PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA SEQUENCING

VERSION 1.0

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1.0 DNA EXTRACTION METHODS

1.1 Chelex DNA Extraction From Whole Blood, Bloodstains, or Blood Scraped or Swabbed Off a Surface, Tissue, or Saliva Stains

1. Fill out the extraction worksheet.
2. Extract either evidence or exemplars. Do not extract both together.
3. Pipette 1 mL of sterile deionized water into each of the tubes in the extraction rack.
4. Mix the tubes by inversion or by vortexing.
5. Incubate at room temperature for 15 - 30 minutes. Mix occasionally by inversion or by vortexing.
6. Spin in microcentrifuge for 2 - 3 minutes at 10,000 to 15,000 x g (14,000 rpm).
7. Carefully remove the supernatant (all but 20 - 30 µL). If the sample is a bloodstain or a swab, leave the substrate in the tube with the pellet. (The supernatant can be frozen and retained for analysis according to the Forensic Biochemistry and Hematology Manual or it can be discarded if it is not needed).
8. Add 175 µL of 5% Chelex. Mix the Chelex by inversion or by vortexing each time to ensure that Chelex beads are transferred as well.
9. Incubate at 56°C for 15 - 30 minutes.
10. Vortex at high speed for 5 - 10 seconds.
11. Incubate at 100°C for 8 minutes using the screw down rack.
12. Vortex at high speed for 5 - 10 seconds.
13. Spin in a microcentrifuge for 2 - 3 minutes at 10,000 to 15,000 x g (14,000 rpm).
14. Pipette 20 µL into a microcentrifuge tube for Quantiblot Analysis to determine human DNA concentration. At the same time prepare 1:10 dilutions for each sample in microcentrifuge tubes for Quantiblot Analysis as well (18 µL TE^{-4} and 2 µL DNA).
15. Store the remainder of the supernatant at 2 - 8°C or frozen.

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1.2 DNA Extraction From Hair

Prior to analysis, the New York City Police Department Trace Evidence Unit or any other reputable outside agency must examine unknown hair samples (head, pubic and axillary) by comparison microscopy. Only those hairs that have been associated through comparison will be accepted for mitochondrial DNA analysis. **A copy of the report should be submitted by the agency requesting analysis.** The New York City Office of Chief Medical Examiner Department of Forensic Biology will proceed with analysis if and when the submitting agency provides a known blood sample or saliva sample from applicable subjects.

1.2.1 Removal of Hair Sample From Microscopic Mount

Note: Removal of hairs from microscopic slides should be performed under a chemical fume hood.

1. Fill a slide chamber with fresh xylene.
2. Place the slide into the chamber and let sit for 1-2 min.
3. Remove the slide from the chamber and place on several sheets of sterile Kimwipe.
4. Using a sterile razor blade or forceps, slide the cover slip off of the slide.
5. Add 10-20 µL of fresh xylene to the embedded hair sample. Allow 10-20 seconds for the Permount to dissolve, then tease out the hair sample with sterile forceps.
6. Place the hair sample into a sterile 1.5 mL centrifuge tube containing xylene, and finger vortex to remove any adhering mounting media.
7. Transfer the hair into a 1.5 mL centrifuge tube containing 100% ethanol.

1.2.2 Preparation of Hair Sample For Extraction

1. Remove the hair using sterile forceps and place onto a sterile piece of weighing paper. Basic microscopic examinations, using a stereoscopic microscope, of the hair sample must be performed before attempts at DNA typing are performed. Physical characteristics of each hair sample should be noted. The following characteristics should be recorded:

    1. Type of hair sample (head, pubic, axillary, etc.)
    2. Color of hair sample
    3. Shed or plucked hair sample
    4. Growth stage of the hair sample
    5. Presence or absence of a root containing sheath cells

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6. Length and approximate volume of hair sample

Volume = $V = \pi r^2 l$
where: r = (0.5)(average diameter)
l = length of hair

7. Note the presence of any extraneous body fluid or tissue on the hair sample

Note: The findings within these examinations should be compared with findings reported by the outside agency.

2. Place the hair in a 1.5 mL centrifuge tube containing 1 mL of 5% (w/v) Terg-a-zyme solution.

3. Transfer sample into a floating rack and agitate for approximately 20 min. in an ultrasonic water bath.

4. Place the hair sample onto a of sterile piece of weighing paper. If surface debris were present in step 1, do not discard the wash buffer containing the debris. Label this tube surface debris remains and retain. The buffer can be discarded if surface debris were not present in step 8.

Note: Surface debris will be examined on a case to case basis. The type of cells present in the surface debris remains will dictate the type of extraction used to isolate DNA. The analyst assigned to the particular case should consult with a manager of the Forensic Biology laboratory prior to extraction of the fraction. The fraction should be packaged and retained with the remainder of the case evidence if the fraction is not to be used.

5. Place the hair sample into a 1.5 mL centrifuge tube containing 1 mL of 5% (w/v) Terg-a-zyme solution.

6. Transfer sample into a floating rack and agitate once again for approximately 20 min in an ultrasonic water bath.

7. Examine the hair under a stereoscopic microscope for the presence of remaining surface debris. If surface debris are still present repeat washing steps 5-7 until the hair sample is void of debris. If surface debris are not present proceed to step 8.

8a. If the hair root contains sheath cells, clip the hair sample approximately 0.5 cm above the root using a sterile pair of scissors. Place the root into a labeled sterile 1.5 mL centrifuge tube containing 1 mL of sterile dH_20. Return the hair shaft in a labeled sterile 1.5 mL centrifuge tube for possible future analysis.

-or-

8b. If the hair root does not contain sheath cells or if the root is not present, clip approximately 2 cm of the shaft and place the sample into a labeled sterile 1.5 mL centrifuge tube containing 1 mL of sterile dH_20.

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Note: If a root containing sheath cells is present, proceed to the **Chelex DNA Extraction From Hair Root** section 1.2.3. If there are no visible sheath cells surrounding the hair root or if the root is absent proceed to the **Organic DNA Extraction From Hair Shaft** section 1.2.4.

1.2.3 Chelex DNA Extraction From Hair Root

1. Fill out the extraction worksheet.

2. Using sterile forceps, add the root portion of the hair to 200 µL of 5% Chelex in a 1.5 mL microcentrifuge tube.

3. Incubate at 56°C (at least 6 to 8 hours) or overnight.

   Note: Check that the hair is completely immersed in the Chelex solution before incubating.

4. Vortex at high speed for 5 to 10 seconds.

5. Incubate at 100°C for 8 minutes using a screw down rack.

   Note: Check that the hair is completely immersed in the Chelex solution before boiling.

6. Vortex at high speed for 5 to 10 seconds.

7. Spin in a microcentrifuge for 2 to 3 minutes at 10,000 to 15,000 x g (14,000 rpm).

8. Pipet 20 µL neat and also a 1:10 dilution into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration.

9. Store the remainder of the supernatant at either 2 to 8°C or frozen. To re-use, repeat Steps 6 through 7.

1.2.4 Organic DNA Extraction From Hair Shaft

1.2.4.1 Grinding the Hair

Micro Tissue Grinders are used to grind hairs for DNA extraction. The grinders consist of matched sets of mortars and pestles, and should be used as such. To facilitate working with the grinders, the grinders may be placed in a small plastic tube, such as a 1.5 mL centrifuge tube. Grinders should only

be used twice. Once to generate an extraction control and a second time to extract the unknown hair sample. Prior to beginning hair extraction procedures, the grinders should be cleaned using the following protocol:

1. Carefully rinse the grinders with dH_2O. Using cotton tip applicators and warm 5% (w/v) Terg-a-zyme™ detergent scrub the pestles and the insides of the mortars.

2. Rinse the grinders with sterile dH_2O and add approximately 200 µL of 1N H_2SO_4. (Note: Gloves, protective eye wear, and a lab coat should be worn, and all work should be performed under a fume hood). Place the pestles in the matching mortars and briefly simulate grinding. Allow the mortars and pestles to soak in 1N H_2SO_4 for a minimum of 20 minutes.

3. Rinse the grinders with dH_2O. Remove the pestles and place the mortars in a microcentrifuge. Pulse spin at high speed to collect the remaining water.

4. Remove the mortars from the microcentrifuge and remove the remaining water with a pipette.

5. Place the grinders in a rack and place in a Stratalinker® for a minimum of 15 minutes.

1.2.4.2 DNA Extraction

1. A reagent blank should be prepared for each grinder to be used. Prepare the reagent blank by placing 200 µL of mtDNA extraction buffer into the micro tissue grinder to be used. Briefly simulate grinding. Remove the pestle and transfer the liquid to a sterile 1.5 mL plastic tube. Set aside until step 6. The extraction control should be labeled as the ***sample unique identifier*-EC.**

2. To the same grinder add 200 µL of stain extraction buffer. Using sterile forceps place the hair fragment into the micro tissue grinder.

3. Move the pestle up and down to force the hair into the bottom of the mortar. Grind until fragments are no longer visible.

4. Remove the pestle from the mortar. If liquid is adhering to the pestle head, gently pass it along the inner lip of the mortar until liquid flows to the bottom of the mortar.

5. Transfer the homogenate liquid to a sterile 1.5 mL plastic tube.

6. Add 1 µL of proteinase K (20 mg/mL) and 8 µL of 1M DTT to each tube.

7. Vortex on low speed and briefly centrifuge. Place the tubes in a water bath and incubate at 56°C for a minimum of 2 hours and maximum of 24 hours.
8. Remove the tubes from the water bath and briefly spin in a microcentrifuge to force the

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condensate into the bottom of the tubes.

9. Add 200 µL of phenol/chloroforrn/isoamyl alcohol (PCIA, 25:24:1) to each tube.

CAUTION: PCIA is an irritant and is toxic. Its use should be confined to a hood. Gloves and a mask should be worn.

10. Vortex 30 seconds to attain a milky emulsion, then spin the tubes in a microcentrifuge 3 minutes at 14,000 rpm.

11. Insert Microcon 100 columns (blue) into labeled microcon tubes for each sample.

12. Prepare the Microcon 100 concentrators by adding 200 µL of sterile dH_2O to the filter side (top) of each concentrator.

13. Carefully remove the aqueous (top layer) phase from each tube, and transfer to the appropriate concentrator. (Avoid drawing any of the proteinaceous interface into the pipette tip.)

14. Spin the Microcon 100 concentrators for 20 minutes at 2500 rpm. (Note: additional spin time may be required to filter the entire volume.)

15. Discard the wash and return the filtrate cups to the concentrators.

16. Add 400 µL of hot (80°C-90°) sterile dH_2O to the filter side of each Microcon 100 concentrator.

17. Spin again at 2500 rpm for 20 minutes.

18. Add 30 µL of hot (80°C-90°) sterile dH_2O to the filter side of each Microcon 100 concentrator.

19. Invert sample reservoir and place into a newly labeled tube. Spin at 3500 rpm for 3 minutes to collect sample.

20. Discard sample reservoir and adjust sample volume to 60 µL with sterile dH_2O.

Note: DNA extracts from hair shafts do not need to be quantitated. They can be amplified using 20 µL of extracted sample without quantitation.

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1.3 Organic DNA Extraction From Human Tissue or Human Bone

1. Obtain human tissue/bone specimens. Fill out an extraction worksheet.
2a. Cut a 0.5 x 0.5 cm tissue sample into small sections and place into a newly labeled 1.5 mL microcentrifuge tube.

-or-

2b. For a bone specimen proceed to the **Extraction of Bone Using the Micro-Mill ® Grinder** section 1.4.

3. Create a master mix for (n + 1) samples with:

400 µL of mtDNA Extraction Buffer
10 µL of 20 % SDS
13.6 µL of proteinase-K (20 mg/mL)

4. Vortex at high speed for 5 to 10 seconds.

5. Add 400 µL of master mix to each tissue/bone sample.

6. Incubate in a rotating water bath at 56°C overnight.

7. Add 300 µL of Phenol:Chloroform:Isoamyl Alcohol (25:24:1). Vortex 30 seconds to attain a milky emulsion, then spin the tubes in a microcentrifuge 3 minutes at 14,000 rpm.

CAUTION: PCIA is an irritant and is toxic. Its use should be confined to a hood. Gloves and a mask should be worn.

8. Insert Microcon 100 columns (blue) into labeled microcon tubes for each sample.

9. Prepare the Microcon 100 concentrators by adding 100 µL of dH_2O to the filter side (top) of each concentrator.

10. Carefully remove the aqueous (top layer) phase from each tube, and transfer to the appropriate concentrator. (Avoid drawing any of the proteinaceous interface into the pipette tip.)

11. Spin the Microcon 100 concentrators for 20 minutes at 2500 rpm. (Note: additional spin time may be required to filter the entire volume).

12. Discard the wash and return the filtrate cups to the concentrators.

13. Add 400 µL of hot (80°C-90°) sterile dH_2O to the filter side of each Microcon 100 concentrator.

14. Spin again at 2500 rpm for 20 minutes (Note: additional spin time may be required to filter the entire volume).

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15. Add 30 µL of hot (80°C-90°) sterile dH_2O to the filter side of each Microcon 100 concentrator.

16. Invert sample reservoir and place into a newly labeled tube. Spin at 3500 rpm for 3 minutes to collect sample.

17. Discard sample reservoir and adjust sample volume to 60 µL for dried bone or 200 mL for fresh bone or tissue using sterile dH_2O.

18. Pipet 20 µL neat and also a 1:10 dilution into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration. Store the remainder of the supernatant at 2 to 8°C or frozen.

Note: Skeletal remains containing dried bone marrow do not need to be quantitated. They can be amplified using 20 µL of extracted sample without quantitation.

1.4 Extraction of Bone Using the Micro-Mill ® Grinder

1a. For bone specimens 3 x 2 cm in dimension, proceed to step 2.

-or-

1b. For bone specimens larger than 3 cm in length, place whole specimen onto a sterile bench pad under a biological hood. Using a Dremel or shock saw equipped with a new or sterile blade (cleaned with 10% bleach and 100% ethanol), cut the bone specimen into an approximate 3 x 2 cm length. **Protective eye ware, lab coats and facial mask should be worn when cutting bone.**

2. Using a Dremel equipped with an emery disk, sand the outer surface of the bone so that the outer surface appears free of dirt and debris. The emery disk should only be used once then discarded.

3. Place the bone in a 50 mL conical vial containing 90% EtOH. Shake the tube 10 times. Decant the EtOH into a waste container and repeat the wash step. Place the bone fragment into a weigh boat and allow it to air dry under a hood.

4. When the bone specimen has dried, record gross weight.

5. Transfer the bone specimen into a sterile 50 mL conical vial (do not cap) and submerge the vial in liquid nitrogen for 1-2 minutes.

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6. Crush the bone in a clean sterile mortar using a pestle and hammer. **[The mortar should be cleaned and sterilized with 10% bleach, 0.1% SDS, sterile dH_2O, and then dried with 100% EtOH].**

7a. If fresh bone marrow is present, separate the marrow from the bone fragments using sterile forceps. Place the pieces of fresh marrow onto a sterile piece of weighing paper. Transfer the fresh bone marrow into a sterile labeled 1.5 mL centrifuge tube. Proceed to the **Organic DNA Extraction From Human Tissue or Human Bone** section 1.3. step 3.

-or-

7b. If the bone is completely dry proceed to step 8.

8. Pulverize the crushed bone fragments in the Micro-Mill Grinder. Before use, the Micro-Mill Grinder must be cleaned and sterilized in the following manner:
   a. Clean the upper grinding chamber with 10% bleach. Rub with cotton swabs and Kimwipes.
   b. Rinse with dH_2O.
   c. Clean the upper grinding chamber with 0.1% SDS. Rub with cotton swabs and Kimwipes.
   d. Rinse with dH_2O.
   e. Dry the upper grinding chamber with 100% EtOH. Rub with cotton swabs and Kimwipes.
   f. Repeat steps a-f for the lower grinding chamber making sure the blade is cleaned thoroughly.
   g. Allow all internal surfaces to dry.

9. When the grinder has been cleaned and sterilized an extraction negative must be taken from the grinder after the final washing step, prior to sample application. This is done by hydrating a cotton tip applicator with dH_2O and vigorously swabbing the interior of the upper and lower grinding chambers. Clip and place the entire cotton tip into a sterile centrifuge tube and label it accordingly. The extraction control should be labeled as the ***sample unique identifier*-EC**.

10. The bone specimen should be dry before milling, otherwise it will clump on the blade of the mill. The specimen should also be frozen with liquid nitrogen to ensure proper milling.

11. Place the fragmented bone into the lower grinding chamber. The sample must have a volume between 20 mL and 50 mL. Lesser or greater volumes will not result in adequate milling.

12. Replace the upper grinding chamber by inserting the pins through the holes in the back plate on the lower grinding chamber. Fasten latches, left one first.

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13. If heat exchanger facilities are to be used, make the necessary connections to the hose ends on the grinding chambers. Set the grinding timer for intervals of 5 min. -or- if heat exchangers are not used, do not set grinding intervals for more than 2 min.

14. After each interval allow the bone dust to settle for 5 min., then open the chamber and check the results. Reassemble and restart the unit if more time is required.

15. When the sample is completely reduced to dust, remove the lower grinding chamber with the top remaining attached. Tilt the unit 45° and tap gently on the bench top to accumulate the specimen to one side. Wait approximately 30 sec to allow bone power to settle, then lift off the top chamber. Pour out the milled material onto a sterile weighing boat or weighing paper.

16. Place the sample into a 15 mL conical vial. Proceed to the **Organic DNA Extraction Procedure for Human Tissue or Bone** section 1.3, step 3.

2.0 Estimation of DNA Quantity from QuantiBlot Analysis

2.1 Sample Blotting

1. Vortex all samples including DNA Standards and Calibrators 1 and 2. Centrifuge briefly to bring the contents to the bottom of the tube. If Chelex extracts are being used, centrifuge for 2 minutes.

2. While wearing gloves, label enough microfuge tubes for all samples and standards.

   Pipet samples and standards into the microfuge tubes, using the following amounts of each:

   a. DNA Standards and Calibrators - 5 µL

   b. All other samples - 20 µL and 1/10 dilutions (with a final volume of 20 µL). When necessary, 1/100 and 1/1000 dilutions may also be run. Prepare all necessary dilutions in TE^{-4}. The samples can be aliquoted ahead of time and stored at 4°C.

3. Heat a shaking water bath to 50°C. The water level should be 1/4 to 1/2 inch above the shaking platform. The temperature should not go below 49°C or above 51°C. **It is essential to check the temperature with a calibrated thermistor probe or thermometer before the hybridization is performed. Also remember to record the temperature.**

   Note: Heat a stationary water bath to between 37°C and 50°C. Warm the QuantiBlot Hybridization Solution and the QuantiBlot Wash Solution in the water bath. **All solids must be in solution before use.**

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4. Once you begin the rest of the QuantiBlot assay, you must finish. Allow approximately 3 hours. Add 150 µL of Spotting Solution to each tube. Vortex and centrifuge briefly to bring the contents to the bottom of the tube.

5. While wearing gloves, cut a piece of Biodyne B membrane to 11.5 x 7.9 cm. Cut a small notch in the upper left corner to mark its orientation. Place the membrane in a container containing 50 mL of Pre-Wetting Solution and incubate at room temperature for 1-30 minutes. Note: Either side of the Biodyne B membrane can be used as the side onto which samples are pipetted. **Always wear gloves when handling the membrane.**

6. Using forceps, remove the membrane from the Pre-Wetting solution. Place the membrane on the gasket of the slot blotter, then place the top plate of the slot blotter on top of the membrane. Turn on vacuum pump to a vacuum pressure of approximately 200 to 250 mm Hg. Turn off the sample vacuum and turn on the clamp vacuum on the slot blot apparatus. Push down to ensure a tight seal.

7. Load the membrane as follows:

Using a new pipet tip for each sample, apply all of each sample into a separate well of the slot blotter. For best results, slowly dispense each sample directly into the center of the wells, with the pipet tip approximately 5 mm above the membrane. **Note: Do not allow the pipet tip to touch the membrane since this may compromise the membrane at that spot.**

Slot	Sample
1A	10 ng standard
1B	5 ng standard
1C	2.5 ng standard
1D	1.25 ng standard
1E	0.625 ng standard
1F	0.3125 ng standard
1G	0.15625 ng standard
1H	extraction negative control (negative)
2A	3.5 ng Calibration 1 Std.

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Slot	Sample
2B	0.5 ng Calibration 2 Std.
2C	0.15625 ng standard
2D-6F	samples and controls
6G	3.5 ng Calibration 1 Std.
6H	0.5 ng Calibration 2 Std.

8. After all the samples have been applied, **slowly** turn on the sample vacuum. Leave the sample vacuum on until all samples have been drawn through the membrane. Inspect each slot that contains a sample for a uniform blue band. If a uniform blue band is not visible, make a note of it.

   Turn off the sample vacuum, the clamp vacuum, then the vacuum source.

9. Disassemble the slot blotter and remove the membrane. Proceed immediately to pre-hybridization. Do not allow the membrane to dry out.

   Clean the apparatus by soaking in enough 0.1% SDS to cover for 5-15 minutes. Following soaking in SDS, rinse the gasket and the side of the top plate that contacts the membrane **thoroughly** with H_2O (include a final rinse with deionized H_2O). Make sure that all the wells of the top plate are rinsed and clean. Rinse the bottom unit of the slot blotter with deionized H_2O and allow to dry at room temperature. **Never use bleach.**

10. Transfer the membrane to 100 mL of pre-warmed QuantiBlot Hybridization Solution in the hybridization tray. Add 5 mL of 30% H_2O_2. Place the lid on the tray. Put the tray into the 50°C shaking water bath. Place a weight (eg. lead ring) on the covered tray to prevent the tray from sliding or floating.

    Shake at 50°C for 15 minutes at 50-60 rpm. Pour off the solution.

2.2 Hybridization

11. Add 30 mL of pre-warmed QuantiBlot Hybridization Solution to the tray. Tilt the tray to one side and add 20 µL of QuantiBlot D17Z1 Probe to the QuantiBlot Hybridization Solution.

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Cover tray with lid and weight.

Shake at 50°C for 20 minutes at 50-60 rpm. Pour off the solution.

12. Add 100 mL of pre-warmed QuantiBlot Wash Solution to the tray. Rinse by rocking for several seconds, then pour off the solution.

13. Add 30 mL of pre-warmed QuantiBlot Wash Solution to the tray. Tilt the tray to one side and add 180 µL of Enzyme Conjugate. Cover tray with lid and weight.

Shake at 50°C for 10 minutes at 50-60 rpm. Pour off the solution.

14. Add 100 mL of QuantiBlot Wash Solution to the tray. Rinse by rocking for 1 minute, then pour off the solution. Repeat for a total two washes.

15. Add 100 mL of QuantiBlot Wash Solution to the tray. Cover tray with lid and weight.

Shake at room temperature for 15 minutes at 100-125 rpm. Pour off the solution. During this time, prepare the Color Development Solution (see below).

2.3 Color Development

16. In a glass flask, prepare the Color Development Solution. Add the reagents in order:

60 mL of Citrate Buffer

3 mL Chromogen

60 µL 3% H_2O_2.

Mix thoroughly by swirling (do not vortex).

Note: Do not prepare the Color Development Solution more than 10 minutes before use. Use a new tube of hydrogen peroxide for each batch of Color Development Solution. Discard the remaining hydrogen peroxide after use. Wrap the Chromogen bottle in parafilm after each use to prevent oxidation.

17. Add 100 mL of Citrate Buffer to the tray. Rinse by rocking for several seconds, then pour off the solution.

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18. Add the Color Development Solution to the tray. Cover tray with lid.

    Develop the membrane by shaking at room temperature for 20-60 minutes at 50-60 rpm. Pour off the solution.

19. Stop the color development by washing in approximately 100 mL deionized H_2O. Repeat several times. After the last wash, store membrane in deionized H_2O. Cover tray with lid and proceed with photography.

2.4 Photography

20. Photograph the membrane while wet. Place the membrane on a dark, flat, non-absorbent surface.

21. Use a Polaroid MP4 camera system with type 667 or 664 film and a Wratten 23A or 22 (orange) filter.

22. Turn on the flood lights. Adjust the height of the camera and focus so that the membrane fills the entire viewing frame.

23. Photograph at 1/125 seconds and f8 for type 667 film. Photograph at 1/2 second and f5.6 for type 664 film.

24. Develop the film for 30-60 seconds. If the photograph is out of focus, not exposed properly, or does not accurately record the bands on the membrane, vary the exposure conditions and re-photograph.

    Indicate the appropriate columns and rows on front of the photograph. Also, initial and date the photograph.

25. Attach the photograph to the QuantiBlot worksheet. Once the QuantiBlot passes review (see below), make photocopies and distribute to the appropriate analysts. File the original QuantiBlot worksheet along with photograph in the appropriate binder. Discard the membrane.

    Note: If an entire QuantiBlot or a portion of it does not pass review (see below), it is the responsibility of the analyst on the QuantiBlot rotation to resubmit those samples that are in question.

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2.5 QuantiBlot Quality Control

1. **The DNA standards should yield band intensities that decrease approximately two-fold for each subsequent dilution (see below for exceptions).**

2. Compare the intensities of calibrator 1 (3.5 ng) and calibrator 2 (0.5 ng) to those of the DNA standards. The intensity of calibrator 1 should be between 2.5 and 5 ng; the intensity of calibrator 2 should be between 0.31 and 0.62 ng. Calibrators 1 and 2 are loaded in duplicate on the membrane. At least three of these four calibrator samples must be consistent with the DNA ladder and each other. **If more than one of the calibrator samples are incorrect, the membrane must be repeated.**

3. Estimate the quantity (ng) loaded for each sample by comparing band intensities of the unknown samples with those of the DNA standards. If the band intensity is between two DNA standards, **match the sample to the DNA standard with the lower amount of DNA.**

   It is recommended to run the neat and 1/10 dilution for each sample. As a general rule in determining the DNA quantity of an unknown sample, the band intensity must be less than the largest DNA standard (10 ng). If the sample band intensity is ≥ 10 ng, multiply the reading of the 1/10 dilution by 10 to determine the DNA quantity for that sample. If the sample band intensity of the 1/10 dilution is ≥ 10 ng, then submit 1/100 and 1/1000 (if necessary) dilutions for analysis. To determine the DNA quantity for these samples, multiply the 1/100 and 1/1000 dilution readings by 100 and 1000, respectively.

4. **Membranes that have compromised DNA standards may pass review, however, care must be taken in the interpretation of the results.** Examples of compromised DNA standards include bands that are not visible, nonuniform signal intensity within a slot, and samples that yield a band intensity that is not consistent with that of the other DNA standards.

   The following guidelines must be followed:

   a. At least one of the 0.15 ng standards must be visible. If not, samples that show band intensities of <0.31 ng and samples with no apparent DNA must be repeated, including the extraction negative control(s).

   b. If one of the DNA standards other than 0.15 ng produces a band intensity that is not consistent with the other DNA standards and the DNA calibrators, then sample readings that

fall at or near the amount of the compromised standard, and are between the adjacent two standards (one on either side of the compromised standard) are not valid for that membrane.

For example, if the 2.5 ng DNA standard is compromised, the sample readings that are <5 ng but >1.25 ng are not valid for that membrane. If the 10 ng DNA standard is compromised, sample readings >5 ng are not valid for that membrane.

c. If more than one of the DNA standards are compromised, the membrane can pass review if four consecutive DNA standards are correct as determined by comparing band intensities to those of the DNA calibrators. In such a case, the membrane will be valid for the determination of DNA sample amounts that yield values at or within those four DNA standards.

For example, if the 0.625, 0.31, and 0.15 ng DNA standards are compromised, the membrane in question is still valid for readings ≥1.25 ng given that the 10, 5, 2.5, and 1.25 ng DNA standards are consistent with the concentrations of DNA calibrators 1 and 2.

5. If the membrane fails review for the determination of DNA amounts, it can still be used as a guide in the resubmission of neat samples and/or dilutions. Also, any bands that are produced on the membrane are indicative of the presence of human DNA*, provided that the corresponding plate negative and extraction negative controls do not produce a signal.

6. Submit a photograph and QuantiBlot Worksheet for review by the QuantiBlot Station supervisor.

* Non-human primate DNA may give comparable results to that of human DNA using this procedure (Perkin Elmer Corp. 1996).

2.6 Quantiblot interpretation

1. The Quantiblot procedure, done properly, gives reproducible and fairly accurate determinations of the total amount of human DNA. The neat sample and 1/10 dilution should give quantitation results that make sense - the neat and 1/10 samples should correlate with one another.

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neat	1/10 dilution	1/10 dilution
	results OK	**review photo to determine best concentration**
≥ 10	5, 2.5, 1.25	0.62
5	0.62	1.25, 0.31
2.5	0.31	0.62, 0.15
1.25	0.15	0.31, blank
0.62	< 0.15	0.15
0.31	blank	
0.15	blank	

2. If you have any pairs of results other than those above, you need to take steps to correct the problem **before** amplification. A review of the photograph may be all that is needed to determine the discrepancy of the results (eg. incorrectly called results, nonuniform signal intensity, thick or thin bands) and obtain a reasonable estimate of the DNA concentration.

   a. If neat and 1/10 dilutions are both ≥ 10 ng, submit additional dilutions for Quantiblot.

   b. If neat and 1/10 dilution are of equal intensity, resubmit neat and 1/10 dilution for Quantiblot.

   c. If neat and 1/10 dilution are too far apart in intensity (eg. 5 and 0.15 ng, 2.5 ng and blank), resubmit neat and 1/10 dilution for Quantiblot.

   d. If neat and 1/10 dilutions are both "*" due to colored impurities, then the sample may need cleaning up using a Microcon spin filter followed by Quantiblot.

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2.7 Troubleshooting of QuantiBlot

(taken from the Perkin Elmer QuantiBlot package insert, see QuantiBlot references)

	Observation	Possible Cause	Recommended Action
1.	No signal or low sensitivity.	Use of a membrane other than Biodyne B.	Use Biodyne B nylon membrane. Do not use membranes that have neutral charge.
		Incorrect NaOH or EDTA concentrations in Spotting Solution.	Prepare Spotting Solution correctly.
		Water bath temperature too high.	Water bath temperature should be 50°C (±1°C).
		DNA Probe was not added at hybridization step.	Add QuantiBlot D17Z1 Probe.
		Enzyme conjugate was not added.	Add Enzyme conjugate: HRP-SA at indicated step in protocol. Use 180 µL of Enzyme Conjugate: HRP-SA.
		Hydrogen peroxide was inactive.	Prepare a new Color Development Solution using a fresh bottle of hydrogen peroxide.
		Presence of $MgCl_2$ in the DNA sample.	Concentrations of $MgCl_2$ >0.3 mM can result in reduced sensitivity. Prepare all DNA dilutions in TE^{-4} Buffer. Any $MgCl_2$ can be removed from samples by microdialysis using Centricon 100 spin units (follow manufacture's directions).

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	Observation	Possible Cause	Recommended Action
2.	Areas of low sensitivity across the membrane.	Membrane slipped up onto the side of the Hybridization Tray during Hybridization or Stringent Wash steps.	Reduce the rotation rate of the water bath to 50-60 rpm. Check that the membrane is fully submerged in the bottom of the Hybridization Tray before shaking.
		Membrane dried-out significantly at some point in the protocol.	Do not allow the membrane to dry at any point in the protocol.
3.	Non-uniform signal intensity within a slot.	Bubbles(s) in slot blot wells when sample was pipetted into well, or when vacuum was applied.	Slowly pipet the Spotting Solution directly over the center of the wells of the slot blot apparatus, with the pipet tip raised approximately 5 mm above the membrane. Turn on the sample vacuum slowly, not all at once. After being drawn through the membrane, the sample should appear as a uniform blue band on the membrane. If the entire sample is not drawn through the membrane, turn off the sample vacuum. Pipet the sample back into the pipet tip; then pipet the sample back into the well of the slot blot apparatus. Turn on the sample vacuum to draw the sample through the membrane.
4.	Filter background	No or low SDS in the Hybridization Solution or in the Wash Solution	Prepare solutions with proper concentrations of SDS.

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	Observation	Possible Cause	Recommended Action
		Membrane was not pre-wetted prior to slot blotting.	Pre-wet the membrane in Pre-Wetting Solution prior to slot blotting.
		Too much Enzyme conjugate: HRP-SA was added.	Use 180 µL of Enzyme Conjugate:HRP-SA.
		Lack of thorough rinsing.	Thoroughly rinse twice, for 1 minute each, using 100 mL of pre-warmed Wash Solution. These two rinse times can be extended beyond 1 minute if necessary.
		Slot blot apparatus not cleaned thoroughly after last use.	Immediately after each use, soak the slot blot apparatus in a large volume of 0.1% SDS solution. Never use bleach.
5.	The DNA Calibrators do not quantitate correctly with respect to the DNA Standards.	DNA Standard serial dilutions prepared incorrectly.	Prepare two-fold serial dilutions of DNA Standard in TE^{-4} Buffer as described. Add 5 µL of each dilution to 150 µL of Spotting Solution for slot blotting.
6.	Signal obtained for non-human DNA samples.*	Water bath temperature too low.	Water bath temperature should be 50°C (±1°C).

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Observation	Possible Cause	Recommended Action
	SSPE concentration too high in Wash Solution.	Check that the 20X SSPE solution and the Wash Solution were prepared correctly.

* DNA from primates species may give signals similar to those obtained from equivalent amounts of human DNA. In Roche Molecular Systems (RMS) laboratories, 30 ng to 300 ng quantities of non-primate DNA samples result in either no signals or signals that are less than or equal to the signal obtained for 0.15 ng of human DNA.

3.0 Amplification of mtDNA

3.1 System Design

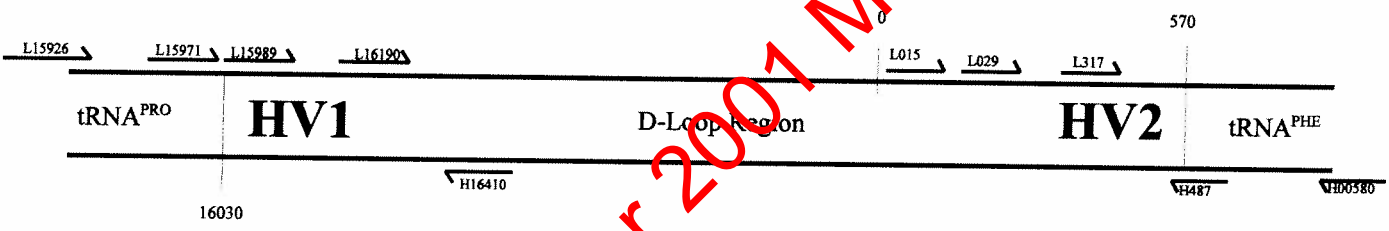

Figure 1: mtDNA Primers and Locations

Region	Primer Position	Sequence
CRF	L 15926	5' TCA AAG CTT ACA CCA GTC TTG TAA ACC 3'
CRR	H 00580	5' TTG AGG AGG TAA GCT ACA TA 3'
HV1-A	L15971	5' TTA ACT CCA CCA TTA GCA CC 3'
HV1-B	L 15989	5' CCC AAA GCT AAG ATT CTA AT 3'
HV1-C	L 16190	5' CCC CAT GCT TAC AAG CAA GT 3'
HV1-R	H 16410	5' GAG GAT GGT GGT CAA GGG AC 3'
HV2-A	L 015	5' CAC CCT ATT AAC CAC TCA CG 3'
HV2-B	L029	5' CTC ACG GGA GCTCTC CAT GG 3'
HV2-C	L317	5' CTT CTG GCC ACA GCA CTT AAA 3'
HV2-R	H 487	5' TGA GAT TAG TAG TAT GGG AG 3'

Table 1: Primer sequences

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Amplified Region	Primer Set (F/R)	Amplified Fragment
Control Region	CRF/CRR	1223 bp
HV1	HV1-A/HV1-R	420 bp
HV2	HV2-A/HV2-R	450 bp

Table 2: Amplification Design

3.2 General PCR Guidelines

For bone void of fresh marrow, teeth void of soft tissue, hair shaft, highly degraded samples, or samples with undetectable levels of DNA, proceed to the **mtDNA Control Region Amplification** section 3.3. Nested PCR will increase the likelihood of obtaining sequenceable DNA from highly degraded samples. For non-degraded tissue, bone containing fresh marrow, and exemplars, proceed to the **Hypervariable Region I or II Amplification** section 3.4.

A positive control (PC), an amplification negative control, an extraction reagent control and any applicable controls must be included with each batch of samples being amplified to demonstrate procedural integrity.

3.3 mtDNA Control Region Amplification

Note: Prior to amplification, dilutions of extracted samples (including extraction reagent controls) should be prepared in 1.5 mL labeled centrifuge tubes to a final concentration of 1 ng/20 µL (if 1 ng is available). If samples are not to be amplified the same day they can be stored frozen in a labeled rack. Prepare dilutions of extracted DNA in dH_20 to a total volume of 20 µL as follows:

Quantiblot (ng/20 µL)	target volume (µL) DNA extract	target volume (µL) sterile dH_2O	total volume
≥25	dilute 1:10 and use dilution		
12.5	1.6	18.4	20
6.2	3.3	16.7	20
5	4	16	20
2.5	8	12	20
1.25	16	4	20
0.62	20	0	20
0.31	20	0	20
≤0.15	20	0	20

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1. For each sample to be amplified, label a 0.2-mL MicroAmp reaction tubes containing mtDNA Control Region amplification mixture. Be sure to include tubes for your positive and amplification negative controls (and any other applicable controls).

Note: Sterile techniques should be employed to assure sample integrity. Only one tube should be opened at any given time to eliminate cross-contamination. Tubes should be opened with a decapping device or Kimwipes. Filtered tips must be used. Bench tops should be sterilized with 10% Bleach and 70% EtOH.

2. Add 3 µL of 25mM $MgCl_2$ to each tube [1.5 mM final concentration].

3. To the positive control tube, add 20 µl of mtDNA PC control DNA.

4. To the amplification negative control tube, add 20 µL of sterile dH_2O.

5. Add the 20 µL of extraction reagent control to the Control Region amplification reaction mix tube.

6. Add the 20 µL of sample DNA to the Control Region amplification reaction mix tubes.

7. Vortex and spin samples.

8. Turn on the GeneAmp 9600 PCR system. Turn the knob on the lid of the cover counterclockwise to slightly raise the lid.

9. Slide cover back and place tubes into sample wells. Slide cover forward and close lid by turning knob clockwise until white markings on the lid and the knob line up.

10. Press option key on keyboard until cursor is under 'Run' then press 'Enter'.

11. Enter program #39 and press 'Enter'.

12. The reaction volume should read 50 µL (if not, change value to 50) press 'Enter' again.

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13. Screen will read 'Close and tighten sample cover'.

14. Cycling parameters are as follows:

Hold:	96°C for 11 minutes
30 cycles:	94°C for 45 seconds
	60°C for 1 minute
	72°C for 5.5 minutes
Hold:	4°C

running time is approximately 4.8 hours.

15. When the run has ended, proceed to the **Hypervariable Region I or II Amplification** section 3.4.

3.4 Hypervariable Region I or II Amplification

Note: Prior to amplification, dilutions of extracted samples (including extraction reagent controls) should be prepared as in the mtDNA Control Region Amplification section 5.3.

***Note: If mtDNA control region amplified products ("first round amplified products") are being combined with the present amplification set, a new mtDNA PE control DNA, amplification negative, and applicable reagent blank are not needed. 1 µL of the first round product from these samples will be combined with 19 µL of dH_20 and added to the reaction. Be aware that amplified products must never enter low DNA areas. These samples must be added to the amplification reaction in an amplified DNA area.**

1. For each sample to be amplified, label a 0.2-mL MicroAmp reaction tube containing mtDNA HV1 or HV2 amplification mixture. Be sure to include tubes for your positive and amplification negative controls (and any other applicable controls).

Note: Sterile techniques should be employed to assure sample integrity. Only one tube should be opened at any given time to eliminate cross-contamination. Tubes should be opened with a decapping device or Kimwipes. Filtered tips must be used. Bench tops should be sterilized with 10% Bleach and 70% EtOH.

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2. Add 3 µL of 25mM $MgCl_2$ to each tube [1.5 mM final concentration].

3. To the positive control tube, add 20 µL of mtDNA PE control DNA (see above *).

4. To the amplification negative control tube add 20 µL of sterile dH_2O (see above*).

5. Add the 20 µL of extraction reagent control to the Control Region amplification reaction mix tube (see above*).

6. Add the 20 µL of sample DNA to the HV1 or HV2 amplification reaction mix tubes (see above*).

7. Vortex and spin samples.

8. Turn on the GeneAmp 9600 PCR system. Turn the knob on the lid of the cover counterclockwise to slightly raise the lid.

9. Slide cover back and place tubes into sample wells. Slide cover forward and close lid by turning knob clockwise until white markings on the lid and the knob line up.

10. Press option key on keyboard until cursor is under 'Run' then press 'Enter'.

11. Enter program #42 and press enter.

12. Reaction volume should read 50 µl (if not, change value to 50) press 'Enter' again.

13. Screen will read 'Close and tighten sample cover'.

14. Cycling parameters are as follows:

Hold:	96°C for 11 minutes
Hold:	94°C for 30 seconds
32 cycles:	94°C for 20 seconds
	56°C for 10 seconds
	72°C for 30 seconds

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Hold: 4°C

running time is approximate 1.7 hours

3.3 Product Gel Electrophoresis

After the HV1 or HV2 amplification, a 1.5% agarose gel is run to determine if the desired 439 bp HV1 fragment or 472 bp HV2 fragment was amplified. The number of samples amplified will determine the size of the gel needed. Fill out a mtDNA product gel worksheet. The small gel can accommodate up to 12 amplified samples plus two lanes for the ladders. If there are more than 12 samples a large gel will need to be poured.

1. Fill out a mtDNA Product Gel Work Sheet.

2. In an Erlenmeyer flask, mix the following reagents:

Reagent	Small gel	Large gel
DNA Typing Grade Agarose	0.75 g	3.0 g
1X TBE (or TAE)	50 mL	200 mL

3. Heat the flask on high in a microwave for 1.5-2.0 minutes, or until agarose is dissolved. Allow the solution to cool, then add ethidium bromide [1ug/mL] (5 µL for the small gel or 20 µL for the large gel).

4. Assemble gel unit and pour gel mixture into tank. Position comb and let gel solidify (approximately 15-30 minutes).

5. Once sample amplification has completed (screen should read 'Hold 4^{0}C...Block Temp 4^{0}C) prepare samples as follows:

   Sample(s): 10 µL amp product + 2 µL loading buffer

   Prepare a low molecular weight and 1 kb DNA ladder as follows:

   Standards: 4 µL ladder + 6 µL sterile dH_2O + 2 µL loading buffer

6. Vortex and spin all samples.
7. Once the gel is set, pour 1X TBE (or TAE) into the buffer chamber, just enough to cover the

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gel (approximately 200 mL for the small gel buffer tank and 2000 mL for the large buffer tank) and remove comb.

8. The gel should be loaded with the ladder flanking the samples. Loading volumes are as follows: 12 uL for the large gel, 12 µL for the small gel using the 8-well comb, and 7 µL for the small gel using the 14-well comb.

9. Run the small gel at 90-100 volts for 30-45* min. or the large gel at 150-200 volts for 45* min. -1 hr. (*or until the bromophenol blue dye front has run approximately 1 cm from the base of the gel).

10. Switch off power supply and remove gel from the tank. Examine the gel under ultraviolet light.

11. Photograph the gel using the following parameters:

film type 667	-or-	film type 665
f-stop 5.6		f-stop 5.6
shutter speed 1 sec.		shutter speed 30 sec. (manual setting)
transmitted UV light 300nm		transmitted UV light 300nm
orange filter		orange filter
30 sec. - 1 min. development		30 sec. - 1 min. development

Vary f-stop and shutter speed to obtain optimal photographs.

12. Examine the photograph for the presence or absence of amplified product. Fill out the results column on your mtDNA Product Gel Work Sheet accordingly; (+) for the presence of amplified product, (-) for absence of amplified product. Interpret results following section 3.4.

3.4 Product Gel Results Interpretation

Note: Following product gel electrophoresis, samples must be purified using Microcon-100 purification filters. Dependant upon amplification success, purified PCR fragments can be reconstituted between 20 µL to 40 µL with dH_2O. Use the following interpretation

guidelines to purify the amplified fragments in the **Microcon-100 Purification of PCR Fragments** section 6:

Situation	Interpretation	Result
Amplified fragment in extraction control	Extraction fails	Re-extract all samples from the applicable extraction
Amplified fragment in amplification control	Amplification fails	Re-amplify all samples
Intense band of amplified product	High concentration of amplified product	Purify product by Microcon-100 filtration. Reconstitute to a final volume of 40 µL
Faint band of amplified product	Low concentration of amplified product	Purify product by Microcon-100 filtration. Reconstitute to a final volume of 20 µL
Absence of band in positive control	Positive Control failed to amplify	Re-amplify all samples
Absence of band in case sample	Case sample failed to amplify	Re-amplify sample. Refer to section 3.5.

3.5 Amplification Troubleshooting

If the Chelex extracted or Organic extracted samples fail to amplify try one or several of the following:

1. Amplify using an additional 10 units of Taq Gold polymerase.
2. Amplify a smaller aliquot of the DNA extract to dilute potential Taq polymerase inhibitors or amplify a larger aliquot of the DNA extract because the sample is highly degraded (both can be done during the same amplification).
3. Re-extract the sample using a smaller area of the stain or less biological material (i.e. bone, muscle or blood sample) to prevent saturation of the Chelex and to dilute potential Taq polymerase inhibitors.
4. Re-extract the sample using a larger portion of the biological material to ensure sufficient DNA is present.

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4.0 Microcon-100 Purification of PCR Fragments

Note: Before sequencing, amplified samples must be purified to remove salts, unincorporated primers and nucleotides. With the exception of controls, do not Microcon any samples that failed to amplify.

1. Insert microcon columns (blue) into labeled microcon tubes.

2. Add 100 µL of sterile dH_2O to each column and load entire amplified product (40 µL) onto the column.

3. Spin at 500 x (2500 rpm) for 15 minutes at room temperature.

4. Add 200 µL of sterile dH_2O to each column and repeat step #3 above.

5. Remove assembly from centrifuge and add 20 µL of sterile dH_2O to each sample reservoir.

6. Invert sample reservoir and place into a newly labeled tube. Spin at 1000 x (3500 rpm) for 3 minutes to collect sample.

7. Discard sample reservoir. There should be approximately 20 µL of liquid in each tube. Refer to the **Product Gel Results Interpretation** section 3.4 to determine the final reconstitution volume. Reconstitute samples in dH_2O. Purified amplified samples should be stored at -4^{0}C until needed.

5.0 BigDye™ Cycle Sequencing

Note: Separate forward and reverse sequencing reactions must be performed on all samples. Dependant upon the number of samples, more than one set of sequencing reactions may be required to obtain forward and reverse strands. Sequencing reaction master mixes should be produced for each primer being used for n + 1 samples.

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1. Prepare the master mix as follows per sample:

Reagent	Reagent stock concentration	Volume per reaction	Final Concentration
Terminator Ready Reaction Mix	N/A	8 µL	N/A
Sterile dH_2O	N/A	6 µL	N/A
Primer working solution:*	1.6 pmol/µL	2 µL	160 fmol/µL
Forward primer HV1A, B or C			
Forward primer HV2A, B or C			
Reverse primer HV1R			
Reverse primer or HV2R			

*Refer to figure 1 of the System Design section 3.1. One primer is used per reaction. Separate reactions are required for forward and reverse sequence generation.

2. Mix thoroughly by vortexing and spin down tubes.

3. For each sample to be sequenced, label a 0.2-mL MicroAmp reaction tubes accordingly. Include tubes for all controls.

4. Add 16 µL of master mix to each tube. Cap all tubes.

5. Using a de-capping device or sterile Kimwipes, uncap one tube at a time and add 4 µL of purified PCR product. Cap the tube and repeat for the remaining samples.

6. Vortex and spin samples.

7. Turn on the GeneAmp 9600 PCR system. Turn the knob on the lid of the cover counterclockwise to slightly raise the lid.

8. Slide cover back and place tubes into sample wells. Slide cover forward and close lid by turning knob clockwise until white markings on the lid and the knob line up.

9. Press option key on keyboard until cursor is under 'Run' then press 'Enter'.

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10. Enter program #44 and press enter.

11. Reaction volume should read 20 µL (if not, change value to 20) press 'Enter' again.

12. Screen will read 'Close and tighten sample cover'.

13. Cycle sequencing parameters are as follows:

25 cycles:	96^{0}C	10 sec.
	50^{0}C	5 sec.
	60^{0}C	4 min.
Hold:	4^{0}C	

running time is approximately 2.0 hrs.

6.0 Centri-Sep Spin Column Purification of Sequenced Products

Prior to sample electrophoresis, sequenced products must be purified in order to remove unincorporated dye terminators. It will save time if you start this procedure while the samples are sequencing.

1. Gently tap columns to insure dry gel material has settled to bottom of spin column. Remove top column cap and add 800 µL of sterile dH_2O. You will need to hydrate one column for each sequencing reaction.

2. Replace top cap and mix thoroughly by inverting column and vortexing briefly. It is important to hydrate all of the dry gel. Allow columns to hydrate for at least 30 minutes at room temperature. As the columns are hydrating you will need to label one sample collection tube (1.5 mL microcentrifuge tube) for each sequencing reaction. You will also need one wash tube for each hydrated column. These do not need to be labeled.

3. Once the columns are hydrated, remove any air bubbles by inverting the column and sharply tapping the column, allowing the gel to slurry to the opposite end of the column. Stand the column upright and allow the gel to settle while in a centrifuge tube rack.

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4. Once the gel is settled, remove first the top column cap, and then remove the column end stopper from the bottom.

5. Allow excess column fluid to drain into a wash tube by first gently tapping the column into the wash tube then allowing to sit for approximately 5 minutes. Remove the column from the wash tube, discard the liquid and reinsert the column into the wash tube.

6. Spin the assembly at 3000 rpm for 2 minutes to remove interstitial fluid. Be sure to note the orientation of the columns.

7. Load entire sequencing reaction volume (20 µL) to the top of the gel. Be careful to dispense sample directly onto the center of the gel bed without disturbing the gel surface.

8. Place column into labeled sample collection tube and spin at 3000 rpm for 2 minutes maintaining original orientation. The purified sample will collect in the bottom of the tube.

9. Discard the column and dry the sample in a vacuum centrifuge (approximately 15 minutes). Do not over dry samples.

10. Prepare samples for electrophoresis by resuspending the dried pellet in 6 µL of blue formamide loading buffer [1:5 blue dextran:formamide] (see 12b.).

11. Vortex samples for 10 - 15 seconds and then spin samples.

12a. Denature samples prior to loading in a 95°C heat block for 3 min. and then place them on ice until loaded.

-or-

12b. If the samples are not going to be loaded immediately, they should be stored as a dried pellet at 4°C for no longer then 14 days. When the samples are ready to be loaded proceed to step 10 - 12a.

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7.0 Sample Electrophoresis on the ABI 377 Sequencer

7.1 Gel Casting

The sequenced products are separated on 5% denaturing Long Ranger gels. The plates that are being used result in a 36 cm separation distance from the wells to the laser read area. The bottom plates have two side indentations that fit against a metal notch in the gel mounting cassette. The top plate has a cut out area at the top to allow buffer-gel contact. For the first time use of new plates, see the Quality Control manual (QC033) for additional plate preparation. During set up, insure that the correct sides are facing the inside (see step 3.).

1. When making **one** plate:

   Pour 10.8 g of pre-weighed urea in 100 mL Erlenmeyer flask.

   Add 10 mL of deionized water

   3 mL of 10x TBE buffer

   3 mL of 50% Long Ranger

   When making **two** plates:

   Pour 18 g of pre-weighed urea in 100 mL Erlenmeyer flask.

   Add 20 mL of deionized water

   5 mL of 10x TBE buffer

   5 mL of 50% Long Ranger

   Add stir bar and place on stirrer with low heat. Do not boil!!!

2. Clean appropriate set of bottom and top glass plates as follows: Clean both sides with liquid detergent and paper towels, rinse with water, rinse with deionized water. Remove from sink and let dry or dry with low lint Kimwipes.

   NOTE: Do use solvents such as isopropanol sparingly on these plates. Do not use ethanol.

3. Prepare lab bench with bench paper and square box. Place bottom plate on box such that the etched letters "L" and "R" for left and right are readable and are on the correct sides. Place 0.2mm spacers on each side of plate with the jagged side facing the top of the plate. Put notched top plate on top of bottom plate such that the etched letters "L" and "R" for left and

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right are readable and are on the correct sides. Place three clamps on each side of the sandwiched plates.

4. The urea in the gel solution must be completely dissolved before proceeding. Pour the gel solution into a 50 mL tube:

   - for **one** plate adjust the volume to **30 mL,**

   - for **two** plates adjust the volume to **50 mL** with deionized water.

   Filter the solution using disposable 100mL 0.2 µm pore size filter units and a vacuum pump. Degas the solution by leaving the filter attached to the running vacuum pump for **5 minutes**. Pour the filtered solution back into the 50 mL tube. Let the solution cool to room temperature.

5. Before proceeding, have a 20 mL glass pipette and a 0.2mm 24 wells gel comb ready to use.

   - For **one** plate (**30mL** volume) add **150 µL** of 10% ammonium persulfate (APS) and **21 µL** of TEMED to the Long Ranger/Urea solution.
   - For **two** plates (**50 mL** volume) add **250 µL** of 10% ammonium persulfate (APS) and **35 µL** of TEMED to the Long Ranger/Urea solution.

Note: APS older than FIVE days should not be used. Check date on tube. If necessary make a new APS solution by adding 5 mL of deionized water to pre-weighed 0.5g of APS. Do not forget to date the tube.

Mix gently and immediately pour the gels using a 20 mL glass pipette to apply solution to the notched area while continuously tapping the plates to prevent air bubble formation. Insure that there is always enough gel solution in the notched area, so that the gel does not run dry. After the gel solution reaches the bottom of the sandwiched plates, insert comb slowly and carefully to prevent splatter and formation of air bubbles. Place a clamp on the comb.

6. Allow gel to polymerize for 1.5 - 2.0 hours.

   NOTE: since the gels are very thin, they dry out rapidly. A gel should be used immediately after the 1.5 hr. polymerization.

   If it is not used immediately, wrap the gel after 1.5h. Remove the gel clips, **do not pull the comb!!** Place paper towels moistened with 1X TBE or deionized water on the bottom and the top ends of the gel, cover the moist paper towels with clear plastic wrap, fold the plastic wrap over and hold in place using gel clips. Wrapped in this manner the gels can be stored over night at room temperature. Do not use a gel older than 24 hrs.

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Note: The occurrence of air bubbles does not mean the whole gel must be discarded. Just avoid this area of the gel while loading. Sometimes air bubbles in the comb area can be removed by pulling the comb out again, adding more gel solution, and inserting the comb again. Depending on the room temperature in the laboratory sometimes the urea in the gel crystallizes out. This process will be reversed if the gel is allowed to warm up.

7.2 Gel Mounting and Instrument Setup

In order to run a gel on the 377, the run must be set up on both the software and the instrument. Additionally, proper documentation of the gel run has to be prepared. The documentation consists of filling out a gel sheet that list the samples to be loaded, the lot numbers of the reagents that were used, and the Analyst's and the Witnesses' initials. The run must be logged in the 377 QC instrument log book.

The program used to operate the run and collect the data is ABI Prism 377 Collection Software. This program has to be started. The software preparation then consists of creating a run folder and naming the gel.

On the instrument, the gel has to be mounted into the electrophoresis unit, checked for artefactual fluorescence, and preheated before the run can be started. The instrument does not have manual control switches, and it operates from a computer terminal, utilizing the 377 Collection Software.

Before starting a run, the computer should be restarted. This removes hard drive memory fragmentation and prevents the software from crashing. Also, to monitor instrument usage and help troubleshooting, a 377 QC instrument log book must be filled out for every electrophoresis run.

1. Place gel in cassette - place the plates back in the gel cassette with the bottom plates down, push the plates towards the bottom of the cassette until the rear plate notches rest firmly against the metal stops. Wipe laser read region clean using dH_2O, then move the beam stopper into the down position, and turn both clamps #6 to hold it.

   Turn cassette clamps #2, #3, #4 and #5 on both sides to lock the plates into position. You are ready to place the cassette gel assembly into the instrument.

2. Making sure bottom buffer chamber is in place, place cassette/gel assembly into machine and turn the four corner knobs to lock cassette into place (plate must be flat against surface of machine). Close instrument door.

3. Double-click *ABI Prism 377 collection* icon on the desktop.

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4. Select 'Sequence Sample' and fill out sample sheet accordingly. Be sure to skip any lanes with air bubbles in the path. Under "DyeSet/Primer" select 'DT {BD Set -Any Primer}' and for the Instrument File select 'dRhod matrix'. Close sample sheet and save.

5. Under **'File'** select 'New'. Click once on 'Sequence Run' icon. This will open a new run folder with current date and time at the top.

6. Under the sample sheet pop-up menu import the sample sheet just created.

7.3 Plate Check/Pre-Running Gel

7. Click 'Plate Check'. If plate is clean (scan lines flat), click 'Cancel' and then 'Terminate' to end plate check. If scan lines are not flat, plate must be removed and cleaned again. Do not proceed to next step until the plate check is clean. Open instrument door.

8. Remove comb, attach upper buffer chamber and fill upper and lower buffer chambers with 1x TBE buffer, checking for any leaks from the upper chamber. Remove any air bubbles in wells and attach cooling plate to front of gel/cassette. Attach all electrodes (3 total: upper chamber, lower chamber, and cooling plate) on the left and attach tubing from the cooling plate on the right. Close instrument door.

9. Before pre-running check to see all proper run modules are set:

Plate Check Module:	Plate Check A	*PreRun Module:*	Seq PR 36A-1200
Run Module:	Seq Run 36E-1200	*Collect time:*	7.0 hours
Sample Sheet:	None	*Well-to-read:*	36 cm
Instrument File:	drhod matrix		
Lanes:	24		

-if 'auto analyze' is checked, deselect 'auto analyze'.

10. Click 'PreRun'. Under **Window**, choose 'Status'. Enter the pre-run actual values (values boxed in green) from 'status' window into log book.

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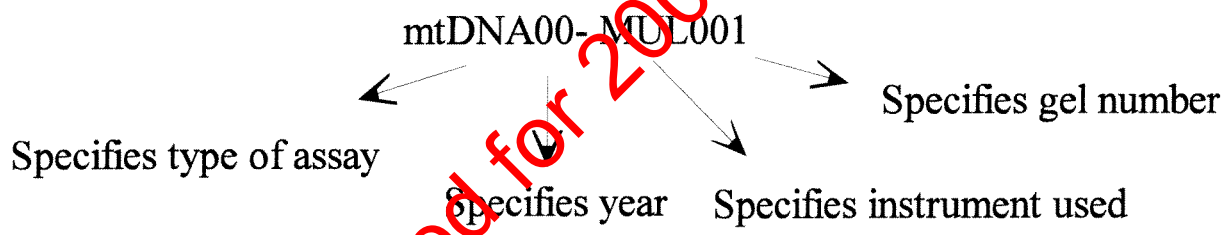
7.4 Sample Loading and Starting Run

11. It will take approximately 15 minutes for the gel temperature to reach the required minimum of 48^{0}C before samples can be loaded. Samples must be heat denatured at 95^{0}C for 1-2 minutes and put on ice for at least 1 minute before loading.

12. Once the gel temperature has reached 48^{0}C, click 'Pause'. "Instrument is paused" box appears. Click 'OK'. Pausing the instrument keeps the gel at a constant temperature as samples are being loaded. Open instrument door.

13. Load 2 µL of the each sample into the appropriate lane. Save the remaining 4 µl until sequencing results are obtained. Close instrument door.

14. Click 'Run'. The instrument will prompt you for a gel file name. Save the gel using the following format:



15. Under **Window**, choose 'Status'. Enter run actual values from 'status' window into the QC log book.

16. After the gel run is finished, the Collection Software quits automatically.

7.5 Gel Disassembly and Clean-Up

1. Open the door, remove the lid from the upper buffer chamber, and remove the buffer from both buffer chambers before proceeding.

2. Disconnect all electrodes, the electrical wire for the heat plate and the tubing.

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3. Un-clamp clamps #3, #4, #5 on each side to release the heat plate. Remove the heat plate. Put the clamps back to hold the gel plates.

   Important: Do not remove the gel cassette with the front heat plate still being attached to it. The weight of the front heat plate could damage the cassette during that step.

   Occasionally, especially after a buffer spill, the heat transfer plate is sticking to the glass plate, and cannot be removed. In that situation, remove the upper buffer chamber first and then slide the heat plate upwards until it can be removed.

4. Un-clamp the upper buffer chamber (clamps #2), remove it, and put clamps back to hold the gel plates.

5. Push the gel cassette towards the back plate, release the four outer clamps that hold the cassette, and remove the cassette/gel assembly from the instrument.

6. Place cassette/gel assembly on a bench top. Remove the gel plates from the cassette, pry open and clean the plates. Rinse the black gel cassette under running tap water to remove any electrophoresis buffer. Pay special attention to the beam stop bar and the clamps - these should not be allowed to become encrusted with dried buffer.

7. Go back to the instrument and remove any TBE buffer from the laser lens, the back plate and the heat plate. Rinse the upper and lower buffer chambers with tap water.

7.6 Troubleshooting Electrophoresis

1. **Communication problems** between the instrument and the Macintosh terminals are caused by corrupted memory files, and can cause several problems, e.g. the status window displays the message "door open" and will not let you start a run. In reality the instrument door is closed. To clear the memory the instrument must be reset:

   - press the rest button on the back of the instrument using a pencil
   - watch the back panel LEDs and wait for them to blink in a four on, four off sequence
   - while the four on, four off sequence is occurring press the rest button a second time

   The next time you start the data collection program a new firmware image will be copied to the instrument (See also 7 - 6 to 7 - 9 in the ABI PRISM 377 DNA sequencer User's Manual).

   After resetting the instrument, the CCD pixel position has to be reentered. Under the collection

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software menu go to **Window**, select **Manual control**. Click on the arrow next to **Fxn name** and from the option list select **CCD pixel position**. The correct value varies from instrument to instrument, and was determined during the installation. The value is listed inside the door of each instrument. Enter the number, click execute, and close the manual control window.

2. Poor resolution is most likely caused by low quality, too old, or simply the wrong gel casting reagents. Check the age of e.g. the APS, remake the 1 X TBE Buffer, make a fresh gel, and try again. When making the gel check if the right urea aliquots, the right spacers, and the right comb were used.

3. **Inconsistent mobilities within gel** are most likely caused by incomplete denaturation of single samples. There is no strong effect if the denaturation temperature is up to 10°C lower than 95°C, but the mobility changes if the sample was mistakenly not denatured at all, or if the sample was allowed to sit more than 30 minutes after the denaturation.

   Another reason for the inconsistent mobility and **smearing** up and down of bands ("black hole") are electrostatic charges caused by "over wiping" the glass plates with Kimwipes. Make a fresh gel and rerun the samples.

4. **Fuzzy bands and hazy gels** can be caused by improper alignment of the gel plates with the laser plane. Reasons can be a mistakenly un-clamped lower gel cassette holding clamp, or a worn out gel cassette holding clamp. Always apply pressure on the gel cassette when releasing the outer clamps in order to avoid a loosening of the clamps.

5. **Horizontal streaks** on a gel are a sign of the laser being on it's way out. It must be replaced as soon as possible.

6. **Vertical streaks** are less disruptive since they do not cause artificial peaks in an electropherogram. Vertical streaks are caused by scratches or lint on the glass plates in the laser read region.

7. "Red rain" is a phenomenon where **red vertical streaks** appear late into the run. These red streaks are caused by bubbles between the glass plate and the gel matrix. The problem occurs when a gel dried out after sitting without being wrapped. Another reason is the appearance of hydrophobic patches on the glass surface. This can be solved by repeating the treatment of the plates with sodium hydroxide (QC033).

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8.0 Sequencing Analysis 3.3

After run is finished, the run folder containing the gel image file, run collection file and run log can be found in the 'current runs' folder on the desktop or on the hard drive.

1. Generate the gel image by double-clicking on the gel image icon. Diamond-shaped markers above the gel image represent which lanes were recognized as 'used' by the collection software. Grey markers represent 'unused' (or empty) lanes; blue or white markers represent 'used' lanes. This is dictated by how the sample sheet was filled in prior to the run.

2. Tracker lines' locations must now be optimized by positioning lines over areas of data that display the strongest fluorescent signal. A slice view window to the left of the gel picture displays the signal from the active tracker line. Use this window to observe any changes in fluorescent signal as tracker lines are moved. To move any tracker line, click on marker of interest then either use left or right ⇦ keys or drag marker using cursor.

3. Prior to lane extraction check to see that the gel preferences are set to the default values. Under the **Edit** menu select 'Gel Preferences' and check the following parameters:

   Multicomponent Gel Image: **Deselected** **3** *Channel Averaging*

   Estimated Maximum Peak Height: **2000** *Use Weighted Average:* **Selected**

   Select *Stop extraction when below confidence level of* **70%**

4. Once lanes are tracked (all markers above 'used' lanes should now be white) extraction of data must now be extracted from each lane which will result in the generation of one sample file for each lane/sample extracted. Choose 'Extract Lanes' from the **Gel** menu. The Extract Lanes dialog box appears. De-select 'Auto-Analyze New Sample Files'. Click 'OK' to accept all other default settings.

5. After extraction, the Sample Manager window should appear (it may also be hidden behind the gel image-click on it to make it the active window). Click on 'Add files'. Locate and open the

folder that contains the sample files just generated (these can be found in the same run folder containing the gel image). Click 'Add All'. All sample files from that run should now be listed in the Sample Manager window.

6. 'A' and 'P' are checked as default. De-select 'P' (this cancels the autoprint command). Check to make sure analysis settings are correct:

DyeSet/Primer file:	**DT{BD set-Any Primer}**
Instrument file:	**drhod matrix**
Basecaller	**semi-adaptive**

The Spacing, Basecaller Settings, Peak 1 Location, Start Point and Stop Point settings are all default values determined by the software.

7. Click 'Start' to begin a preliminary analysis. The color of the check box under 'A' indicates whether analysis was successful (green), failed (red), or has not been started (no color).

8. Since the entire 7.0 hr. scan is represented in this preliminary analysis, the majority of the analysis contains non sequence data. This nonsense data prior to and after the desired region must be removed before the final analysis. After the preliminary analysis double click on the sample file name to select the sample. Select the raw data icon on the bottom left corner of the display menu. All four colors will be represented in the raw data. Using the mouse, position the curser at the first primer peak and record the x-axis value. Repeat this for the last primer peak. You now have recorded a raw data value for the start and stop analysis point. This will exclude all nonspecific sequence data. Repeat this for all samples.

9. Close the raw data window and return to the sample manager sheet. For each sample, enter the corrected start and stop scan unit values and re-analyze the sample file following step 6 above.

10. **[Amplification Negative Check]** After analysis select the sample file name for the amplification negative. The amplification negative passes under the following conditions: a) the amplification negative does not analyze, b) the amplification negative analyzes but the quality of analysis is below comparison purposes, or c) the amplification negative analyzes, but the sequence is not in concordance with any of the evidence samples and its quantity is below 10%

of the lowest evidentiary sample signal in the amplification. If the amplification negative meets one of the criteria outlined above, the amplification passes and should be noted on the top of the mtDNA editing sheet by circling the "P" for Pass. If the amplification passes proceed to the following step. If the amplification negative does not meet one of the criteria outlined above, the amplification fails and should be noted on the editing sheet by circling the "F" for Failed. All samples must be re-amplified.

11. Under **File**, choose 'Page Setup', select the 'landscape' format, '4' panels per page, and '1500' points per panel. Click 'OK'. Under **File**, choose 'Print'. Check 'Electropherogram' and click 'OK'. In the print window under **Pages** only print from pages 1 to 1, click 'Print'. Printouts of unedited sequences are required for the case file.

12. Do the same for the remaining samples. Printouts are not necessary for samples that have not been analyzed.

8.1 Sequence Analysis 3.3 Trouble Shooting

1. **Poor sequence spacing.** It may be necessary to manually adjust the spacing and re-analyze the sample if it appears the spacing assigned by the software is poor (peaks are too close or too broad). This can be performed in two ways:

   a. return to the sample manager window and adjust the "base spacing" default accordingly to the sequence quality. Increase the value by ±0.5 units and reanalyze the sample until the desired results are obtained.

   -or-

   b. return to the sample manager window and change the Basecaller setting from semi-adaptive to ABI 100. Reanalyze sample.

   c. It may also be necessary to re-electrophorese the sample if steps a-b does not improve sequence spacing.

3. **Low Signal**. Samples displaying observable sequence product but low signal can be re-

analyzed in the following manner:

a. Re-analyze the sample under lower stringent conditions. The analysis threshold can be decreased by selecting 'Gel Preferences' under the **Edit** menu. Select the following parameters:

Multicomponent Gel Image: **Deselected** **1** *Channel Averaging*

Estimated Maximum Peak Height: **500** *Use Weighted Average:* **Deselected**

Select *Stop extraction when below confidence level of* **70%**

This will enable the software to analyze the sample using a non-averaged single tracking lane at a lower signal threshold.

-or-

b. Re-electrophorese the sample with the remaining 4 µL of sequence product.

9.0 Sequence Alignment and Interpretation

9.1 Creating a Batch Worksheet in Factura

1. In the **Sequence Navigator *f*** folder on the hard drive, double click on the **Factura** icon. This will open a 'Batch-untitled' worksheet.
2. Under the **Worksheet** menu, choose 'Add Sequences...' . Find and import all sample files of the gel run. Click 'Add All'. This will add all analyzed samples.
3. Under **File**, choose 'Save As...' and name the worksheet in the following format: "Batch-[*gel name*]". Make sure you are saving the worksheet in the current run folder with your sample files. Click 'Save'. Under **File**, choose 'Quit'.

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9.2 Sequence Navigator

1. Double-click on the HV1 or HV2 consensus icon, also in the **Sequence Navigator *f*** folder. This will open a layout containing the Anderson reference sequence and a positive control reference sequence.. Under **File**, choose 'Save As...' and rename the layout in the following format: "[*gel name*.alignment]".

2. Under the **Sequences** menu, choose 'Import Batch Worksheet...' . Find the batch worksheet just saved in the current run folder. Click 'Import'. This imports the base sequences of all analyzed samples from that gel run. The left panel (or ID panel) contains the lane numbers and sample file names. The right panel displays all the sample base sequences.

3. Under the **View** menu, choose 'Preferences (ID Panel)...' click the radio button labeled "Nickname (or Filename)" and in the bottom right entry field change the number of characters in from 9 to 25. This will allow you to change the name in the ID panel by setting a 'nickname' for each sequence.

4. Select the first sample by clicking the ID number in the left panel. This will highlight the name in black. Under the **Sequences** menu choose 'Set Sequence Nickname...' . Under 'Nickname' type in: "[*name of sample* -**A** or **R**]" for primer-A strand or reverse primer strand dependant upon the sequence direction. Click 'OK'. Do the same for the remaining samples.

Note: **Samples sequenced with the reverse primer are the *forward strands*. Samples sequenced with the A primer are the *reverse strands*.**

5. Before the sequences can be edited, they must be offset to be placed in their respective region of the Anderson reference sequence. Shift-click all sequences just imported. Under **Sequences**, choose 'Offset Sequences...' and offset selected sequences to the "right" by the value 15970 for HVI, or by the value 14 for HV2. This will shift all imported sequences to the right by 15970 or 14 bases respectfully.

6. Prior to analysis the reverse primer strand (forward strand) sequences must first be reverse/complemented before they can be aligned with the corresponding reverse strand (forward primer) sequences. Shift-click the ID numbers of all the reverse strands to select

them. Under the **Sequences** menu, choose 'Reverse Complement Sequence'. Selected sequence(s) are reverse/complemented with indicators appearing next to the ID number(s). These reverse sequences now show the exact base sequence of the forward strand and can now be directly compared.

Proceed to section 9.2.1 for positive control alignments. Proceed to section 9.2.2 for sample alignments.

9.2.1 Positive Control Check

The positive control's forward and reverse sequence must first be compared with the **positive control reference sequence** to confirm that the correct sequence has been obtained. This assures the reaction integrity.

1. Click-drag the reverse primer strand (now reverse-complemented) of the positive control so that it is directly beneath the positive control A-primer strand.
2. The reverse primer strand ID should still be highlighted. Shift-click ID name for the A-primer strand. Both sequences should be highlighted.
3. Under the **Align** menu, choose 'Clustal...' . Click 'OK' to accept default values. This aligns the forward and reverse sequence to each other for comparison.
4. With both sequence ID numbers still highlighted, choose 'Create Shadow(s)' from the **Sequences** menu and select 'Compute Ambiguity Sequence' from the pop-up menu. The shadow sequence appears on the layout below the other sequences. Name this sequence by first clicking on the ID name and then selecting 'Set Sequence Nickname...' under the **Sequences** menu. Under 'Nickname' type in: "[**PC.comp**]" Click-drag this shadow sequence so that it is directly beneath the positive control A-primer and reverse primer sequences. This will make any editing of bases easier to follow.
5. Clip (delete) poor sequence quality base calls associated with the 5' and 3' ends of each strand.

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This is done by click-holding the mouse button at the first base and dragging it to the last desired base. The selected sequence will be highlighted yellow. Remove the unwanted sequence by hitting the "delete" key on the keyboard. The 5' and 3' ends of HV1 and HV2 are not polymorphic so valuable information will not be lost.

6. **Interpretation of [PC.comp] shadow sequence**. Identical-base calls are seen as dashes (–), while any ambiguities between the two sequences will be represented by a (*). If no ambiguities are noted, proceed to step 6. If ambiguities are noted, they can be reconciled by importing the electropherograms and manually calling/editing the base at that site. [In order for a base to be called or edited the editing must fall under the general guidelines of the **Interpretation of Results: criteria for accepting or editing a base call** section 9.4]. To import both electropherograms, click the ID numbers of both sequences. In the first sequence, drag the cursor starting at 10 bases left of the (*) to 10 bases right of the (*). Under the **Sequences** menu, select 'Display Electropherograms'. Electropherogram windows for the selected sequences appear below the layout. Manual editing should be done directly to the displayed electropherogram beginning at the first 5' ambiguity and ending with the last 3' ambiguity. This will update the sequence in the layout automatically. All editing must be recorded in lower case letters and on the mtDNA editing sheet. The exact base position will remain undetermined until alignment to the Anderson Consensus Sequence. As ambiguities are resolved in the positive control sequence the (*) will change to (-) in the [PC.comp] shadow sequence denoting sequence complementation. No sequence ambiguities should be remaining in the final edited sequence. If this is the case, proceed to the next step. If there are sequence ambiguities between the forward and reverse strands of the positive control, the run fails and all samples must be re-amplified. If the ambiguities are attributed to an electrophoresis problem, all samples can be re-electrophoresed prior to re-sequencing or re-amplification.

7. If there is complete sequence complementation between the forward and reverse strands of the positive control, the two sequences must now be compared to the positive control reference sequence. Holding down the shift key, click on the positive control reference sequence along with both of the positive control sequences.

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8. Under the **Align** menu, choose 'Clustal...' . Click 'OK' to accept default values. This aligns both the forward and reverse sequence to the positive control reference sequence.

9. With all three sequence ID numbers still highlighted, choose 'Create Shadow (s)' from the **Sequences** menu and select 'Compute Ambiguity Sequence' from the pop-up menu. The shadow sequence appears on the layout below the other sequences. Name this sequence by first clicking on the ID name and then selecting 'freeze shadow sequence' under the **Sequence** menu. Under the **Sequences** menu, choose 'Set Sequence Nickname...'. Under 'Nickname' type in: "[**PC ref.align**]" Click-drag this shadow sequence so that it is directly beneath the positive control reference sequence but above the positive control forward and reverse sequences. No editing can be performed at this point. If there is complete complementation between the reference and the control sequences, the positive control passes. If there are ambiguities between the two sequences, the control fails and all samples must be re-amplified.

10. In order to properly document any base editing, all control sequences must be aligned to the Anderson Consensus Sequence to establish the Anderson Consensus base number of the edited base(s). To align the positive control sequences to the Anderson sequence, shift-click both the forward and reverse PE sequences, the PC reference sequence, and the HV1 or HV2 Anderson consensus sequence. Under the **Align** menu, choose 'Clustal...' . Click 'OK' to accept default values. All three positive control sequences (which should be identical at this point) are now aligned to the exact Anderson Consensus position. There is no need to create a shadow with the Anderson sequence at this point since the polymorphisms of positive control reference sequence are established.

11. Clip (delete) any non-overlapping 5' and 3' sequence from the PC reference sequence and realign the control sequences by selecting 'Clustal' alignment under the **Align** menu.

12. Record the exact base position for each edited base (if any) performed to the positive control A-primer and reverse primer stands on the mtDNA editing sheet.

13. For final presentation purposes (and to not clutter up the alignment sheet with repetitive sequences) the only sequence required on the alignment page is the [**PC ref.align**] shadow

sequence. This will assure the reviewer of the positive control sequence integrity. To remove the A-primer and reverse primer PE control sequences and [PC.comp] shadow sequence, shift/click each and select 'Remove Sequences' under the **Sequence** menu.

9.2.2 Sample Alignment

Note: If signal dropout is observed after the polymorphic C stretch in either the HV1 or HV2 sequence, proceed to the **Sequencing Beyond the C Stretch in Samples Displaying Sequence Heteroplasmy** section 9.3.

1. Click and drag the reverse primer strand (now reverse complemented) of the first sample directly below its' A-primer strand.
2. Highlight the reverse primer strand ID and A-primer strand ID by shift-clicking each. Both sequences should be highlighted.
3. Under the **Align** menu, choose 'Clustal...' . Click 'OK' to accept default values. This aligns the forward and reverse sequence to each other for comparison.
4. With both sequence ID numbers still highlighted, choose 'Create Shadow(s)' from the **Sequences** menu and select 'Compute Ambiguity Sequence' from the pop-up menu. The shadow sequence appears on the layout below the other sequences. Name this sequence by first clicking on the ID name and then selecting 'Set Sequence Nickname...' under the **Sequences** menu. Under 'Nickname' type in: "[***sample name.*comp**]" Click-drag this shadow sequence so that it is directly beneath the A-primer and reverse primer sequences. This will make any editing of bases easier to follow.
5. Clip (delete) poor sequence quality base calls associated with the 5' and 3' ends of each strand. This is done by click-holding the mouse button at the first base and dragging it to the last desired base. The selected sequence will be highlighted yellow. Remove the unwanted sequence by hitting the "delete" key on the keyboard. The 5' and 3' ends of HV1 and HV2 are not polymorphic so valuable information will not be lost.

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6. Identical-base calls are seen as dashes (–), while any ambiguities between the two sequences will be represented by a (*). If ambiguities are noted, they can be reconciled by importing the electropherograms and manually calling/editing the base at that site. [In order for a base to be called or edited the editing must fall under the general guidelines of the **Interpretation of Results: criteria for accepting or editing a base call** section 9.4]. To import both electropherograms, click the ID numbers of both sequences. In the first sequence, drag the cursor starting at 10 bases left of the (*) to 10 bases right of the (*). Under the **Sequences** menu, select 'Display Electropherograms'. Electropherogram windows for the selected sequences appear below the layout. Manual editing should be done directly to the displayed electropherogram beginning at the first 5' ambiguity and ending with the last 3' ambiguity. This will update the sequence in the layout automatically. The exact base position will remain undetermined until alignment to the Anderson Consensus Sequence. As ambiguities are resolved in the sample the (*) will change to (-) in the "**[*sample name*.com]**" shadow sequence denoting sequence complementation.

Note: **Do not edit any base which cannot be resolved by any of the criteria for accepting or editing a base call from the Interpretation of Results: criteria for accepting or editing a base call section 9.4. If an ambiguity cannot be resolved alter both bases in the forward and reverse strand as "n". Record the base position in the editing sheet and interpret the situation in the comments section. If sequence data contains 2 or more ambiguities between the two sequences, the integrity of the sequence data must be assessed. Re-electrophorese, re-sequence or re-amplify the sample if needed.**

6. After the samples' A-primer and reverse primer sequences have been compared and, when possible, any ambiguities reconciled, the next step is to align and compare the sample with the Anderson Consensus sequence. In order to properly document any base editing and for final sequence interpretation, the forward and reverse sequence must be aligned to the Anderson Consensus sequence. To align the A-primer and reverse primer sequence to the Anderson sequence, shift-click both the A-primer and reverse primer sequences and also the Anderson reference sequence. Under the **Align** menu, choose 'Clustal...' . Click 'OK' to accept default

values. Both the A-primer and reverse primer sequences are now aligned to the exact Anderson Consensus position.

7. Choose 'Create Shadow(s)' from the **Sequences** menu and select 'Compute Ambiguity Sequence' from the pop-up menu. The shadow sequence appears on the layout below the other sequences. Change the shadow ID by first clicking on the ID name and then selecting 'freeze shadow sequence' under the **Sequence** menu. Under the **Sequences** menu, choose 'Set Sequence Nickname...'. Under 'Nickname' type in: **"[*sample name.*polymorph]"** Click-drag this shadow sequence so that it is directly above A-primer sequence.

8. Repeat steps 1-7 for all remaining samples.

9.2.2.1 Interpretation of Results: Comparison to the Anderson Consensus Sequence

a. For each FB case a separate mtDNA Polymorphism Result Table Sheet must be filled out.

b. The same-base calls will be represented in the corresponding shadow sequence as dashes (–); any ambiguities between the two sequences will be represented by a (*). Click on the first (*) in the shadow sequence until it is highlighted. Type in the specific base polymorphism using capitals. In the advent of a deletion type "D". On the Polymorphism Result Table record the sample name, Anderson Consensus base position, the Anderson base and the sample polymorphism in the appropriate column.

c. In the case of an insertion, a (-) is inserted in the Anderson Consensus sequence which offsets the base positioning by N+1. To adjust for the offset in sequence, locate the insertion. Take note of the Anderson Consensus base number prior to the insertion. Record the insertion on the mtDNA Polymorphism Result Table sheet as the [Anderson Consensus base.1] or "N.1". If there are tandem insertions record the first as N.1 and the second as base position N.2. After recording the inserted bases, delete them from the forward strand, reverse strand, and Anderson Consensus sequence. This will circumvent the frame shift in the Anderson Consensus sequence.

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ie. Two insertions occurs after Anderson Consensus base #16861.

FBXX-0001

Sample	Region	Anderson Base Number	Anderson Base Sequence	Polymorphism
rib bone	HV1	16861.1	I	A
rib bone	HV1	16861.2	I	T

Note: It is expected that approximately 20 bases at the beginning and end of the Anderson sequence will not match the sequence of any of the samples. Since the first and last 20 bases of the Anderson sequence reflect the non fluorescently labeled primer sequences used in the reaction, these bases will not be interpreted due to the lack of overlapping sequences.

8. After all editing and polymorphisms have been noted in the shadow sequence, the only sequence required on the alignment page is the **[*sample name*. polymorph]** shadow sequence. Freeze this sequence by first clicking on the ID name and then selecting 'freeze shadow sequence' under the **Sequence** menu. The A-primer and reverse primer strand sequences (along with their shadow sequences) are no longer needed on the final alignment printout. Shift-click all of the A-primer and reverse primer strands and select 'remove sequence' from the **Sequence** menu. The unfrozen **[*sample name*.comp]** shadow sequences will be removed simultaneously.

10. Save the alignment project by selecting 'Save' from the **File** menu.

11. Select 'page setup' from the **File** menu, select the 'portrait' format, '4' panels per page, 1500 points per panel and then click 'OK' to print.

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9.2.2.2 Interpretation of Results: Comparison of Unknown Samples with Known Samples

The sequences are now to be compared to other relevant sequences in the case. Interpret all results using the following table:

SITUATION	INTERPRETATION
Identical sequences	Cannot exclude
One heteroplasmic base in both samples **at the same position**	Cannot exclude
One heteroplasmic base in one sample, not observed in the other	Cannot exclude
One base difference between two samples with no evidence of heteroplasmy	Inconclusive
Two heteroplasmic bases at the same positions in both samples	Cannot exclude
One heteroplasmic base at the same position, one base showing heteroplasmy in one sample but not in the other, with a common nucleotide in each	Cannot exclude
One heteroplasmic base at the same position, one different base at another position with no evidence of heteroplasmy	Inconclusive
Two or more base differences with no evidence of heteroplasmy	Exclusion

9.3 Sequencing Beyond the C Stretch in Samples Displaying Sequence Heteroplasmy

Signal dropout is a phenomena caused by sequence heteroplasmy. Sequence heteroplasmy occurs in approximately 20% - 25% of the population in HV1 and HV2. The region which this is most often noted is in the heteroplasmic C-stretch of HV1 beginning at base number 16180. When a T→C transition occurs at the highly polymorphic base position 16189, length heteroplasmy is most often noted.. These heteroplasmic samples resemble mixtures beyond the C stretch and therefore display

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obscured sequencing results beyond this point. Figures 2 and 3 below represent signal dropout due to length heteroplasmy in HV1 and HV2 respectively. In such cases it is difficult to unambiguously determine the number of cytosine residues present. Therefore, no attempt is made to count the number of cytosine residues within the C-stretch, and all comparisons will assume that the same number is present. The same holds true when a T→C transition occurs at position 310 of HV2. Additional sequencing must be performed using internal primers (B and C) to resolve sequence data beyond this point. The positive control and amplification negative need to be processed from the gel containing heteroplasmic samples since the forward and reverse strands will be used in conjunction with re-sequenced samples.

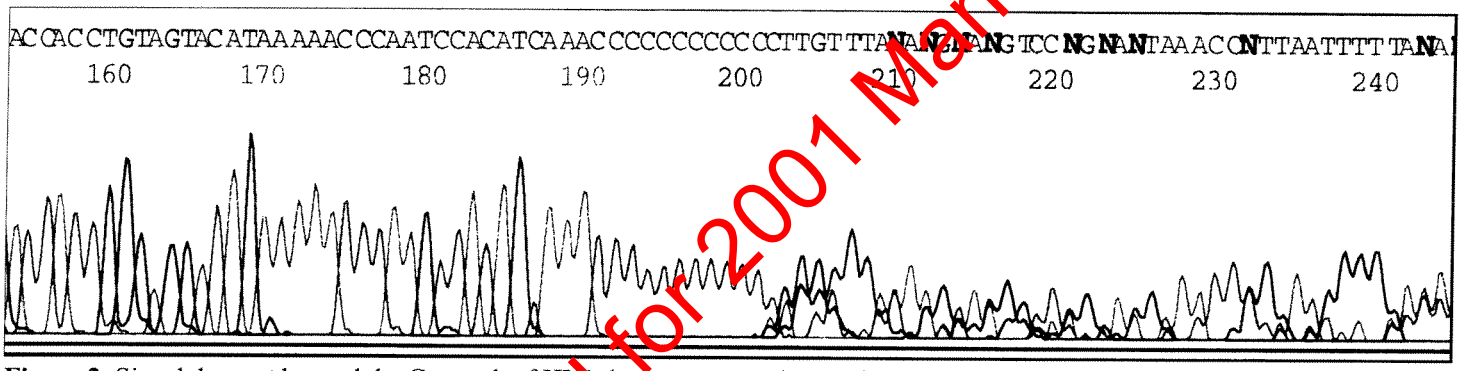

Figure 2: Signal dropout beyond the C-stretch of HV1 due to sequence heteroplasmy.

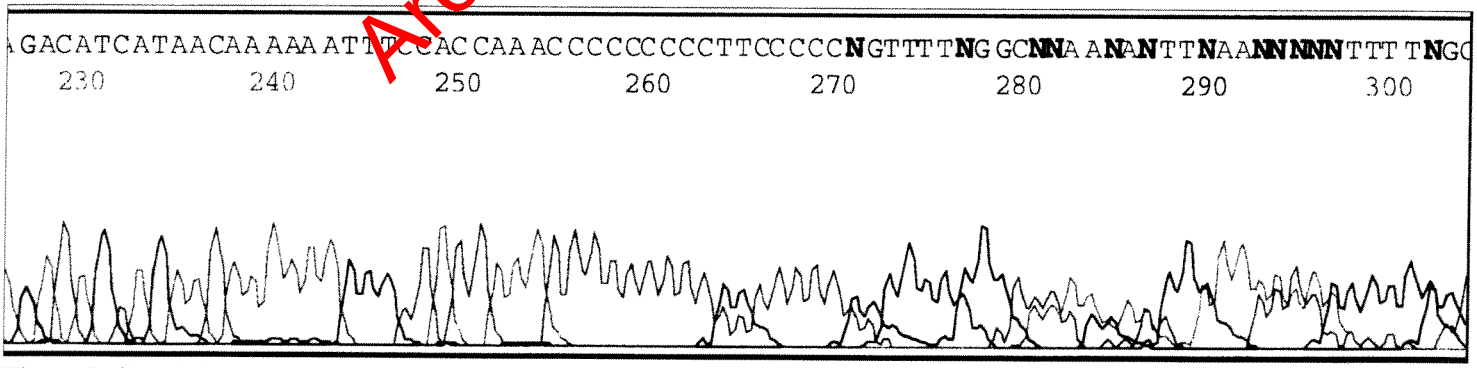

Figure 3: Signal dropout beyond the C-stretch of HV2 due to sequence heteroplasmy.

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1. Re-sequence heteroplasmic samples using the B and C primers for either HV1 or HV2. Follow the same procedures as a non-heteroplasmic sample starting with the **BigDye™ Terminator Cycle Sequencing** section 5.0 through the **Sequencing Analysis 3.3** section 8.0. A sequencing positive and negative control must be processed.

2. In the **Sequence Navigator *f*** folder on the hard drive, double click on the **Factura** icon. This will open a 'Batch-untitled' worksheet.

3. Under the **Worksheet** menu, choose 'Add Sequences...' . Find and import all sample files of the gel run containing the B and C primer. Click 'Add All'.

4. Under the **Worksheet** menu, choose 'Add Sequences...' . Find and import sample files from the previous gel run which displayed sequence heteroplasmy (signal dropout). This gel will have the A and R primers. The batch worksheet should now have all four sequences (A, B,C, and R).

5. Under **File**, choose 'Save As...' and name the worksheet in the following format: **"Batch-[*gel name*]"**. Make sure you are saving the worksheet in the current run folder with your sample files. Click 'Save'. Under **File**, choose 'Quit'.

Note: on the mtDNA sequence editing sheet for the gel containing the B and C primers, note the gel name of samples being imported from a previous gel under the comment section.

6. Double-click on the HV1 or HV2 consensus icon, also in the **Sequence Navigator *f*** folder. This will open a layout containing the Anderson reference sequence and a positive control reference sequence.. Under **File**, choose 'Save As...' and rename the layout in the following format: **"[*gel name*.alignment]".**

7. Under the **Sequences** menu, choose 'Import Batch Worksheet...' . Find the batch worksheet just saved in the current run folder. Click 'Import'. This imports the base sequences of all analyzed samples from that gel run. The left panel (or ID panel) contains the lane numbers and

sample file names. The right panel displays all the sample base sequences.

8. Under the **View** menu, choose 'Preferences (ID Panel)...' click the radio button labeled "Nickname (or Filename)" and in the bottom right entry field change the number of characters in from 9 to 25. This will allow you to change the name in the ID panel by setting a 'nickname' for each sequence.

9. Select the first sample by clicking the ID number in the left panel. This will highlight the name in black. Under the **Sequences** menu, choose 'Set Sequence Nickname...' . Under 'Nickname' type in: "[*name of sample* -**A, B, C** or **R**]" for A-primer, B-primer, C-primer or reverse primer strand.

10. Before the sequences can be edited, they must be offset to be placed in the region of the Anderson Consensus sequence. Shift-click all sequences just imported. Under **Sequences**, choose 'Offset Sequences...' and offset selected sequences to the right by the value 15970 for HVI, or by the value 14 for HV2. This will shift all imported sequences to the right by 15970 or 14 bases respectfully.

11. Prior to analysis the reverse primer strand forward strand) sequences must first be reverse/complemented before they can be aligned with the corresponding C-primer strand. Shift-click the ID numbers for all the reverse primer strands to select them. Under the **Sequences** menu, choose 'Reverse Complement Sequence'. Selected sequence(s) are reverse/complemented with indicators appearing next to the ID number(s). These reverse primer sequences now show the exact base sequence of the C-primer strand sequence and can now be directly compared.

12. Since the sequence is uninterpretable beyond the polymorphic C-stretch in the A-primer and B-primer strands, it can be removed. Click on the first base after the final C in the stretch and drag to the end of the sequence. The selected sequence should be highlighted yellow. Press the delete key to remove the stretch of sequence. Repeat for the remainder of the forward sequences.

13. The A and B-primer strands must now be aligned. Shift-click the ID name for the A and B

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strands. Under the **Align** menu choose 'Clustal...'. Click 'OK' to accept the default values.

14. With both sequence ID numbers still highlighted, choose 'Create Shadow(s)' from the **Sequences** menu and select 'Compute Ambiguity Sequence' from the pop-up menu. The shadow sequence appears on the layout below the other sequences. Name this sequence by first clicking on the ID name and then selecting 'Set sequence Nickname...' under the **Sequence** menu. Under 'Nickname' type in: "[***sample name-A/B.*comp**]" Click-drag this shadow sequence so that it is directly beneath the A and B strand sequences. This will make any editing of bases easier to follow. If ambiguities are present proceed to step 15. If not, skip to step 16.

15. Identical-base calls are seen as dashes (–), while any ambiguities between the two sequences will be represented by a (*). If ambiguities are noted, they can be reconciled by importing the electropherograms and manually calling/editing the base at that site. [In order for a base to be called or edited the editing must fall under the general guidelines of the **Interpretation of Results: criteria for accepting or editing a base call** section 9.4]. To import both electropherograms, click the ID numbers of both sequences. In the first sequence, drag the cursor starting at 10 bases left of the (*) to 10 bases right of the (*). Under the **Sequences** menu, select 'Display Electropherograms'. Electropherogram windows for the selected sequences appear below the layout. Manual editing should be done directly to the displayed electropherogram beginning at the first 5' ambiguity and ending with the last 3' ambiguity. This will update the sequence in the layout automatically. All editing must be recorded in lower case letters and on the mtDNA editing sheet. The exact base position will remain undetermined until alignment to the Anderson Consensus Sequence. As ambiguities are resolved in the sample the (*) will change to (-) in the **"[*sample name A/B.*comp]"** shadow sequence denoting sequence complementation.

Note: Do not edit any base which cannot be resolved by any of the criteria for accepting or editing a base call from the Interpretation of Results: criteria for accepting or editing a base call section 9.4. If an ambiguity cannot be resolved alter both bases in the

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forward and reverse strand as "n". Record the base position in the editing sheet and interpret the the situation in the comments section. If sequence data contains 2 or more ambiguities between the two sequences, the integrity of the sequence data must be assessed. Re-electrophorese or re-amplify the sample if needed.

16. A consensus of sequences A and B must now be created. Shift-click the A and B strands and then select 'Create consensus sequence' from the **Sequences** menu. The shadow of the consensus sequence appears on the layout below the other sequences. Name this sequence by first clicking on the ID name and then selecting 'freeze shadow sequence' under the **Sequence** menu. Under the **Sequences** menu, choose 'Set Sequence Nickname...' . Under 'Nickname' type in: "[***sample name-A/B.*cons**]" Click-drag this shadow sequence so that it is directly beneath the A and B sequence sequences.

17. Prior to their analysis the uniterpretable sequence data prior to the polymorphic C-stretch in the C and R strands must be removed. Click the first base in the C strand and drag to the left-most base prior to the C-stretch. Remove the sequence by selecting the delete key. Repeat for the R strand.

18. The internal C strand and reverse strand must now be further offset to place them into close proximity to their base position in the Anderson consensus sequence. Shift-click the C and R strand. Under **Sequences**, choose 'Offset Sequences...' and offset selected sequences to the right by an additional 210 bases to the right for HV1, or by an additional 320 to the right for HV2.

19. The internal C stand and the R strand must now be aligned. Shift-click the ID name for the C and R strands. Under the **Align** menu choose 'Clustal...'. Click 'OK' to accept the default values.

20. With both sequence ID numbers still highlighted, choose 'Create Shadow(s)' from the **Sequences** menu and select 'Compute Ambiguity Sequence' from the pop-up menu. The shadow sequence appears on the layout below the other sequences. Name this sequence by

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choosing 'Set Sequence Nickname...' under the **Sequences** menu. Under 'Nickname' type in: "[***sample name-C/R.*comp**]" Click-drag this shadow sequence so that it is directly beneath the C and R-primer sequences. This will make any editing of bases easier to follow. If ambiguities are present proceed to step 21. If not, skip to step 22.

21. Identical-base calls are seen as dashes (–), while any ambiguities between the two sequences will be represented by a (*). If ambiguities are noted, they can be reconciled by importing the electropherograms and manually calling/editing the base at that site. To import both electropherograms, click the ID numbers of both sequences. In the first sequence, drag the cursor starting at 10 bases left of the (*) to 10 bases right of the (*). Under the **Sequences** menu, select 'Display Electropherograms'. Electropherogram windows for the selected sequences appear below the layout. Manual editing should be done directly to the displayed electropherogram, which will automatically update the sequences in layout. All editing must be recorded in lower case letters and on the mtDNA editing sheet. The exact base position will remain undetermined until alignment to the Anderson Consensus Sequence. As ambiguities are resolved in the sample the (*) will change to (-) in the **"[*sample name C/R.*comp]"** shadow sequence denoting sequence complementation. Repeat for all samples.

Note: Do not edit any base which cannot be resolved by any of the criteria for accepting or editing a base call from the Interpretation of Results: criteria for accepting or editing a base call section 11.4. If an ambiguity cannot be resolved alter both bases in the forward and reverse strand as "n". Record the base position in the editing sheet and interpret the the situation in the comments section. If sequence data contains 2 or more ambiguities between the two sequences, the integrity of the sequence data must be assessed. Re-electrophorese or re-amplify the sample if needed.

22. A consensus of sequences C and R must now be created. Shift-click the C and R strands and then select 'Create consensus sequence' from the **Sequences** menu. The shadow of the consensus sequence appears on the layout below the other sequences. Name this sequence by first clicking on the ID name and then selecting 'freeze shadow sequence' under the **Sequence** menu. Under the **Sequences** menu, choose 'Set Sequence Nickname...' . Under 'Nickname'

type in: "[***sample name-C/R.*cons**]" Click-drag this shadow sequence so that it is directly beneath the A and B sequence sequences.

23. To create one united sequence of all four strands, a consensus of the "[***sample name-A/B.*cons**]" and "[***sample name-C/R.*cons**]" shadow sequences must be created. To do this the two consensus sequences must be overlapped. Shift-click the "[***sample name-A/R.*cons**]" shadow sequence and "[***sample name-C/R.*cons**]" shadow sequence and select 'Overlap' from the **Align** menu. Following this select 'Create consensus sequence' from the **Sequences** menu. The shadow of the consensus sequence appears on the layout below the other sequences. Name this sequence by first clicking on the ID name and then selecting 'Freeze shadow sequence' under the **Sequence** menu. Under the **Sequences** menu, choose 'Set Sequence Nickname...'. Under 'Nickname' type in: "[***sample name.*cons**]" Click-drag this shadow sequence so that it is directly beneath all the sequences used to create it.

24. The only sequences required for interpretation at this point are the A, B, C, R and "[***sample name.*cons**]" sequences. Shift-click all sequences but these and select **'Remove sequences'** from the Sequences menu.

25. In order to properly document any base editing and for final sequence interpretation, the A,B, C, R and "[***sample name.*cons**]" strands must be aligned to the Anderson Consensus sequence. (These 5 strands should be identical at this point). To align the A,B, C, R and "[***sample name.*cons**]" strands to the Anderson sequence, Shift-click all strands along with the Anderson reference sequence. Under the **Align** menu, choose 'Clustal...' . Click 'OK' to accept default values. All strands are now aligned to the exact Anderson Consensus position.

26. Choose 'Create Shadow(s)' from the **Sequences** menu and select 'Compute Ambiguity Sequence' from the pop-up menu. The shadow sequence appears on the layout below the other sequences. Change the shadow ID by first clicking on the ID name and then selecting choose 'Set Sequence Nickname...' under the **Sequences** menu. Under 'Nickname' type in: **"[*sample name.*polymorph]"** Click-drag this shadow sequence so that it is directly above the A-primer sequence.

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9.3.1 Interpretation of Results: Comparison to the Anderson Consensus Sequence

a. For each FB case a separate mtDNA Polymorphism Result Table Sheet must be filled out.

b. As in before, the same-base calls will be represented in the corresponding shadow sequence as dashes (–); any ambiguities between the two sequences will be represented by a (*). Click on the first (*) in the shadow sequence until it is highlighted. Type in the specific base polymorphism using capitals. In the advent of a deletion type "D". On the Polymorphism Result Table record the sample name, Anderson Consensus base position, the Anderson base and the sample polymorphism in the appropriate column.

c. In the case of an insertion, a (-) is inserted in the Anderson Consensus sequence which offsets the base positioning by N+1. To adjust for the offset in sequence, locate the insertion. Take note of the Anderson Consensus base number prior to the insertion. Record the insertion on the mtDNA Polymorphism Result Table sheet as the [Anderson Consensus base.1] or "N.1". If there are two tandem insertions record the first as N.1 and the second as base position N.2. After recording the inserted bases, delete them from the forward strand, reverse strand, and Anderson Consensus sequence. This will circumvent the frame shift in the Anderson Consensus sequence.

ie. Two insertion occurs after Anderson Consensus base #16861.

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Sample	Region	Anderson Base Number	Anderson Base Sequence	Polymorphism
rib bone	HV1	16861.1	I	A
rib bone	HV1	16861.2	I	T

Note: It is expected that approximately 20 bases at the beginning and end of the Anderson sequence will not match the sequence of any of the samples. Since the first and last 20 bases of the Anderson sequence reflect the non fluorescently labeled primer sequences used in the reaction, these bases will not be interpreted due to the lack of overlapping sequences.

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23. For final presentation purposes (and to not clutter up the alignment sheet with repetitive sequences) the only sequence required on the alignment page is the **"[*sample name.* polymorph]"** shadow sequence. After all editing and polymorphisms have been noted in the shadow sequence, the A, B, C, R and **"[*sample name.* cons]"** sequences are no longer needed on the final alignment printout. Freeze the **"[*sample name.* polymorph]"** shadow sequence by first clicking on the ID name and then selecting 'freeze shadow sequence' under the **Sequence** menu. To remove the A, B, C, R and **"[*sample name.* cons]"** sequences, shift-click the respective sequences and select 'Remove sequence' from the **Sequence** menu.

24. Save the alignment project by selecting 'Save' from the **File** menu.

25. Select 'page setup' from the **File** menu, select the 'portrait' format, '4' panels per page, 1500 points per panel and then click 'OK' to print.

26. The sequences are now to be compared to other relevant sequences in the case. Interpret all results using the following table:

SITUATION	INTERPRETATION
Identical sequences	Cannot exclude
One heteroplasmic base in both samples **at the same position**	Cannot exclude
One heteroplasmic base in one sample, not observed in the other	Cannot exclude
One base difference between two samples with no evidence of heteroplasmy	Inconclusive
Two heteroplasmic bases at the same positions in both samples	Cannot exclude
One heteroplasmic base at the same position, one base showing heteroplasmy in one sample but not in the other, with a common nucleotide in each	Cannot exclude
One heteroplasmic base at the same position, one different base at another position with no evidence of heteroplasmy	Inconclusive
Two or more base differences with no evidence of heteroplasmy	Exclusion

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9.4 Interpretation of Results: criteria for accepting or editing a base call

Base calls at each position made by 'Sequencing Analysis' must be either accepted or edited.

a. A base call is accepted if the base is clearly apparent, is called by the analysis program and is confirmed when both the forward and reverse sequence show clearly defined complementary peaks.

b. Edit base call to authentic base if base is clearly apparent but is obscured or has a lower signal than other base(s) which is/are clearly background.

c. Edit base call to authentic base if base is clearly apparent but is off-scale and an underlying base is recognized by the software.

d. Edit base call to authentic base when clear mitochondrial sequence is visible below an artifactual signal due to excess fluorescence of a single base over a range of bases.

e. Delete additional bases that are present in sequence data (often due to peak broadening near the ends of sequence data).

f. Insert authentic base that has been omitted from sequence even though a discreet peak in electropherogram.

g. A base call on the forward strand with a definitive peak that is clearly different from a base call on the reverse strand, also with a definitive peak, should be kept as called but be included in the report as "N" (unconfirmed) in the report.

h. Two or more peaks of approximately equal intensity and spacing at a single base position must be called "N".

i. Data should be discarded when sequence information is off-scale, at baseline, or contains excessive background.

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10.0 mtDNA Control Interpretation Guidelines

10.1 Negative Controls

The extraction negative control and amplification negative control are a check for the possible contamination of the reagents used during the mtDNA sequencing test by other human DNA. The extraction negative control is performed by carrying out the extraction in a tube containing no sample. The amplification negative control contains no added DNA and checks for contamination at the amplification step.

The extraction negative control and amplification negative control are amplified and sequenced along with the test samples. The appearance of signals in the sequencing of these controls indicates any or all of the following:

a. The sample preparation reagents may have been contaminated.

b. Cross-contamination between samples may be occurring during preparation.

c. Human DNA or amplified DNA may be getting into the samples from some other source.

d. Sample overflow from the adjacent lane might have happened during gel loading.

Clearly, if the test sample does not show any signal in common with the controls, the test sample is not affected by the same source of contamination.

If the extraction negative and amplification negative controls show very weak signals (or "noise") and the test samples show distinct peaks that meet the reporting criteria, the contamination problem is not serious. If the extraction negative, amplification negative or substrate controls show sequenceable data, the contamination problem is more serious. **See Table 2 for interpretation guidelines.**

The appearance of signals in the extraction negative and amplification negative does not necessarily

mean that the types obtained for the test samples are incorrect because of the following:

a. The contamination might be due to a single event limited to the control.

b. The level of contamination might be inconsequential compared to the amount of DNA being amplified and typed in the test samples.

However, further testing may be necessary to support these possibilities.

Control	Results	Interpretation of Test Sample
Positive Control	Matches reference control sequence (100% homology)	All test samples conclusive.
Positive Control	Does not match reference control sequence	All test samples in conclusive. All samples must be re-amlified or re-sequenced.
Extraction Negative	Sequence data obtained	All test samples inconclusive.
Extraction Negative	noise visible, no sequence data obtained	Test samples are conclusive.
Amplification Negative	sequence data obtained	Refer to section 8.0 step 10.
Amplification Negative	noise visible, no sequence data obtained	All test samples conclusive.

Table 2: Guideline to the Interpretation of Signal/Noise in the Extraction negative and Amplification negative controls

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10.2 Sequencing Positive Control

The positive control DNA (mtDNA PC) is used with each batch of samples typed to demonstrate that the amplification and sequencing reactions performed optimally.

10.2.1 mtDNA PC Reference Sequence HV1 (Base Count 420)

5' CAAAGCTAAG ATTCTAATTT AAACTATTCT CTGTTCTTTC ATGGGGAAGC
AGATTTGGGT ACCACCCAAG TATTGACTCA CCCATCAACA ACCGCTATGT
ATTTCGTACA TTACTGCCAG CCACCATGAA TATTGTACGG TACCATAAAT
ACTTGACCAC CTGTAGTACA TAAAAACCCA ATCCACATCA AAACCCCCTC
CCCATGCTTA CAAGCAAGTA CAGCAATCAA CCCCCAACTA TCACACATCA
ACTGCTACTC CAAAGCCACC CCTCACCCAC TAGGATACCA ACAAACCTAC
CCACCCTTAA CAGTACATAG CACATAAAGC CATTTACCGT ACATAGCACA
TTACAGTCAA ATCCCTTCTC GTCCCCATGG ATGACCCCCC TCAGATAGGG
GTCCCTTGAC CACCATCCTC 3'

Sample	Region	Anderson Base Number	Anderson Base Sequence	Polymorphism
PC	HV1	16224	T	C
PC	HV1	16246	A	T
PC	HV1	16311	T	C

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10.2.2 mtDNA PC Reference Sequence HV2 (Base Count 440)

5' CTCTCCATGC ATTTGGTATT TTCGTCTGGG GGGTGTGCAC GCGATAGCAT
TGCGAGACGC TGGAGCCGGA GCACCCTATG TCGCAGTATC TGTCTTTGAT
TCCTGCCTCA TCCTATTATT TATCGCACCT ACGTTCAATA TTACAGGCGA
ACATACTTAC TAAAGTGTGT TAATTAATTA ATGCTTGTAG GACATAATAA
TAACAATTGA ATGTCTGCAC AGCCGCTTTC CACACAGACA TCATAACAAA
AAATTTCCAC CAAACCCCCC CTCCCCCGCT TCTGGCCAGA GCACTTAAAC
ACATCTCTGC CAAACCCCAA AAACAAAGAA CCCTAACACC AGCCTAACCA
GATTTCAAAT TTTATCTTTT GGCGGTATGC ACTTTTAACA GTCACCCCCC
AACTAACACA TTATTTTCCC CTCCCACTCC CATACTACTA 3'

Sample	Region	Anderson Base Number	Anderson Base Sequence	Polymorphism
PC	HV2	[illegible]	A	G
PC	HV2	263	A	G
PC	HV2	315.1	I	C

If any positive control does not produce a correct and readable type, the amplification must be repeated and the test samples are considered inconclusive.

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10.3 Anderson HV1 Consensus Sequence (Base Count 450)

5' TTAACTCCAC CATTAGCACC CAAAGCTAAG ATTCTAATTT AAACTATTCT
CTGTTCTTTC ATGGGGAAGC AGATTTGGGT ACCACCCAAG TATTGACTCA
CCCATCAACA ACCGCTATGT ATTTCGTACA TTACTGCCAG CCACCATGAA
TATTGTACGG TACCATAAAT ACTTGACCAC CTGTAGTACA TAAAAACCCA
ATCCACATCA AAACCCCCTC CCCATGCTTA CAAGCAAGTA CAGCAATCAA
CCCTCAACTA TCACACATCA ACTGCAACTC CAAAGCCACC CCTCACCCAC
TAGGATACCA ACAAACCTAC CCACCCTTAA CAGTACATAG TACATAAAGC
CATTTACCGT ACATAGCACA TTACAGTCAA ATCCCTTCTC GTCCCCATGG
ATGACCCCCC TCAGATAGGG GTCCCTTGAC CACCATCCTC 3'

10.4 Anderson HV2 Consensus Sequence (Base Count 477)

5'CTATCACCCT ATTAACCACT CACGGGAGCT CTCCATGCAT TTGGTATTTT
CGTCTGGGGG GTATGCACGC GATAGCATTG CGAGACGCTG GAGCCGGAGC
ACCCTATGTC GCAGTATCTG TCTTTGATTC CTGCCTCATC CTATTATTTA
TCGCACCTAC GTTCAATATT ACAGGCGAAC ATACTTACTA AAGTGTGTTA
ATTAATTAAT GCTTGTAGGA CATAATAATA ACAATTGAAT GTCTGCACAG
CCACTTTCCA CACAGACATC ATAACAAAAA ATTTCCACCA AACCCCCCCT
CCCCCGCTTC TGGCCACAGC ACTTAAACAC ATCTCTGCCA AACCCCAAAA
ACAAAGAACC CTAACACCAG CCTAACCAGA TTTCAAATTT TATCTTTTGG
CGGTATGCAC TTTTAACAGT CACCCCCCAA CTAACACATT ATTTTCCCCT
CCCACTCCCA TACTACTAAT CTCATCA3'

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11.0 Troubleshooting Sequencing of mtDNA

A. T to C transition at position 16184 (homopolymer C stretch causing HV1 C-stretch length variation), with unreadable "wave-like" pattern following.

- repeat sequencing reaction of amplified product using primer HV1-C (F16190).

B. C insertion at position 309.1 (homopolymer C stretch causing HVII C-stretch length variation), with unreadable "wave-like" pattern following.

- repeat sequencing reaction of amplified product using primer HV2-C (L317).

C. High background ("noise" or signal underlying sequence data), poor resolution (discreet peaks not distinguishable).

- repeat sequencing reaction
- repeat microcon of amplified product
- repeat amplification

D. Off-scale peaks throughout entire sample.

- rerun samples diluted 1:10 with blue formamide

E. Off-scale peaks at start of sequence followed by signal that "falls off" or becomes very weak.

- repeat sequencing reaction using less amplified product

C. Low or faint signal across entire gel (including positive control) that is an obvious technical gel problem.

- rerun gel

D. Faint or no signal for evidence samples with good sequence data for positive control

- repeat sequencing reaction with more amplified product

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12.0 References

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