal Cycler 9700 Temperature Calibration, Temperature Non-Uniformity, and System Performance Diagnostic Tests

2.1.8.1. If the test fails, repeat the procedure to make sure the digital thermometer was not misread or that errors were not made entering data.

2.1.8.2. If the test fails again, contact Applied Biosystems Technical Support.

2.1.9. End the test by selecting Exit (F5).

TNU Performance	
Sample temp = xx.x°C Cover temp = xxx°C	
Place Probe in well A1	
Press Run	
Run	Cancel

F1 F2 F3 F4 F5

2.1.10. Press Run (F1) and the following screen appears with the setpoint value displayed.

TNU Performance	
Sample temp = xx.x°C Cover temp = xxx°C	
Setpoint is 37°C	
Sample must be within 1°C of setpoint	
	Cancel

F1 F2 F3 F4 F5

*The sample block must be within 1.0°C of the setpoint and the cover must be within 1°C of 35°C. It may take a few minutes for the system to reach the setpoint temperature.

2.1.11. The TNU Performance screen counts down the time until the setpoint is stabilized. When the “Stabilization at setpoint” value decrements to zero, read the digital thermometer.

2.1.12. Using the numeric keys, type the value displayed on the digital thermometer in the “Enter actual block temperature” field displayed on the screen.

*The digital thermometer displays a four-digit value; round this off to three digits before typing it in the Calibration Verification screen.

- 2.1.13. Press enter on the keypad. The TNU Performance screen appears with the setpoint value displayed at 94°C. When the sample temp reaches 94°C the setpoint value changes to 37°C and the following screen appears.

TNU Performance				
Sample temp = xx.x°C Cover temp = xxx°C				
Place probe in well A12				
Press Run				
Run		Cancel		

F1 F2 F3 F4 F5

*During the 94°C setpoint test this will be reversed. The first setpoint value displayed is 37°C, when this sample temp setpoint is reached the setpoint value changes to 94°C and the TNU Performance screen appears.

- 2.1.14. Slide the heated cover back and place the probe in A12. Make sure the probe is seated properly. Close the cover carefully by sliding the heated cover forward, and pulling the lever down. Repeat steps 2.1.10 through 2.1.13. Complete the steps for all 8 wells: A1, A12, C4, C9, F4, F9, H1, H12.
- 2.1.15. After reading the last well (H12) press Enter and the 9700 system will begin the second reading (94°C setpoint). The TNU Performance screen appears with the setpoint displayed at 94°C the cover temp will increase and must be within 1°C of 105°C. It may take a few minutes for the system to stabilize at the setpoint temperature.
- 2.1.16. When the system has stabilized the TNU Performance screen with the “place probe in well A1” prompt appears. Repeat steps 2.1.9 to 2.1.13.
- 2.1.17. After the last reading for setpoint 94°C is completed, the system 9700 evaluates the uniformity of the sample block temperature for the setpoint values that were entered and displays the results. A summary screen appears at the conclusion of the test.

Well	94°C	37°C		Well	94°C	37°C
A1	xx.x	xx.x		F4	xx.x	xx.x
A12	xx.x	xx.x		F9	xx.x	xx.x
C4	xx.x	xx.x		H1	xx.x	xx.x
C9	xx.x	xx.x		H12	xx.x	xx.x
Accept			More			Cancel

F1 F2 F3 F4 F5

- 2.1.18. Record the results on the Temperature Non-Uniformity Test Data Sheet (210-F601), then select Accept (F1).
- 2.1.19. When the system completes the Temperature Non-uniformity test, the TNU Performance screen appears with either “Pass” or “Fail” after each setpoint.
- 2.1.19.1. If the temperature of the sample block wells is uniform then the test has passed.
- 2.1.19.2. If the temperature variation of the sample block wells exceeds performance expectations the test has failed.

Appendix E – Thermal Cycler 9700 Temperature Calibration, Temperature Non-Uniformity, and System Performance Diagnostic Tests

2.1.19.2.1. If the test fails, repeat the procedure to make sure the digital thermometer was not misread or that errors were not made entering data.

2.1.19.2.2. If the test fails again, contact Applied Biosystems Technical Support.

2.1.20. End the test by selecting Cancel (F5).

2.1.21. If all measurements are completed, remove the probe and the Temperature Verification Frame from the sample block and turn off the digital thermometer.

2.1.22. Clean the wells of the sample block using cotton swabs.

3. SYSTEM PERFORMANCE DIAGNOSTICS: RATE TEST AND CYCLE TEST

3.1. Testing the integrity of the cooling and heating system

Perform the procedure as described in the following steps:

3.2. Before beginning the tests, an empty 96-well plate with full plate cover or caps must be in the sample block.

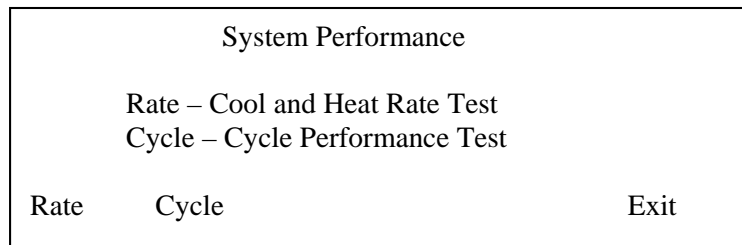
3.2.1 Turn on the GeneAmp PCR System 9700. The main menu appears:

3.2.1.1 Select Utilities (F4)

3.2.1.2 Select Diagnostics (F1)

3.2.1.3 Select System (F2)

3.2.2 The System Performance Screen appears.



F1

F2

F3

F4

F5

3.2.3 Select Rate Test (F1) to verify that the Peltier units are operating correctly. The System Performance screen displays the following message: "WARNING!! Install the appropriate empty consumables into the sample block. Refer to System Performance Section of the Block User Manual."

3.2.4 Select Cont. (F1) after the plate and cover has been installed and the Cool and Heat test screen appears.

3.2.5 The instrument then runs through a series of tests where the sample block ramps to and stabilize at 35°C, 94°C and 4°C, which is displayed on the screen. At the conclusion of the test, the Cool and Heat screen appears displaying the results and whether the test results passed or failed. Record this information in the Cool and Heat Rate Test area on the worksheet (210-F601). If the test fails contact Applied Biosystems Technical Support.

Cool and Heat Rate Test	Pass
Heating rate: x.xx °C/s	
Cooling rate: x.xx °C/s	
Print	Cancel

F1 F2 F3 F4 F5

3.2.6 The following table lists the passing ranges for the Rate test:

	Heating Rate	Cooling Rate
Gold 96-well	3.0 to 6.0 °C/second	3.0 to 6.0 °C/second
Aluminum 96-well	1.5 to 2.5 °C/second	1.4 to 2.5 °C/second

3.2.7 Return to the System Performance screen by selecting Cancel (F5) and select Cycle Test (F2) to verify that the PCR cycling function operates properly. The System Performance screen displays the following message: “WARNING!! Install the appropriate empty consumables into the sample block. Refer to System Performance Section of the Block User Manual.” Select Cont. (F1) after the plate and cover has been installed.

3.2.8 The Cycle Test executes a standard PCR cycle reaction (6 cycles), measures, and reports the average cycle time, and the cycle to cycle variation. At the conclusion of the test, the Cycle Test screen will display the results and whether the test results passed or failed. Record this information in the Cycle Performance area on the worksheet (210-F601). If the test fails contact Applied Biosystems Technical Support.

Cycle Performance	Pass
Average Cycle Time: xxx.x sec	
Cycle time STD: x.x sec	
Print	Cancel

F1 F2 F3 F4 F5

3.2.9 The following table lists the passing ranges for the Cycle Test.

Average Cycle Time	<= 160 sec
Cycle Time STD	< 5 sec

3.2.10 Select Cancel (F5) to exit the test.

APPENDIX F – UV STERILIZATION**UV Lamp Intensity Calibration**

Periodically, over the lifespan of the UV bulb the amount of energy delivered will vary. The amount of energy delivered must be estimated so that sufficient irradiation of laboratory equipment will produce a sterilizing effect. This is accomplished by measuring the amount of time the UV equipment takes to deliver 0.9999 J/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 $\mu\text{J}/\text{cm}^2$.
- Repeat this measurement for a total of 3 times and enter the times in the table.

Date	Calibration Time		Energy Rate Delivery (J/cm ² /minute)	Conical Tubes 15 J/cm ² Time	Micro Tubes 6 J/cm ² Time	Microcons 4 J/cm ² Time
	Minutes	Seconds				

This table is provided as an example, the actual 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 G – 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 (P-2, P-10, P-20, P-100, P-200, P-1000)
 Microcentrifuge
 Hood
 Bleach, 10% commercial (7 mM sodium hypochlorite solution)
 Gloves
 Freezer, -20°C
 Isopropanol
 Kim-wipes
 Laboratory coat
 Racks, tube
 Safety glasses
 Sleeve Protectors
 Water, ultra-pure
 TE Buffer (10 mM Tris, 1 mM EDTA, pH 7.5)
 Tips, aerosol-resistant (e.g., for P-10, P-100, P-1000 pipettes)

CONCENTRATION DETERMINATION

Primer concentration is verified by UV absorbance measurement

- Resolublize the primer in TE buffer so that the final concentration is 200µM.
- Vortex the stock 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 50µl ultra-pure water and discard.
- Add 2.5 µl of the stock 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 50 µl of the sample back 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 stock solution; however, the aliquot concentration will be re-verified prior to use. Aliquots will be prepared with ultra-pure water.

SPECIFICITY DETERMINATION

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

- Set up amplification using a Negative Control and HL-60 Control DNA in the following quantities:
 - Control Region Amplification
 - 500 pg
 - 250 pg
 - 50 pg

- Primer Set Amplification
 - 200 pg
 - 100 pg
 - 20 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 for each concentration of HL-60 Control DNA sample.
- Cycle sequence the amplified products using the optimal amount of amplification 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.

VIRGINIA
DEPARTMENT
OF
FORENSIC SCIENCE

**APPENDIX H – PROCEDURE FOR DRYING DOWN AND RESOLUBILIZING
MITOCHONDRIAL DNA EXTRACTS**

1. REAGENTS, MATERIALS AND EQUIPMENT

- DNA concentrator/evaporator
- Microcentrifuge
- Pipettes
- Plastic ziploc bags
- Microcentrifuge tubes, 1.5 mL
- Kimwipes
- Ultra-Pure Water

2. 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 mtDNA sample has been dried down close the microcentrifuge cap and store in a plastic ziploc bag or similar storage medium.

3. PROCEDURE FOR RESOLUBILIZING EXTRACTS

- To resolubilize the dried down extracted mtDNA sample and Reagent Blank, 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.