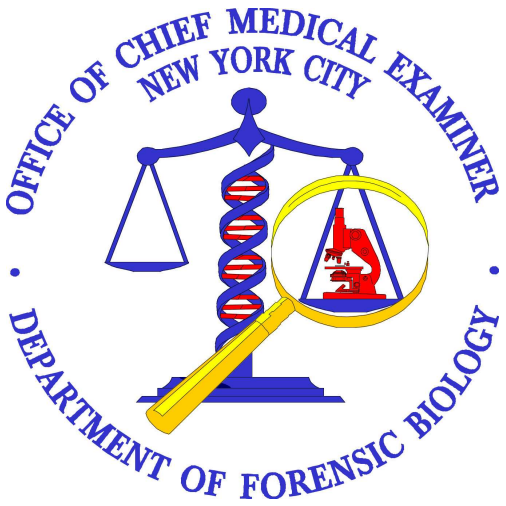


FORENSIC BIOLOGY PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

Primary Approving Authority:
Eli Shapiro, Technical Leader – Mitochondrial DNA Operations

Procedure	Effective Date	Comments
Mitochondrial DNA Guidelines	07/24/2010	Initial Version of Procedure
Hair Evidence Examination	07/24/2010	Initial Version of Procedure
Washing Hairs for Mitochondrial or Nuclear DNA Testing	07/24/2010	Initial Version of Procedure
Organic Extraction for Mitochondrial or Nuclear DNA Testing	11/02/2010	
Duplex Mitochondrial DNA PCR Amplification-Roche	07/24/2010	Initial Version of Procedure
Mitochondrial DNA Product Gel Analysis	07/24/2010	Initial Version of Procedure
Quantitation Using Agilent 2100 Bioanalyzer	07/24/2010	Initial Version of Procedure
Mitochondrial DNA Linear Array Analysis	09/03/2010	
Exo-Sap-It Sample Cleanup & Cycle-Sequencing Worksheet	07/24/2010	Initial Version of Procedure
Cycle-Sequencing	07/24/2010	Initial Version of Procedure
SDS Cleanup	07/24/2010	Initial Version of Procedure
Centri-Sep Sample Filtration	07/24/2010	Initial Version of Procedure
ABI 3130xl Sequencing	07/24/2010	Initial Version of Procedure

Procedure	Effective Date	Comments
Mitochondrial DNA Sequencing Analysis	07/24/2010	Initial Version of Procedure
Sequence Nomenclature and Alignment	07/24/2010	Initial Version of Procedure
Editing Guidelines	07/24/2010	Initial Version of Procedure
Interpretation Guidelines	09/03/2010	
Statistical Analysis	07/24/2010	Initial Version of Procedure
Creation of a Casefile CD	07/24/2010	Initial Version of Procedure
References	07/24/2010	Initial Version of Procedure
Appendix A	07/24/2010	Initial Version of Procedure
Appendix B	07/24/2010	Initial Version of Procedure
Appendix C	07/24/2010	Initial Version of Procedure
Appendix D	07/24/2010	Initial Version of Procedure

MITOCHONDRIAL DNA GUIDELINES		
DATE EFFECTIVE 07-24-2010	APPROVED BY ELI SHAPIRO	PAGE 1 OF 5

PURPOSE: Guidelines for mitochondrial DNA testing to ensure clean laboratory practices, unambiguous sample identification, and relevant control runs.

A. GENERAL PROCEDURES:

To reduce the possible contamination in the laboratory that could occur: (i) between the analyst and the samples (ii) from one sample to another, or (iii) from extraneous sources of DNA within the laboratory.

1. Lab coat, gloves, and mask, eye protection, and/or face shield, must always be worn while in the exam and pre-amplification room. Lab coat, gloves, eye protection must be worn in the post amplification area. All gowning must be done in the vestibules of exam, pre-amp or post amp rooms.
2. Lab coats can be reused for a period of one week. Afterwards, they should be thrown out. Masks/face shields can also be reused for a period of one week. Goggles can be exposed to UV light in the Stratalinker to extend their time of use.
3. When working in the exam or pre-amplification laboratory, gloves must be rinsed in 10% bleach before each procedure and in-between the handling of separate samples.
4. Pipettes must be wiped down with 10% bleach before each procedure, and between the pipetting of separate samples.
5. All hoods must be wiped down with 10% bleach before and after each procedure, followed by a 70% Ethyl Alcohol rinse, and UV light, if available, should be applied for 30 minutes before and following each procedure.
6. All racks, tube-openers and any other plastic implements (but not the pipettes) must be exposed to UV light in the Stratalinker for a minimum of 30 minutes before they can be used for amplification or extraction.
7 Any 96-well tube racks taken from the pre-amp room to the post-amp room must be placed into the post-amp bleach bath, rinsed, and dried prior to being returned to the pre-amp room.
8. All 1.5ml and 0.2ml tubes can be kept in plastic Nalgene boxes or comparable containers, and should only be removed with bleached and dried gloves while fully gowned. Prior to placement of tubes into these containers, the tubes used for, washing, extraction and amplification must be exposed to UV light in the Stratalinker for 30 minutes.
9. Counters, sinks, refrigerator/freezer handles and door handles inside the laboratory and gowning room should be wiped down with 10% bleach on a monthly basis.

B. NOMENCLATURE

The following are suggested naming conventions for use throughout the sample processing. The goal of this nomenclature is to ensure that sample names are unique identifiers.

1. Samples re-extracted for the purposes of duplication (new cutting): The suffix : ”dup” will be added to the sample name to separately identify the re-extraction sample from the original, and this suffix will be applied to these duplication samples throughout the processing

2. Samples reamplified in order to improve on the quality of the results or for other purposes: The suffix “reamp” will be added to the sample name.

3. At the 3130xl run step:
   - The suffix “recyc” will be added to each sample name for samples that are re-sequenced (e.g. sample-recyc).
   - The suffix “conf” will be added to each sample name for samples that are re-sequenced to confirm sequence or length heteroplasmy (e.g. sample-conf).
   - The suffix “reinj” will be added to each sample name for samples that are re-injected (e.g. sample-reinj).
   - The primer used will be added as suffix to each sample name. This suffix will always be added last, e.g. sample-B4, sample-recyc-B4, sample-conf-B4, sample-reinj-B4.

4. Contig name:
   - A contig name should be: FBYY-12345-HVI, or FBYY-12345-HVII, or FBYY-12345-HVI dup, FBYY-12345-HVII dup
   - In certain cases more than one sample will be typed and identifiers could be added after FBYY-12345, e.g. FBYY-12345(V)-HVI, FBYY-12345-PM7-HVI, FBYY-12345-PM1-HVI, FBYY-12345-Q1-HVI, FBYY-12345-Q2-HVI

5. Sequencher ID
   - The sequencher ID of an analyzed run will be identical to that run ID, e.g. SYY-123.
   - The sequencher ID of a case will be identical to the FB case number, e.g. FBYY-12345.
   - The sequencher ID for a Missing person case will be the FB# space MP (e.g. FBYY-12345 MP)

6. Amplification and Cycle-sequencing worksheets should be reviewed for typographical errors. This paperwork review can be performed by any analyst.

C. REPEAT ANALYSIS OF SAMPLES

Repeat testing of a sample can start at different stages, as listed below. Appropriate controls must be used.

1. Extraction stage: A new extraction negative control must be run.
2. Amplification stage: New amplification negative and positive control must be included. The extraction negative control does not need to be repeated if it previously passed.
3. Cycle sequencing: Positive and negative controls must be tested for each primer used. The original extraction negative does not have to be repeated if it passed for all needed sequences. The original amplification negative does not have to be repeated if it passed for all needed sequences; a cycle sequencing negative (cAN) should then be used (20 µl H_2O) for each primer used. The original positive control should be used and suffixed recyc for each primer used; however, any positive control can also be used as long as the contig of interest can be built with that positive control. Note that if a sample needs to be re-sequenced with a primer because the positive control at that primer failed, then every control or sample in that run needs to be re-sequenced with that primer.

D. BATCHING AND DUPLICATION GUIDELINES

Duplication of samples is only necessary from when samples are batched.

Exemplar samples batched and extracted for nuclear DNA may be duplicated with a second nuclear DNA extraction and STR typing.

For mtDNA, duplication of a given sample can be accomplished by running one informative primer for that sample in either HVI or HVII.

1. Evidence samples

a) There will be no batching of evidence samples at the DNA extraction and amplification stages (e.g. each sample will have its own extraction and amplification negative controls). Therefore, duplication of evidence samples at the extraction level is not required. Duplication at the extraction level can be done for case-related reasons (see supervisor).

b) Batching of evidence samples is allowed at quantification steps. Duplication of sample at the quantification level is not required.

c) Evidence samples within a given case (e.g. all hair samples from a single case) may be processed at the same time at quantification and sequencing steps. Duplication of sample at the quantification level is not required. Duplication can be done at the cycle sequencing step if the evidence sample does not match any other sample in the case. Duplication at the cycle sequencing step can be done for other case related reasons (see supervisor).

2. Exemplar samples

a) Batching of exemplar samples from different cases will be allowed at all steps of mtDNA analysis including the DNA extraction stage.
b) HVI-HVII amplification and sequencing of exemplar samples from the same case (e.g. family members, duplication samples) should be performed at least once separately.
c) Suspect exemplars will be duplicated if that sample matches an evidence sample.
d) Victim exemplars will be duplicated if they do not match any other sample in the case and if that exclusion is informative.
e) Missing Persons' exemplars and unidentified remains do not need to be duplicated.
f) Any exemplar may be duplicated for case related reasons or to streamline testing.

3. Exemplar with Evidence samples

With the exception of quantification evidence and exemplar samples must always be tested separately in time and/or space. Batching of evidence with exemplar samples is allowed during the DNA quantitation step. Batching of evidence with exemplar samples is also allowed during product gel or Agilent analysis provided that sample aliquots are done on each sample type (evidence or exemplar) at separate times. Quantification steps do not need to be duplicated.

Revision History:

July 24, 2010 – Initial version of procedure.

HAIR EVIDENCE EXAMINATION		
DATE EFFECTIVE 07-24-2010	APPROVED BY ELI SHAPIRO	PAGE 1 OF 4

General Guidelines

Refer to Evidence and Case Management Manual for general laboratory guidelines for evidence examination, note-taking, itemization, and preparation for evidence examination.

A. Hair Evidence Examination

1. Document all packaging on appropriate worksheet(s) and open packaging in a dead-air hood (preferred) or comparable (e.g. enclosed space).

2. Document the hair examination. Note the hair approximate length and whether or not the hair is mounted.
3. **If hair is <1 cm in length, see supervisor. If the hair will be consumed indicate in case notes that the sample will be consumed for testing and proceed.**
4. For hairs that are loose, proceed with step 5. For hairs that are mounted, proceed with demounting (See "Washing Hairs for Mitochondrial and Nuclear Testing", part A) and then return to step 5.
   Note: It is at the analyst's discretion to photo document hair mounted on a slide at this step. In this case follow step 5 below prior to demounting hair.
5. Take a picture of the full hair:
   - Digital or Mideo picture can be made.
   - If the hair is unmounted, it can be placed in a weigh boat.
   - Place hair on appropriate background for photo documentation. Brown, black, or darker colored hairs should be placed on a white sheet of clean paper. Blonde, white or light colored hairs should be placed on a darker background.
   - Take a digital/Mideo photograph of the full hair, including a ruler/measurement in the frame. For digital pictures, be sure the digital camera is set to Macro (flower) and the flash is off before taking the picture. For Mideo pictures, see Mideo Macro/Microscopic Digital Imaging System below (see part B).
     i. Save /export pictures
     ii. Print digital and Mideo images for case file (see part C).

B. Mideo Macro/microscopic digital imaging system

1. Make sure the computer is on and all of the proper cables are connected between the Firewire camera and the computer.

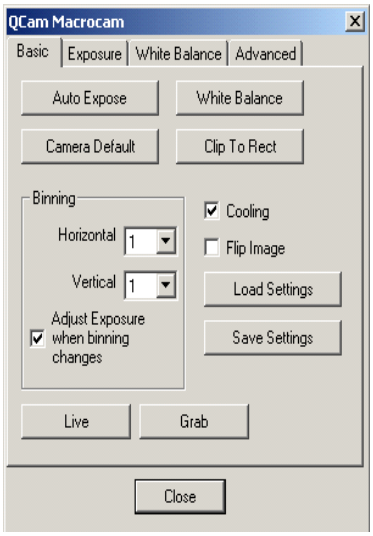
2. Double-click on the desktop shortcut **EZDocPlus**.

3. The main program screen will appear. Click on "Camera" and select "Micro Cam M". The QCam Microcam Control Panel will appear.

4. Make sure at this point that the stereo microscope is on, the light source is active, and the specimen is in focus. When viewing solid, dark objects, it is best to use the ocular light ring to illuminate the sample. When viewing slides or thin tissue samples, use the direct light from the lamp base of the microscope.

5. On the **Microcam Control Panel (shown to the right)**, perform the following:

   a. Click the **Live** button.
   b. Adjust the binning so that the setting is 3 for both the horizontal and vertical.
   c. Check the **Flip Image** box.
   d. Click the **Auto Exposure** button.
   e. Adjust the intensity of the light and click **Auto Exposure** if the image is too bright or too dark.

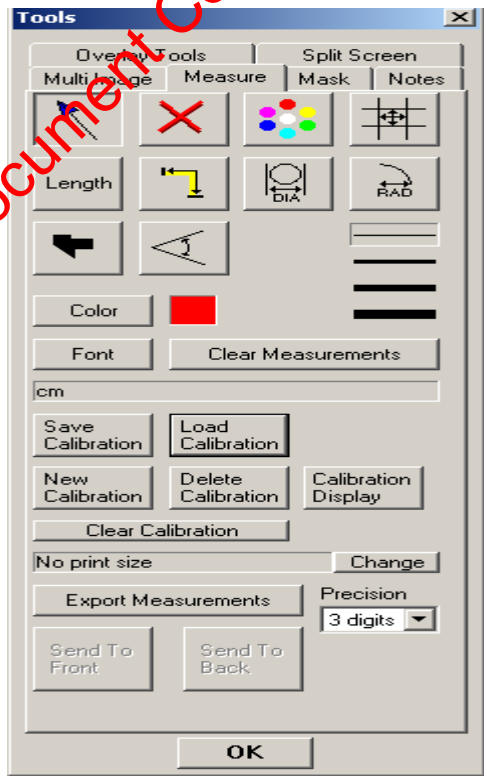


<u>NOTE:</u> If the background color is not white or off white, place a sheet of paper in view of the lens and click on the White Balance button. Repeat step 5d once completed.

6. Once the image on the screen is in focus and there is proper contrast, click the **Grab** button on the control panel. This will freeze the image on the screen.

7. Under the **Tools** menu, select **Overlays**. Once the panel opens up, perform the following:

   a. Click on the **Measure** tab.
   b. Click the **Load Calibration** button.
   c. Select the calibration based on the current microscope magnification level.
   d. FOR LINEAR OBJECTS
      1) Click on the **Length** button.
      2) Select any of the length tools to measure the length of the imaged object.
   e. FOR NON-LINEAR OBJECTS
      1) Click on the **Multilength** button
      2) Trace the non-linear object length by left-clicking the mouse at desired turns and corners. Hit "Enter" on the keyboard when finished.



8. Save the image by going to the **File** menu and selecting **Export Image.**

9. Save image with case identifying name (e.g. FB07-04117 Item 1A-1)

C. Printing digital and mideo images for case file

1. Open Microsoft PowerPoint.
2. Go to File menu and select Page Set-up. Change slide orientation from landscape to **portrait**.
3. Import pictures
4. Add Sample ID. Add comments if needed.
5. Save as FB# in Photo Archives

Revision History:

July 24, 2010 – Initial version of procedure.

WASHING HAIR FOR MITOCHONDRIAL OR NUCLEAR DNA TESTING

DATE EFFECTIVE	APPROVED BY	PAGE
07-24-2010	ELI SHAPIRO & EUGENE LIEN	1 OF 3

PURPOSE: To prepare hairs for DNA extraction.

A. Demounting:

1. If the hair is loose, then proceed to the appropriate hair washing procedure. If a "possible root" is observed, the sample should be cut and washed for nuclear DNA testing extraction (see part C).

2. If the hair is mounted:

i. Process only one mounted slide at a time.

ii. Turn on the heat plate and adjust the heat dial between 100-110^{o}C. Place the slide on a heat plate until the mountant softens and using forceps remove the cover slip. The mountant softens quickly and hairs will scorch if left on the heat plate too long.

iii. The hair will be attached to either the coverslip or the slide. Remove hair and place into a xylene bath for up to 5 minutes or until the mountant completely dissolves. Hairs and slides/coverslip containing hairs can be kept in the xylene bath for longer than 5 minutes if necessary.

iv. Using clean forceps, carefully remove the hair from the xylene bath

v. It is at the discretion of the analyst to make a picture of the full hair at this time.

vi. Proceed to the appropriate hair washing procedure.

B. Washing the hair for mtDNA testing extraction

1. Using forceps and a scalpel cut a 2 cm region of the hair or hair shaft. A picture of the cutting should be taken at this time. If the hair is also to be tested for nuclear DNA, the mitochondrial DNA cutting should be away from the root. Place the unused portion of the hair onto the backing of a post-it note and return to the packaging

2. **If "possible tissue" attached to hair is observed, see your supervisor. In some cases the hair will not be washed, proceed to step 11 and enter N/A as TergAZyme and Saline lot #.**

3. Prepare 5% TergAZyme solution by adding 15ml of GIBCO water to 0.75g of TergAZyme. Mix well. Record TergAZyme lot #.

4. Using clean forceps, place the hair fragment cutting into a 1.5 ml tube with 1 ml of 5% TergAZyme solution. Vortex the tube for 1 minute at high speed, and place into the sonicator for 15 minutes. After sonication, vortex the sample again for 1 minute at high speed.

5. Prepare a 50 ml Falcon tube and filter cup. Label the tube and filter cup tab with the sample name. Pre-wet the filter cup membrane with 1 ml of Gibco dH_2O.
6. Remove the hair from the TergAZyme with clean forceps, and place the hair into the filter cup in the center of the membrane.
7. Wash the hair with 1 ml of Gibco dH_2O. Allow the liquid to pass through the filter.
8. Wash the hair with 1 ml of 0.85% saline. Allow the liquid to pass through the filter. Record Saline Lot #.
9. Wash the hair with 1 ml of 100% ethanol. Allow the liquid to pass through the filter.
10. Remove the filter cup containing the hair and place on a Kimwipe to let the ethanol evaporate. Once the filter membrane is dry, the hair will be dry as well.
11. Transfer the cut hair fragment to the bottom of a clean 1.5 ml tube. Label the tube with sample name.
12. Store the tube containing the hair fragment in the appropriate "To Be Extracted" cryobox in the pre-amplification laboratory freezer.
13. Proceed to Mitochondrial extraction for mitochondrial DNA testing procedure.

C. Washing the hair for nuclear DNA testing extraction

1. For nuclear DNA extractions, using forceps and a scalpel cut up to 1.5 cm of the proximal region of the hair, including the root. Place the unused portion of the hair onto the backing of a post-it note and return to the packaging. A picture of the root should be taken at this time.

   If "possible tissue" attached to hair is observed, see your supervisor. In some cases the hair will not be washed, proceed to step 11 and enter N/A as Saline lot #.
2. Prepare a 50 ml Falcon tube and filter cup set by labeling the tube and filter cup tab with the sample name. Pre-wet the filter cup membrane with 1 ml of 0.85% saline. Document Saline lot #.
3. Using clean forceps, place the cut hair into the filter cup in the center of the membrane.
4. Wash the hair with 1 ml of 0.85% saline. Allow the liquid to pass through the filter. Repeat that step.
5. Wash the hair with 1 ml of 100% ethanol. Allow the liquid to pass through the filter.
6. Remove the filter cup containing the hair and place on a Kimwipe to let the ethanol evaporate. Once the filter membrane is dry, the hair will be dry as well.

7. Transfer the cut hair fragment to the bottom of a clean 1.5 ml tube. Label the tube with sample name.
8. Store the tube containing the hair fragment in the appropriate "To Be Extracted" cryobox in the pre-amplification laboratory freezer
9. Proceed to Mitochondrial extraction for nuclear DNA testing procedure.

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July 24, 2010 – Initial version of procedure.

ORGANIC EXTRACTION FOR MITOCHONDRIAL OR NUCLEAR DNA TESTING		
DATE EFFECTIVE 11-04-2010	APPROVED BY ELI SHAPIRO & EUGENE LIEN	PAGE 1 OF 3

Refer to the current *Protocols for Forensic STR Analysis* manual for extraction, quantitation, amplification, and STR procedures currently on-line for other Nuclear DNA Operations.

PURPOSE: To isolate nuclear or mitochondrial DNA from the hair using an enzymatic digestion of the hair followed by an organic extraction.

A. Extraction for Mitochondrial and Nuclear DNA testing

1. Prepare hair for digestion by removing the appropriate microcentrifuge tube from the "To Be Extracted" cryobox. Fill in Organic Extraction Worksheet.
2. Prepare the incubation solution in a 1.5ml tube using the following table. Label this tube with the extraction date and time as ENEGAMMYY-HHMM.

Reagents	**1 hair + extraction negative**
Proteinase K (20mg/ml)	30 µl (15*2)
DTT (1M)	75 µl (37.5*2)
20% SDS	7.5 µl (3.75*2)
Organic Extraction Buffer	188 µl (94*2)

3. When extracting clumps of hair, or multiple hairs together, the total volume of the incubation solution can be increased 2- to 10-fold, to accommodate the size of the sample. Adjust the reagent volumes to accommodate these changes. Note such volume changes on the extraction worksheet.
4. Have the extraction tube set-up witnessed.
5. Aliquot 150 µl of the incubation solution into the 1.5ml tube containing the hair and leave the remaining solution in the original 1.5ml tube as the negative control.
6. Incubate samples for 30 min. in a 1400 rpm shaker at 56°C. Record the thermal mixer number on the worksheet, and the thermal mixer temperature setting and temperature in the thermal mixer logbook.
7. After 30 min., hairs should be dissolved. If not, incubate for a total of 1-2 hours. If hairs have not dissolved, add 1µl of 1M DTT and incubate overnight. Make a note of this on the extraction worksheet. Hairs and control samples should be both treated the same way. After overnight incubation, record the thermal mixer setting and temperature in the thermal mixer logbook.
8. When the hair sample is completely dissolved, proceed with the extract to the purification step (see part B)

9. The hair sample might not completely digest even after the overnight incubation. If the hair is chemically treated, straightened, or dyed, it might resist digestion. The incubation process might remove the pigment or coloring from a hair and leave it opaque. If this happens, make a note of this on the extraction sheet. Centrifuge sample for 3-5 minutes at full speed. Collect the supernatant (extract) in a new tube, carefully without disturbing the pellet. Add the suffix "-R" to the sample name and label on the original tube containing the hair remain (pellet). Hair remains will be stored with the other sample. Proceed to purification step with the extract (see part B).

B. Purification of DNA for Mitochondrial and Nuclear DNA testing

1. During the incubation, prepare and label for each sample: one Eppendorf Heavy Phase Lock Gel (PLG) tube, one microcon filter, three microcon collection tubes, and one 1.5 ml tube for final extract. PLG tubes can be centrifuged for 30 seconds at maximum speed prior to sample addition.
2. After incubation, have the purification tube set-up witnessed.
3. Transfer each extracted sample to appropriate labeled PLG tube. PLG tubes make the phase separation between organic and aqueous layers of an organic extraction easier. To each PLG tube add an equal volume of Phenol: Chloroform: Isoamyl Alcohol (25:24:1 PCIA). The PCIA volume to be added should be 150µl unless the extraction volume has been increased in step A3. **PCIA is an irritant that is toxic. Its use should be confined to a certified fume hood. Gloves and a mask should be worn**.
4. Shake or briefly vortex the tube to achieve a milky emulsion.
5. Centrifuge the tube in a microcentrifuge for 2 minutes at maximum speed.
6. Insert Microcon 100 filter cup (blue) into labeled microcon tubes for each sample.
7. Prepare the Microcon 100 concentrator by adding 100 µl of TE^{-4} to the filter side (top) of the concentrator.
8. Transfer the aqueous phase (top layer) from the PLG tube to the prepared Microcon 100 concentrator. Do not disturb the PLG layer. Discard the PLG tube containing the organic layer into the organic waste bottle in the fume hood.
9. Spin the Microcon 100 concentrator for 25 minutes at 500 rcf.
10. Transfer the Microcon 100 filter cup into a new labeled Microcon tube and add 400 µl of TE^{-4} to the filter side (top) of the concentrator.
11. Spin again at 500 rcf for 25 minutes. After this spin, if liquid is still observed on the membrane, spin again at 500 rcf for an additional 10 minutes. After this spin, if liquid is still observed on the membrane, continue spinning for a longer time.
12. Add 20 µl of TE^{-4} to the filter side (top) of the concentrator.

13. Invert the blue concentrator cup and place into appropriate microcon collection tubes. Spin at 1000 rcf for 3 minutes to collect samples.
    - <u>For mitochondrial DNA testing,</u>
        - Using a pipetman, measure volume collected and record it.
        - Transfer samples to a 1.5 ml microcentrifuge tube for storage
        - Adjust samples volume to 50 µl using TE^{-4} record these volumes.
        - Proceed with HVI-HVII amplification with 20µl of samples
    - <u>For nuclear DNA testing,</u>
        - Using a pipetman, measure the volume collected
        - The volume should be close to 20ul, in control and hair samples. If the volume is > 30µl, prepare a new microcon filter and tube (see part 6 above) and spin at 500 rcf, control and hair samples, for an additional 10 minutes. After this spin, if liquid is still observed on the membrane, continue spinning for a longer time.
        - Transfer samples to a 1.5 ml microcentrifuge tube for storage
        - Send 2.5 µL of samples (neat) for nuclear quantification. If quantitation results show an insufficient amount of nuclear DNA for STR testing, the extract may then be used for mtDNA analysis.

DUPLEX MITOCHONDRIAL DNA PCR AMPLIFICATION-ROCHE

DATE EFFECTIVE	APPROVED BY	PAGE
07-24-2010	ELI SHAPIRO	1 OF 4

PURPOSE: To increase the amount of available mtDNA for the purposes of analysis, by performing an *in vitro* replication of template DNA using oligonucleotide primers, thermostable DNA polymerase and deoxynucleoside triphosphate bases (dNTPs) within a thermal cycler. The Roche Linear Array kit reagents are used in this procedure.

PROCEDURE:

A positive control, an amplification negative, and an extraction negative control (if applicable) should be included with each batch of samples being amplified to demonstrate procedural integrity. The positive control is a laboratory grade cell line, for which the mtDNA type is known.

Follow the mtDNA pre-amplification guidelines for handling the tubes and cleaning of the work surfaces. The following steps have to be performed in the appropriate dedicated areas. Evidence samples and exemplar samples should not be handled at the same time.

A. Preparing the DNA aliquots for HVI-HVII amplification

- When amplifying extracts which have nuclear DNA quantification data, the target amount of extract to be amplified is 100 pg. If samples are amplified high or low, add the appropriate suffix to the sample name.
- When amplifying samples that have not been quantified (e.g., hair shaft samples), use 20ul of the extract.
- Table I refers to the preparation of the control samples for the amplification.

Table I – Control samples for amplification.

Sample	DNA (extract)	TE^{-4}
HL60 Positive Control DNA (100 pg/20 µl)	20 µl	---
Amplification Negative Control	---	20 µl
Extraction Negative Control, when sample amplified neat	20 µl	---
Extraction Negative Control , when a dilution/concentration of sample extract is amplified	Submit 20 µl of the extraction negative at the same dilution/concentration factor or more concentrated than the sample	

B. Amplification Setup

1. For each amplification set, fill out the corresponding worksheet, have the paperwork reviewed for typographic errors, and record the appropriate lot numbers. Label 0.2 ml PCR reaction tubes with sample label name and with date and time for the positive and negative controls.

2. If samples require dilution, prepare the aliquots in clean, UV treated 1.5mL tubes, and place the neat samples back into storage.

3. Master mixes preparation:

   Prepare a Master Mix of Reaction Mix and Primer Mix, using the following calculations:

   - Reaction Mix: number of samples N x 20 µl Reaction Mix = __ µl
   - Primer Mix: number of samples N x 10 µl Primer Mix = ___ µl

   For $\leq$ 6 samples, use N, for $\geq$ 6 samples, use N+1. To save on reagents, individual aliquots of Reaction and Primer Mix can be made.

   - Add Reaction Mix and Primer Mix together to prepare Master Mix.
   - Vortex the Master Mix and centrifuge briefly
   - Aliquot 30 µl of the Master Mix into the bottom of each labeled 0.2 ml reaction tube.

   At this stage have the amplification set-up witnessed.

4. Add samples into the 0.2ml tubes. **Use a sterile filter pipette tip for each sample addition. Open only one tube at a time for sample addition.** The final aqueous volume in the PCR reaction mix tube will be 50 µl. Transfer the appropriate volume of target DNA or TE^{-4} to each respective sample tube. After the addition of the DNA, cap each sample before proceeding to the next tube. **If necessary, spin down the tubes at 1000 rcf for a few seconds**

5. When finished, place the rack with the 0.2ml tubes in the pre-amp room dumbwaiter. Send the samples up to the post-amp room.

C. Thermal Cycling

1. Turn on the Perkin Elmer 9700 Thermal Cycler.

2. Use the following settings to amplify the samples

9700 Thermal Cycler User: mtDNA File: lamtdna	The amplification file is as follows- Soak at 94°C for 14 minutes 34 cycles: - Denature 92°C for 15 seconds - Anneal at 59°C for 30 seconds - Extend at 72°C for 30 seconds Incubation at 72°C for 10 minutes Storage soak at 4°C indefinitely

3. Place the tubes in the tray in the heat block, slide the heated lid over the tubes, and fasten the lid by pulling the handle forward. Place the microtube rack used to set up the samples for PCR in the post-amp room bleach bath

4. Start the run by performing the following steps:

   a. The main menu options are RUN CREATE EDIT UTIL USER. To select an option, press the F key directly under that menu option.

   b. Verify that the user is set to "mtDNA" if not, select the USER option (F5) to display the "Select User Name" screen.

   c. Use the circular arrow pad to highlight "mtDNA." Select the ACCEPT option (F1).

   d. Press the RUN button (F1) and select the "lamtdna" file.

   e. Verify that the reaction volume is set to 50 µl and the ramp speed is set to **9600 (very important)**.

f. If all is correct, select the START option (F1).

The run will start when the heated cover reaches 103°C. The screen will then display a flow chart of the run conditions. A flashing line indicates the step being performed, hold time is counted down. Cycle number is indicated at the top of the screen, counting up.

g. Be sure to record the use of the thermal cycler in the logbook under the appropriate name. For example, when using Thermal cycler 705A-TC1, you would go to that logsheet, record the date, a brief description of what it was being used for (in this case it would be lamtDNA), your initials, followed by type of evidence, which would be either evidence, quality control, or exemplar.

h. Upon completion of the amplification, press the STOP button repeatedly until the "End of Run" screen is displayed, and remove your samples. Select the EXIT option (F5). Wipe any condensation from the heat block with a Kimwipe and pull the lid closed to prevent dust from collecting on the heat block. Turn the instrument off.

i. After removing your samples, place them in the appropriate 2-8° refrigerator for storage. Samples should be separated according to sample type (exemplar, evidence, or quality control). Record the date and time of when samples were amplified on the cover of the 0.2 mL PCR storage box.

IMPORTANT:
Turn instruments off **ONLY** when the Main Menu is displayed, otherwise there will be a Power Failure message the next time the instrument is turned on. It will prompt you to review the run history. Unless you have reason to believe that there was indeed a power failure, this is not necessary. Instead, press the STOP button repeatedly until the Main Menu appears.
In case of a real power failure the 9700 thermal cycler will automatically resume the run if the power outage did not last more than 18 hours. The Uninterruptible Power Supply (UPS) present in the amplification room will power the thermal cyclers for about 2-3 hours in the case of a total power outage. The history file contains the information at which stage of the cycling process the instrument stopped. Consult with the QA team and/or the Technical Leader on how to proceed.

Revision History:
July 24, 2010 – Initial version of procedure.

MITOCHONDRIAL DNA PRODUCT GEL ANALYSIS

DATE EFFECTIVE	APPROVED BY	PAGE
07-24-2010	ELI SHAPIRO	1 OF 8

PURPOSE: To quantitate the amount of amplified mtDNA by direct comparison to a molecular weight ladder containing known amounts of DNA following DNA size separation by agarose gel electrophoresis.

PROCEDURE:

Following amplification, a 1.5% agarose gel is run to determine if the amplification was successful (see Interpretation of Results section). The number of samples amplified will determine the size of the gel needed. Fill out a "mtDNA product gel worksheet."

Take into account that three wells of each gel must be used for the ladder control samples. Therefore, a 20-well gel can accommodate 17 samples, a 10-well gel can accommodate 7 samples, etc.

A. Preparing the Gel

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

Reagents	Small Gel (10-well)	Medium Gel (14-well)	Large Gel (16- or 20-well)
DNA Typing Grade Agarose	0.75 g	1.5 g	3.0 g
1X TBE	50 ml	100 ml	200 ml

2. Heat the agarose solution to boiling in a microwave or on a hot plate with a stir bar, until the agarose is completely dissolved.

3. Assemble the gel apparatus and pour the liquid agarose into the tray. After pouring the gel, look for bubbles, and if present, attempt to remove them by using a fresh, clean pipette tip. The gel should be 1/4" thick or less. Position the comb and let the gel solidify. For best results, always use the narrowest-gauge comb that is available.

4. Once the gel is set, remove the comb and pour enough 1X TBE buffer into the apparatus to just cover the gel.

B. Preparing the samples

1. Remove amplified samples from storage. Vortex and give them a quick spin such that all of the liquid is spun down to the bottom of the sample tube.

2. Mix 4 µl of each amplified sample with 1 µl of the Orange Dye Lane Marker in a clean 1.5mL microcentrifuge tube. If a sample is being rerun at 1 µl, be sure to add only 1 µl of amplified sample, 1 µl of the Orange Dye Lane Marker, and 3 µl of dH_2O.

3. Prepare three molecular weight ladder (Roche XIV) dilutions by adding the following:

   a. Mix **2 µl of ladder**, 2 µl of dH_2O, and 1 µl Orange Loading Dye
   b. Mix **3 µl of ladder**, 1 µl of dH_2O, and 1 µl Orange Loading Dye
   c. Mix **4 µl of ladder**, and 1 µl Orange Loading Dye

4. Vortex and spin down all of the samples. Have another analyst witness the tube setup.

C. Loading and Running the gel

1. The gel should be loaded with the three ladder samples in the middle of the amplified samples, and from left to right (well #1 is on the far left, and so on).

2. Load the full 5 µl into each well, making sure that the bottom of the wells are not punctured, and that all of the samples and ladder fill into their respective wells.

3. Run the small gel chamber at 100 volts for about 1 hour, the medium gel at 125 volts for about 1.5 hours, or the large gel at 150 volts for about 2.5 hours (or until the Dye Lane Marker has run approximately 10 cm). DNA is negatively charged and migrates to the positive electrode (anode). Make sure that the gel is loaded and the electrodes connected in such a way that the DNA migrates toward the positive electrode.

4. Switch off the power supply and remove the gel from the tank.

5. Place the gel in the ethidium bromide bath (0.5 µg/ml) for 30 minutes. **CAUTION**- ethidium bromide (EtBr) is extremely toxic! Always wear gloves. EtBr liquid waste should not be poured down the drain. Discard all liquids in contact with EtBr into chemical waste bottles. Dispose of gloves and plasticware in contact with EtBr into biological waste containers.

D. Bio-Doc-It Photography

1. Place the gel in the Bio-Doc-It chamber and close the door. Turn on the Power and UV switches. **The UV selector must be at 302nm! (If the bands appear weak or faded, first check to see if the correct UV selector is chosen).**

2. Adjust the zoom lens (the middle lens) until the entire screen is filled with the gel image. Then, adjust the aperture (the top lens, closest to the camera) to achieve good contrast. Finally, adjust the focus lens (the bottom lens) to properly resolve the gel bands. (Small adjustments might be necessary to the zoom after focusing.) Typically, the optimal settings for UV Gel photography with the Bio-Doc-It are:

Aperture: 1-2 Zoom: 15 Focus: <1

3. To further increase the contrast or achieve better resolution, press the [+] and [-] buttons on the Bio-Doc-It control panel. Press [LIVE] to return to a live image.

   Once the desired image is obtained on the LCD screen, it needs to be captured as a digital image. Insert a floppy disk into the drive on the BioDoc-It. Once the image is in focus and proper contrast is achieved, press “Capture” on the control panel and then press “Save”. The image will save as a JPEG file on the disk; this will take about 30-40 seconds. (Please note that if you see an error message stating that there isn’t enough memory to compress the file, turn the Bio-Doc-It power switch off, and unplug it from its electrical outlet. Wait one minute before plugging it back in and turning the power back on. If the message comes up again, refer to user manual for more trouble shooting guidelines).

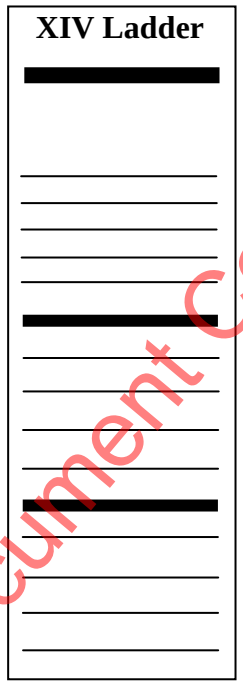
4. When finished, turn off the UV switch and the Bio-Doc-It power. Remove the gel from the chamber, and place it in a Pyrex dish filled with dH_2O. Clean the inside of the chamber with a quick 10% bleach rinse followed by a rinse with deionized water. For disposal of the gel, wrap in aluminum foil and discard in biological waste container.

5. Remove the disk, and open the file on a network computer. Transfer the file to the BioDoc-It Archive (M:\MITO_DATA\Photo Archives\BioDoc-It Archive); save the image according to its reference number assigned by the BioDoc-It instrument. Open Microsoft Office Powerpoint and drag the image into a blank layout. Adjust the image so it fills the slide, and then using the text box, label the lanes, and include the picture reference number on the image as well as a cross reference to the associated gel worksheet. Print out the gel image and label the printed photograph with your initials and the date. Once finished, save the slide in the BioDoc-It Archive, and assign it with the same reference number described above.

6. Finish filling out the worksheet by interpreting the gel values (follow the interpretation guidelines below).

INTERPRETATION OF RESULTS:

A. DNA molecular weight marker*



bp's	2 ul	3 ul	4 ul
2642	234 ng	351 ng	468 ng
1500			
1000			
500	53 ng	79 ng	105 ng
400	20 ng	29 ng	39 ng
300	15 ng	22 ng	29 ng
200	10 ng	14 ng	19 ng
100			

* Table is a graphic representation of the XIV molecular weight ladder with corresponding basepairs (bp) and concentrations of DNA based on either the 2 µl, 3 µl, or 4 µl volume input.

B. Quality of the amplified DNA product

The 444 bp HVI- and 415 bp HVII-specific bands should be between the 400 and 500 bp bands of the DNA molecular weight ladder.

C. Quantitation of the amplified DNA product

Use only the 500, 400, 300 and 200bp ladder bands for the quantitation. The intensity of the fluorescence of the bands in the 2 µl, 3 µl, and 4 µl ladder samples will relate to the concentration of the DNA in the samples using the guidelines in section A above. Match each sample to the closest ladder band and fill in the values on the mtDNA Product Gel Worksheet. Do not round the values. Do not assign values in between two bands. If the band intensity is between two DNA standards, match the sample to the DNA standard with the lower amount of DNA. For sample values of >105 ng/4 µl, rerun using only 1 µl of the sample.

AMPLIFICATION AND PRODUCT GEL TROUBLESHOOTING:

PROBLEM: Faint or no amplified product visible on gel for DNA samples or positive controls. Fragments corresponding to the DNA Molecular Weight Marker XIV ladder visible.	
Possible Cause (Amplification Problem)	**Recommended Action**
No DNA added or insufficient DNA added to Master Mix.	Ensure positive control was diluted properly and the correct concentration and volume was added to reaction: quantitate DNA and repeat amplification with 100 pg DNA.
Insufficient or no mtDNA HVI/HVII Primer Mix or no mtDNA Reaction Mix added to make Master Mix.	Repeat amplification ensuring 10 µl of mtDNA HVI/HVII Primer Mix and 20 µl of mtDNA Reaction Mix is added to each reaction.
PCR instrument failure, wrong program or emulation mode not selected.	Notify Quality Assurance Unit. See PCR instrument system manual and check instrument calibration; repeat amplification and ensure that proper settings are made.
Mistake during the amplification set up such as not adding one of the components or not starting the thermal cycler.	Prepare new samples and repeat amplification.

PROBLEM: Faint or no amplified product visible on gel for DNA samples or positive controls. Fragments corresponding to the DNA Molecular Weight Marker XIV also faint or missing.	
Possible Cause (staining problem)	**Recommended Action**
Ethidium Bromide concentration is too low in bath.	Make a fresh Ethidium Bromide bath, re-soak gel.

PROBLEM: Misshaped bands for DNA samples, positive controls or Molecular weight Marker.	
Possible Cause (Gel problem)	**Recommended Action**
Agarose not dissolved, wells not formed properly.	Prepare new gel and rerun samples.

PROBLEM: Signals in both or multiple negative controls with sizes corresponding to HVI and/or HVII products visible on gel.	
Possible Cause	**Recommended Action**
Contamination.	Refer to Interpretation Guidelines.

PROBLEM: Positive control fails but sample signal level is fine.	
Possible Cause	**Recommended Action**
Mistake during the amplification setup such as not adding enough of the positive control DNA.	Prepare new samples and repeat amplification step.
Positive control lot degraded.	Notify Quality Assurance Unit to investigate lot number, prepare new samples and repeat amplification step.

PROBLEM: Presence of unexpected or additional signals in the positive control.	
Possible Cause	**Recommended Action**
Contamination by other samples; contaminated reagents.	Notify Quality Assurance Unit to investigate the amplification reagents, prepare new samples and repeat amplification step.
Non-specific priming.	Notify Quality Assurance Unit to check thermal cycler for correct annealing settings, prepare new samples and repeat amplification step.

PROBLEM: Strong signal from the positive controls, but no or below threshold signal from DNA test sample.	
Possible Cause	**Recommended Action**
The amount of DNA was insufficient or the DNA is severely degraded.	Amplify a larger aliquot of the DNA extract.

PROBLEM: Strong signal from the positive controls, but no or below threshold signal from DNA test sample.	
Possible Cause	**Recommended Action**
Test sample contains PCR inhibitor (e.g. heme compounds, certain dyes).	Consult with a supervisor and select from the following: 1) Amplify a smaller aliquot of the DNA extract to dilute potential Taq Gold polymerase inhibitors. 2) Re-extract the sample using a smaller piece of hair or stain material to dilute potential Taq Gold polymerase inhibitors. 3) Re-extract the samples using the organic extraction procedure. Purify the extracted DNA using a Microcon 100 ultrafiltration device as described in the Microcon section.

Revision History:

July 24, 2010 – Initial version of procedure.

QUANTITATION USING AGILENT 2100 BIOANALYZER		
DATE EFFECTIVE 07-24-2010	APPROVED BY ELI SHAPIRO	PAGE 1 OF 10

PURPOSE- To quantify the amplified product of the mitochondrial hypervariable regions I and II, in order to establish the input of DNA for linear array analysis and/or cycle sequencing. The DNA 1000 assay is capable of analyzing amplified DNA fragments in the range of 25-1000 bp, and in the concentration range of 0.5-20 ng/µl.

A-**Preparing the worksheet**:

1- open the Agilent Workbook from:M:\FBIOLOGY_MAIN\FORMS\mtDNA\quant\Agilent
2- save as: Qmmddyy-hhmm in M:\FBIOLOGY_MAIN\MTDNA\Sheet Archives\Quant_sheets
3- In the "Agilent" worksheet enter tube label-dilution and sample name-dilution in wells A1 to D3 (e.g., Positive-CTR-100909-1000-d2 for sample name, PC100909-1000-d2 for tube label, Extraction-NEG-100909-1000-d1 for sample name, EN100909-1000-d1or ENEG-d1 for tube label).
4- Exemplar samples and positive controls should be run at 2-fold (d2) and 5-fold (d5) dilutions. Hair and evidence samples should be run d1 (neat) and d2. Negative controls should be run d1 (neat).
5- Exemplar and evidence samples may be quantitated on the same Agilent run; however, they must be aliquoted for quantitation separately.
6- Save and print.
7- Select the "export" worksheet. In file menu select "Save as" , "CVS(coma delimited)" . File name Qmmddyy-hhmm.. Save in M:\FBIOLOGY_MAIN\MTDNA\Sheet Archives\Quant_sheets. Copy on USB drive.

B-Preparing the samples

1- Follow the worksheet to prepare dilutions. Add H_2O first in all tubes where needed.
2- Vortex and centrifuge tubes between serial dilutions
3- Aliquot all volumes less than 2 µl using a 2 µl pipette.
4- When pipetting sample for dilution or quantitation, pick up from the top of the solution (directly on the meniscus) to avoid carrying sample on the outside of the tip.
5- Use 1µl for quantification. Vortex and centrifuge every tube before use.

C-Procedure

- If the Gel-Dye mix is not prepared, proceed to SECTION A.
- If the Gel-Dye mix is already prepared, proceed to SECTION B.
- For analysis of data only, proceed to SECTION C.

POWDER-FREE GLOVES ARE REQUIRED FOR THE HANDLING OF AGILENT CHIPS. POWDER FROM LATEX GLOVES COULD CLOG THE MICRO-CHANNELS ON A CHIP.

SECTION A- **Preparing the gel-dye mix**

1. Allow the DNA dye concentrate (blue tube) and the DNA gel matrix (red tube) to come to 37°C in the heat block.

2. ALWAYS PROTECT THE DYE CONCENTRATE FROM THE LIGHT. Vortex the DNA dye concentrate (blue tube) and spin down. Add 25 µl of the dye concentrate (blue tube) to the DNA gel matrix vial (red tube).

3. Vortex the mixture for 10 seconds to ensure complete mixing, and transfer the entire mixture to the top receptacle of a spin filter.

4. Centrifuge for 15 minutes at 6000 rpm. Discard the filter and label the gel-dye mix tube with the lot numbers of the DNA dye concentrate, the DNA gel matrix, and your initials and the date.

5. One tube of gel-dye mix is enough for 10 runs, and will last for 4 weeks. Discard the gel-dye mix 4 weeks after the date of preparation. Protect the gel-dye mix from light, and store at 4°C.

SECTION B- **Loading and running of the Agilent Bioanalyzer 2100**

2100 Expert System Setting (left inside of the window, click on system) are saved by default as:
1. Data Files Name: serial number, data, time are checked.
2. Data Files directory: “Create Daily subdirectories” is checked.
3. Data File format: “Binary format” is checked.
4. Nothing is checked in “Run and Results”, “Auto Export,” and, “Default Export Directories”.

ALL PIPETTING INTO THE CHIP MUST BE DONE DIRECTLY ON THE GLASS AT THE BOTTOM OF THE WELL, NEVER ON THE SIDES OF THE WELL.

1. Allow the gel-dye mix to equilibrate to room temperature.

2. Have a witness check samples and worksheet. Fill the lot#, analyst initials, date, time, Agilent machine, manually on the worksheet. Fill in Agilent usage log.

3. Open a new DNA chip

4. Pipette 9 µl of gel-dye mix into the bottom of the well marked **G**. Make sure there are no bubbles, if any use a 1µl pipette tip to remove them. Place it in the priming station, and fill out usage log. Make sure the base plate of the station is set to position C, and the clip on the syringe trigger is set to the lowest position. Make sure the syringe piston is pulled back to the 1 ml mark, and close the lid of the priming station. (Listen for the "click.")

5. Grab the syringe with your index fingers under the fins on the syringe body and thumbs on the plunger. Swiftly and steadily, press down on the plunger until it locks under the silver trigger. Make sure your thumbs are not in the way of the trigger lock or it will not work. Let the chip pressurize for 60 seconds.

6. Release the syringe with the trigger, and make sure the syringe comes back to 0.3-0.4 ml. Wait for 5 seconds, pull slowly the syringe back to 1 ml, and open the chip priming station. Turn the chip over and inspect the capillaries for proper filling.

7. Pipette 9 µl of gel-dye mix into the two wells marked **G**. Make sure there are no bubbles, if any use a 1µ tips to remove them.

8. Vortex and spin down the DNA marker (green tube), and pipette 5 µl of marker into each of the 12 sample wells and ladder well. <u>Each well must be filled, even if it will not be used.</u>

9. Vortex and spin down the DNA ladder (yellow tube), and pipette 1 µl of ladder into the lower right well, marked with the ladder symbol.

10. Add 1 µL of amplified DNA to each well. If a well is not used, add 1 µl of dH_2O into the well.

11. Place the chip in the IKA vortexer and vortex at ~2200 rpm for 60 seconds.

12. Run chips within 5 minutes.

13. Start the collection software by clicking on the symbol on the desktop.

14. Click on “instrument” on the left panel and enter the sample names in the Sample Name column or place the cursor in one cell of the Sample Name column, click on the right button of the mouse, select import, choose the cvs text file you created for that run (see A7). The software might give a warning “failed to import the text file”. Press ok.

15. Once the machine is highlighted in the upper left-hand corner of the screen, open the lid. The icon should now show the lid open as well. Insert the DNA 1000 chip and carefully close the lid. The machine icon will now change to a blue chip on the screen. Make sure the “Assay Class” in the “Assay Details” panel (middle right panel) is “DNA 1000”. If the assay class is different than DNA 1000 see a supervisor before starting the run.

16. Adjust the sample # in the Data Acquisition Parameters field, if necessary.

17. Click on the START button.
The run will begin with a pre-heat and should take approximately 35-40 minutes for a full chip.

18. The ladder sample will process first. It is a good idea to monitor this sample to make sure the upper and lower markers come out correctly (15 bp and 1500 bp).

19. Enter manually the run name as yyyy-mm-dd-hh-mm-ss on the worksheet.

20. **Immediately** after the run (less than 5 minutes), remove the sample chip. The electrodes need to be cleaned with the clear electrode cleaner chip within 5 minutes after the run. To do this, begin by filling one of the large wells with 350 µl of deionized water. Place the electrode cleaner in the Agilent 2100 Bioanalyzer and close the lid for 10 seconds (and **not more than 10s**). Open the lid, remove the electrode cleaner chip, and let the pin set dry for another 10 seconds (and **not more than 10s**), then close the lid. Drain and dry the electrode cleaner chip.

SECTION C. **Data collection, analysis, electronic filing**

When the Bioanalyzer 2100 run is complete, go to the Data & Assay field. The main window will show the gel image, sample list, and chip summary. Any problems detected by the software will be indicated with yellow triangles above the lanes in the gel image (see troubleshooting).

1- Ladder:
Select the Ladder sample on the sample list. The main window should show the the following peaks (11 ladder peaks plus lower-LM- and upper-UM- markers):

15bp Lower Marker
25bp Ladder
50bp Ladder
100bp Ladder
150bp Ladder
200bp Ladder
300bp Ladder
400bp Ladder
500bp Ladder
700bp Ladder
850bp Ladder
1000bp Ladder
1500bp Upper Marker

2—Samples:
Click on the individual sample on the sample list. The positive control and sample lanes should show two peaks, indicating the HVII and HVI amplified products (around 420-490 bp) for samples amplified with HVI and HVII multiplex primers. All samples should have the lower marker (~15bp) and upper marker (~1500bp).

3-Manual editing:

If the upper marker (UM) or lower marker (LM) is present but not labeled properly, right click on the peak cell “size bp” in the table and select “manually set upper marker” or “manually set lower marker,” respectively.

If a ladder peak, HVI and/or HVII are present but not labeled or if an extra peak is present: right click on the peak, select “manual integration”, add or remove peak at that position (bp).

Smaller amplified product peaks in samples with severely unbalanced HVI and HVII peak heights due to potential length heteroplasmy may be manually edited.

4-Export data to the network:
The data are automatically saved in a "yyyy-mm-dd" folder as "DES547045xx\yyyy-mm-dd_hh-mm-ss.xad" (a shortcut on the desktop). If from AG1: DES547045**xx** is DES54704**15**, if from AG2:DES547045**xx** is DES54704**24**.

a- Open the yyyy-mm-dd_hh-mm-ss.xad file, make edits if necessary, save the .xad file as DES547045xx\yyyy-mm-dd_hh-mm-ss-**analystinitials.**xad. Transfer the .xad file (s) in M:\MITO_DATA\Agilent Archive\yyyy\ yyyy-mm-dd-hh-mm-ss folder.

b- Create a PDF file by going to the "file" menu and selecting "print." When the print window opens, select "Run Summary", "electropherograms" and "Results Table", choose "all wells" if it is a full chip, or fill in the well numbers of used wells for a partial chip. Select "Include Ladder", one per page, PDF. Click on "… " to select the drive. Select the external USB drive or the appropriate folder on the network. Add analystinitials before ".pdf" in the path name. Click save. Open the PDF file, print and, initial each page.

c- Check that PDF and.xad file (s) are present in M:\MITO_DATA\Agilent Archive\yyyy\ a new yyyy-mm-dd-hh-mm-ss folder.

2100 expert software

If analysis or review is done at a different time than the run or from another computer, open the 2100 Expert software.
Select "data" in the "contexts" column on the left side of the window.
Go to file
Open
Select the folder with the run you want to review/analyze in M:\MITO_DATA\Agilent Archive\2009\xxxxxxxxxxxx
Select the appropriate.xad file of the run
Click open

After analysis/review: IF ANY EDITS/CHANGES ARE MADE, do not forget to re-save the .xad file with "**reviewerinitials**" at the end of its name. Create a new PDF file. Print and initial.

Section D. **Data Entry, Review, Filing, Rerun**

DATA TAB ENTRY
Open the Agilent workbook Qmmddyy-hhmm in M:\FBIOLOGY_MAIN\MTDNA\Sheet Archives\Quant_sheets.

Save it in M:\MITO_DATA\Agilent Archive\yyyy\ yyyy-mm-dd-hh-mm-ss folder as yyyy-mm-dd-hh-mm-ss (from the run)

Open the "Data entry" tab
Run#: enter "yyyy-mm-dd_hh-mm-ss"
Instrument #: enter AG1 or AG2

Ladder:
Select on the pull down menu next to "ladder" between "no editing needed" or "editing". If peak (s) has been edited enter in the next cell the location in bp followed by the one symbols from the table:

bp * : peak position manually called
bp ** : peak position edited out manually
bp ^ : spikes position manually removed
LM*: sample lower maker manually called
UM*: sample upper maker manually called

Ladder markers:
Select on the pull down menu next to "ladder" between "no editing needed" or if the lower marker has been manually called enter (LM*) if the upper marker has been manually called enter (UM*).

*Sample name*s: this column will be populated automatically.

Samples
HVII and HVI peaks should be discrete and approximately 400 to 500bp respectively or the peak will be inconclusive. Samples with multiple peaks or peak imbalance due to potential sequence length heteroplasmy may be manually edited.

Sample edits:
If a sample is edited, enter the location in bp followed by one of the symbols from the table above. If special edits are needed, document them in the sample edits column and explain the edits in the notes at the bottom of the review worksheet.

Dilution factor:
Select the dilution factor from the pull down menu (Neat=d1; 0.5=d2; 0.2=d5; 0.1=d10; 0.01=d100; etc…).

Value HVII and value HVI:
Enter the concentration value of HVII and HVI from the run PDF print results.
If the value is >0.5-20<, the value will appear in red.
If a peak is inconclusive for any other reason than an out of range value, enter INC in the value cell of that peak.
For negative controls, enter '- in both HVI and HVII.

REVIEW TAB ENTRY
Open the "Review" tab
Fill the "Comments column following the guidelines in the table below:

one dilution			
HVII	**HVI**		**Comments**
[0.5-20]	[0.5-20]	HVII and HVI mean concentration will be used for further testing	**USE**
INC	[0.5-20]	non-INC peak concentration value will be used for further testing	**USE**
[0.5-20]	INC	non-INC peak concentration value will be used for further testing	**USE**
INC	INC	Rerun	**RQ**

2 dilutions				
				Comments
dilution A	pass	dilution A within ± 2.5 X dilution B	use lower value	**USE**
dilution B	pass			-
dilution A	pass	dilution A outside ± 2.5 Xdilution B	Rerun	**RQ**
dilution B	pass		Rerun	**RQ**
dilution A	pass	n/a	use for further testing	**USE**
dilution B	INC		n/a	-
dilution A	INC	n/a	n/a	-
dilution B	pass		use for further testing	**USE**
If both dilutions are INC				
dilution A	INC	n/a	Rerun at appropriate dilution	**RQ, dx**
dilution B	INC		Rerun at appropriate dilution	**RQ, dx**
appropriate dilution example 1				
dilution 2	<0.5	n/a	Rerun at appropriate dilution	**RQ, d1**
dilution 5	<0.5		Rerun at appropriate dilution	**RQ, d1**
appropriate dilution example 2				
dilution 2	>20	n/a	Rerun at appropriate dilution	**RQ, d10**
dilution 5	>20		Rerun at appropriate dilution	**RQ, d100**
If both dilutions are INC for any other reason than concentration value out of range, see supervisor				

- If the ladder fails (e.g., discrete bands) or upper or lower markers are not present (e.g., can't be edited) the run is inconclusive, all samples have to be requantified, enter **RQ** in each sample comments cell.
- If one or both markers are not called, the run is inconclusive, all samples have to be requantified, enter **RQ** in each sample comments cell.
- If a sample peak concentration value is out of range it will appear as INC

If a sample was called INC for any other reason than the value range it will appear as INC. A comment can be added to explain why the peak was called INC in the "notes" of the review sheet.

In both cases the concentration of only one peak could be used for further testing instead of the mean concentration of both peaks and **USE** added in the comments column (see table above).
Save the workbook.
Print the "Data entry" and the "Review" worksheets.

On the review worksheet: Enter manually analyst initials, initials and date any notes that were added at the bottom of the page.

- If the ladder fails manually circle "fail" in front of the ladder lane, if not circle "Pass".
- If the ladder marker (s) fail(s) circle "fail" in front of the ladder marker, if not circle "Pass".

REVIEW

Give "Agilent", Data entry", "Review" worksheets, the initialed PDF prints for review.
The reviewer will review the paperwork, any comments based on the parameters in section D, and the "data entry" worksheet.
After review, the reviewer will circle pass or fail for the run, initial and date the "Review" worksheet.

FILING and RERUNS

After review, the analyst will:

1. File in the Agilent binder in one plastic protect sheet the "Agilent" worksheet, reviewed "Data entry" worksheet, reviewed "Review" worksheets and the initialized PDF prints.
2. Copy of the reviewed review worksheet will be distributed for every case file concerned.
3. Set up a new Agilent workbook for the necessary reruns.

E. Further testing

Linear Array Product Quantitation Analysis

A. Open the *Agilent Linear Array Worksheet*, found in M:\FBIOLOGY_MAIN\FORMS\mtDNA
B. Enter the concentrate with "USE" in the comment column of each sample in the concentration column of the *Agilent Linear Array Worksheet*.
C. Go to the linear array part of the manual.

Cycle Sequencing Amplification

A. Open the *3130* spreadsheet, found in M:\FBIOLOGY_MAIN\FORMS\mtDNA
B. Enter the concentration value with "USE" in the comment column of each sample in the quant value column of the *3130 Worksheet*.
C. Go to 3130 worksheet part of the manual.

For troubleshooting, refer to the Agilent 2100 Bioanalyzer Maintenance and Troubleshooting Guide Edition 11/November 2003.pdf archived in MITO_DATA folder.

Revision History:

July 24, 2010 – Initial version of procedure.

MITOCHONDRIAL DNA LINEAR ARRAY ANALYSIS

DATE EFFECTIVE	APPROVED BY	PAGE
09-03-2010	ELI SHAPIRO	1 OF 12

PURPOSE

For the detection of sequence variation targeting 18 positions within the hypervariable regions I (HVI) and II (HVII) of the human mitochondrial DNA (mtDNA) genome. Large sample sets can be screened using immobilized oligonucleotide strips via a biotin/streptavidin-linked hybridization assay (Roche Linear Array Mitochondrial DNA HVI/HVII Region-Sequence Typing Kit is used), allowing the identification and comparison of questioned samples and known samples.

PROCEDURE

The following procedure will take approximately 2-2.5 hours. Make sure before beginning the procedure that sufficient amounts of Linear Array Wash Buffer, Citrate Buffer, and Chromogen are available. If not, contact the QA team to make or order more. The Linear Array procedure can accommodate 24 samples per tray, and no more than two trays can be run simultaneously in one shaking waterbath. If less than 24 samples are being run, leave empty wells between samples whenever possible to avoid spillover contamination. **A single tray should contain only exemplar samples or evidence samples. Do not run exemplars and evidence together in the same tray. Do not run evidence or exemplar sample types from the same case in one Linear Array experiment (e.g. staggering the run of 2 trays in one Linear Array experiment; one tray contains evidence from a given case, the other tray contains an exemplar sample from the same case).**

A. Hybridization

1. Turn on the shaking water bath to 55°C, at 50-70 rpm. Do not leave uncovered. Be sure that there is enough water covering the base of the bath before proceeding.

2. Turn on the circulating stationary water bath to 45°C. Warm the **Wash Buffer**.

3. Remove samples from storage, vortex and spin down in a centrifuge. Arrange your amplified samples in a tube rack, and fill out the Linear Array worksheet.

4. Using forceps, remove an appropriate number of Linear Array strips; arrange on a kimwipe, and label the clear end with a pen containing non-soluble ink so that the **black bar is on the left side** of the strip. Place the array strips in the 24-well tray in the same orientation. Cover the tray with the lid. Take care not to touch the stripes on the strips with gloves or scrape with forceps.

5. Label an appropriate number of 1.5 ml microcentrifuge tubes.

6. Have a witness check tubes set up

7. Add: - 15 µl of Denaturation Solution
- Up to 21.4 µl of PCR product

Whenever possible, the target amount of 75 ng of amplified mtDNA should be used for the Linear Array assay.

If the concentration of amplified DNA in an extract is < 10ng / 4 µl (< 2.5ng / µl), do not use for Linear Array. Reamplify the sample with more input DNA or proceed directly to DNA sequencing.

8. Add 3 ml of pre-warmed **Wash Buffer** to each Linear Array strip.

9. Add the denatured samples to the liquid within each appropriate well.
Do not add the sample directly onto the strip.

10. Mix by rocking gently. Cover the tray with the lid, foil, and add two weights on the top. Place in the shaking water bath for **15 minutes**.

When **5 minutes** are left on the previous step, prepare the **Enzyme Conjugate** solution using the following formula:

Number of samples x **3.3 ml Wash Buffer**
Number of samples x **12 µl Enzyme Conjugate**

Swirl to mix, cover with foil, and add a weight to the top to prevent from tipping over. Place in the circulating water bath to keep warm.

11. Remove the tray from the shaking water bath, uncover, and pour off the Wash Buffer from the labeled end of the strips. Wipe the condensation from the lid and edges of the tray.

12. Add 3 ml of pre-warmed **Wash Buffer** to each strip. Rinse by carefully rocking for 10 seconds and pour off from the labeled end of the strips. Wipe the condensation from the lid and edges of the tray.

13. Add 3 ml of the **Enzyme Conjugate** solution to each strip with a disposable pipette.

14. Cover with lid and foil, add weights, and place the tray in the shaking water bath for **5 minutes**.

15. Remove the tray from the shaking water bath, uncover, and pour off the Enzyme Conjugate solution from the labeled end of the strips. Wipe the condensation from the lid and edges of the tray.

16. Add 3 ml of pre-warmed **Wash Buffer** to each strip. Rinse by rocking for 10 seconds, and pour off from the labeled end of the strips.

17. Add 3 ml of pre-warmed **Wash Buffer** to each strip.

18. Cover with lid and foil, add weights, and place in the shaking water bath for **12 minutes**.

19. Remove the tray from the shaking water bath, uncover, and pour off the Wash Buffer from the labeled end of the strips. Wipe the condensation from the lid and edges of the tray.

20. Add 3 ml of pre-warmed **Wash Buffer** to each strip. Rinse by rocking for 10 seconds, and pour off from the labeled end of the strips.

21. Add 3 ml of **Citrate Buffer** to each strip.

22. Cover with lid and foil, add weights and shake at **Room Temp for 5 minutes** at 100 rpm.

23. **In a glass container**, prepare the **Color Development** solution using the following formula:

    Number of samples x **3.3 ml Citrate Buffer**
    Number of samples x **4 µl 3% Hydrogen Peroxide**
    Number of samples x **0.15 ml Chromogen**

    Swirl to mix and use immediately.

24. Remove the tray from the room temperature shaker, uncover, and pour off the Citrate Buffer from the labeled end of the strips. Wipe the condensation from the lid and edges of the tray.

25. Add 3 ml of the **Color Development** solution to each strip by using a disposable pipette.

26. Cover with lid and foil, add weights, and shake at **Room Temperature for a minimum of 10 minutes** at 100 rpm. After 10 minutes, briefly check color development. If strip is not fully developed, continue development for an additional 5 minutes. Color development is normally complete after 15 minutes; however, further incubation can be done at the analyst's discretion. Color development must be stopped if there is any indication of the appearance of blue non-specific background staining. Document the total time of color development on the Linear Array worksheet.

27. Remove the tray from the room temperature shaker, uncover, and pour off the Color Development solution from the labeled end of the strips. Wipe the condensation from the lid and edges of the tray.

28. Stop the color development by adding **5 ml of dH_2O** to each strip. Rinse by rocking for 20-30 seconds.

29. Pour off the dH_2O from the labeled ends of the strips. **Repeat the dH_2O rinse process twice more.** When finished, either immediately proceed to the photography stage (preferred) or store the strips in a hybridization tray filled with dH_2O (not more than several hours) and photograph later. Record the date, time and a brief description of samples on the lid.

30. Clean trays with ethanol or isopropanol and rinse with dH_2O. If after rinsing with ethanol or isopropanol and the dye is still present, repeat the cleaning, but let stand for half an hour, and then rinse.

B. Photography

Using forceps, arrange the developed Linear Array strips on an acrylic tray, using the supplied ruler from Roche as a guide. The black bar on the left side of the strip should be used to align the strips to the ruler. Be sure to keep the strips moist.

No more than 12 strips should be photographed at a time. If there are more than 12 strips present, the second photograph needs to include a positive and a (amplification or extraction) negative control strip taken from the first set of strips.

1. Once arranged on the tray, place the tray onto the copy stand. Turn on spotlights as necessary.

2. Attach the digital camera to the copy stand by screwing it in place. Adjust the height and focus of the camera so that all of the strips and ruler are visible and clearly focused. Optimal settings include setting the camera to the macro mode (which is the flower image beneath the OK button); manual focusing the image by pressing half way down on the shutter release button; and by also adjusting the white balance by selecting Menu: White Balance: White Bal. Preset, select Measure, and then frame the reference object (with any light sources turned on) and press OK.

3. Connect the camera to the computer, open MS Powerpoint and insert the image into a blank slide. Using a text box label the image with the picture's reference number as LAmmddyy-hhmm from the associated Linear Array worksheet date and time. Save .ppt file to into M:\MITO_DATA\Photo Archives\Linear Array Photo Archive folder using the picture's reference number. Print image, initial and date the picture, and proceed to interpret the results.

INTERPRETATION OF RESULTS

A Allele Calling

1. All interpretations must be made from the photo documentation of the Linear Array strips.

2. Fill in the corresponding bands on the Linear Array worksheet based on the ruler markings.

3. **Weak bands are considered any bands that have an intensity significantly less than the other bands *within the same hypervariable region on the same strip*.** All weak bands should be recorded as "wX", where X is the actual probe signal.

4. In the case where two bands are present at one probe location, record both bands as "X/Y" where X is the first band numerically and Y is the second.
The weak band rule may still apply, such that "wX/wY", "wX/Y" and "X/wY" are all the possibilities.

5. If no bands are present at a probe location, enter "0" for that location in the spreadsheet. If no bands are present in any of the probe locations, all locations should be marked with a dash "-".

B. Control Sample Guidelines

1. Check the negative controls to make sure there are no bands present.

2. The HL60 positive control, run with each amplification set, should yield the following probe signals:

Probe	16093	1A	1C	1D	1E	IIA	IIB	IIC	IID	189
HL60	1	1	1	2	2	2	6	1	1	1

3. See "INTERPRETATION GUIDELINES - GUIDELINES FOR CONTROLS" on how to proceed if there are any problems.

C. Interpretation and further testing strategy

1. See "INTERPRETATION GUIDELINES - LINEAR ARRAY" for comparison of allele calls.

2. Based on the case context, the following samples need to proceed to cycle sequence analysis:
   - Included exemplars
   - One representative sample for all probative evidence
   - Samples with partial profiles
   - All negative and positive controls if any sample from the batch is being sequenced

   No sequencing is required for:
   - Excluded exemplars
   - Apparent mixtures
   - Redundant evidence samples

IMPORTANT: if any linear array re-hybridization is required, make sure sufficient amplification product for cycle sequencing is preserved. This is especially valid for the negative controls and samples with low DNA yields that cannot easily be re-amplified.

Depending on the case it might be necessary to omit the linear array repeat.

D. Sequence concordance

1. For all single source samples with probe signals, the linear array results are correlated to the underlying sequence variants for concordance checking.

2. This is done in an automated fashion using the Linear Array Summary & Stats spreadsheet found on the network.

3. On the first page of the spreadsheet, fill in the sample name.

   A. Fill in the probe signals interpreted from the previous section into the corresponding cells.

      The following rules must be applied:

      1) Input weak bands with a "w" followed by the numerical type.

      2) Input multiple bands, e.g., x/y, on two separate sheets, one with the "x" and one with the "y," with the following exception. If a w2/w3 call is present at 1C, then enter "w2/w3" at this location.

      3) Input null bands with a zero..

   B. Print out the spreadsheet to include in the casefile.

4. The spreadsheet will provide the following sequence for the HL60 positive control:

	Probe Designation	Sequence Variation
HVI	16093	16093 T
	1A	16126 T 16129 G
	1C	16304 T 16309 A 16311 T
	1D	16362 C
	1E	16270 C 16278 T
HVII	IIA	73 G
	IIB	146 T 150 T 152 C
	IIC	189 A 195 T 198 C 200 A
	IID	247 G
	189	189 A

5. Assay results can be translated into sequencing results based on the spreadsheet calculations, and compared against the other samples. Null alleles are not convertible.

For reference, the probe designations and sequence variations follows on the next page.

	Probe Designations	Sequence Variation Detected
HVI	16093 1	A T **T** T C
	16093 2	• • **C** • •
	1A1	T G **T** A C **G** G T
	1A2	• • **C** • • • • •
	1A3	• • • • • **A** • •
	1C1	A G **T** A C A T **A** G **T** A C
	1C2	• • **C** • • • • • • • •
	1C3	• • • • • • • • • **C** • •
	1C4	• • • • • • • **G** • • •
	1Cw2/w3	• • **C** • • • • • • **C** • •
	1D1	C G **T** C C
	1D2	• • **C** • •
	1E1	C A **C** T A G G A T A **C** C A
	1E2	• • • • • • • • • • **T** • •
	1E3	• • **T** • • • • • • • • • •
HVII	IIA 1	G **A** T G
	IIA 2	• **G** • •
	IIB1	C C **T** C A T **C** C **T** A T
	IIB2	• • **C** • • • • • • • • •
	IIB3	• • • • • • • • **C** • •
	IIB4	• • **C** • • • • • **C** • •
	IIB5	• • • • • • **T** • • • •
	IIB6	• • • • • • **T** • **C** • •
	IIB7	• • **C** • • • **T** • **C** • •
	IIC1	G A **A** C A T A C **T** T A **C** T **A** A A
	IIC2	• • • • • • • • **C** • • • • • •
	IIC4	• • • • • • • • **C** • • **T** • • • •
	IIC5	• • **G** • • • • • • • • • • **G** • •
	IID1	T T **G** A A
	IID2	• • **A** • •
	189 1	G A **A** C A T A C C/T*
	189 2	• • **G** • • • • • •

* Both 189 1 and 189 2 probes are degenerate at position 195 with respect to the presence of either a C or T base.

TROUBLESHOOTING

Problem: No bands on a Linear Array strip where there should be.

Possible causes	Solutions
Quality of reagents is inappropriate	Check expiration dates on all reagents, and make new if necessary. Make sure Enzyme Conjugate and Color Development Solutions were not made more than 5 minutes prior to use.
All of the proper reagents were not added	Check that all reagents were added at accurate volumes. Make sure that Citrate and Wash buffers were not switched at any steps.
No DNA present	Check quantitation assay. Check other strips: problem may be isolated to one well or PCR sample.
Temperature	Check temperature of water bath, and ensure that all steps were done at the proper temperature.
DNA was not denatured properly	Make sure Denaturation Solution was added to sample. Make or purchase new Denaturation Solution.
DNA degradation	Do not allow DNA to incubate with Denaturation Solution for more than 60 minutes.

Problem: High Background

Possible causes	Solutions
Color Development step not performed at room temperature or temperature of the room is too high.	Repeat typing and ensure that color development step is performed on an orbital shaker at room temperature, between +15 and +25°C.
Insufficient or improper water washes following Color Development.	Ensure that the strips were washed 3 times for at least 20-30 seconds per wash: wash times can be increased or additional washes can be performed.
Excess Color Development Solution remaining in tray following aspiration of pouring-off of solution.	Ensure Color Development Solution is adequately removed.
Developed strips exposed to strong light and /or not kept wet during interpretation or imaging.	De-ionized water can be applied with a squirt bottle during photography if necessary. Align all strips prior to turning on photography lights.
Inadequate agitation of the strips during Hybridization, Conjugation, and or Wash steps.	Check speed of rotating water bath (50-70 rpm). Verify that solutions are washing over strips. Repeat typing.
Excess Amount of Enzyme Conjugate: SA-POD added to Enzyme Conjugate Solution.	Check calculations and repeat typing, ensuring correct amount of Enzyme Conjugate:SA-POD added to Enzyme Conjugate Solution.
Tray not properly cleaned prior to use.	Ensure trays were not cleaned with detergents or bleach; thoroughly clean and dry the affected trays. Clean trays with ethanol or isopropanol and rinse with dH_2O. Discard tray if discolored even after proper cleaning.
Wash bottles are contaminated.	Thoroughly clean and dry wash bottles.

Revision History:

July 24, 2010 – Initial version of procedure.

September 3, 2010._.Revised version of procedure: Paragraphs B3 and B4 have been rewritten into one paragraph B3 only to reflect our current procedure

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

EXO-SAP-IT SAMPLE CLEANUP & CYCLE-SEQUENCING WORKSHEET		
DATE EFFECTIVE 07-24-2010	APPROVED BY ELI SHAPIRO	PAGE 1 OF 3

PURPOSE: Prior to cycle sequencing, unincorporated primers and nucleotides present in the amplification reaction are deactivated by the addition of ExoSAP-IT.

PROCEDURE:

1. Open the appropriate cycle sequencing/3130xl Workbook located on the Forensic Biology network. Click on **Enable Macros**. Then click on **Don't Update**.

2. Fill in the tube label and sample description for each sample. Every run should include a positive control and an amplification negative control. When using a new Cycle Sequencing amplification negative control (cAN), its date and time should be the same as the worksheet date and time. **Note: It is very important for these entries to be in 3130xl format; do not use spaces or the following characters: \ / : * " > < | ? ')**

3. Fill in the primer samples needed to be run in the "Primers" section of the worksheet. **Note: the primer name must be entered using uppercase letters preceded by recyc-, conf-, or reinj-, when necessary (e.g. A1, recyc-B1, conf-A1, etc…).**

4. Fill in the IA column: use dashes for controls and the initials for the interpreting analyst assigned to the case. Note: when filling in dashes, first type an apostrophe in the field followed by the dash.

5. Based on each sample's previous runs, fill in the appropriate values for each column in the sample worksheet. The target amount for cycle sequencing is 5 ng of amplified product. Samples with less than 5 ng of amplified product in 3μl may be cycle-sequenced using 3u of the sample using "3130xl Manual Entry" worksheet.

   For a detailed description of the calculations performed in this spreadsheet, refer to Appendix D – Detailed CycSeq/3130xl Spreadsheet Calculations.

6. Fill in the "3130 Run ID" field as the *Instrument initial Year-Run Number* (e.g. B08-015).

7. Save the Workbook in the Sheet Archives as "*Instrument initial Year-Run Number* (e.g. B08-015) of the MTDNA subdirectory as "Excel Macro-Enabled".

8. Click on the "Samples" tab and **Print Preview**. Select Set Up, and choose Landscape and 75%. **Print** out worksheet. **This will be your Cycle Sequencing worksheet.**

9. Have the paperwork reviewed for typographic errors.

10. Based on the cycle sequencing worksheet, the ExoSAP-IT column will display the volume of ExoSAP-IT that is to be added to that sample. Add the appropriate volume of ExoSAP-IT to each sample. Vortex and briefly spin down sample.

    There should be 1ul of ExoSAP-IT added for every 5ul of sample in the amplification tube.

11. Use the following settings to incubate the samples:

9700 Thermal Cycler User: mtDNA File: exosap-it	The ExoSAP-IT file is as follows: - Soak at 37°C for 15 minutes - Soak at 80°C for 15 minutes Storage soak at 4°C indefinitely

12. Place the tubes in the tray in the heat block, slide the heated lid over the tubes, and fasten the lid by pulling the handle forward.

13. Start the run by performing the following steps:

    a. The main menu options are RUN CREATE EDIT UTIL USER. To select an option, press the F key directly under that menu option.

    b. Verify that the user is set to "mtDNA." If not, select the USER option (F5) to display the "Select User Name" screen.

    c. Use the circular arrow pad to highlight "mtDNA." Select the ACCEPT option (F1).

    d. Select the "exosap-it" file, and press the RUN button (F1).

e. Verify that the reaction volume is set to 50 µl and the ramp speed is set to **9600 (very important)**.

f. If all is correct, select the START option (F1).

The run will start when the heated cover reaches 37°C. The screen will then display a flow chart of the run conditions. A flashing line indicates the step being performed; the hold time is counted down. Cycle number is indicated at the top of the screen, counting up.

Upon completion of the amplification, remove samples and press the STOP button repeatedly until the "End of Run" screen is displayed. Select the EXIT option (F5). Wipe any condensation from the heat block with a Kimwipe and pull the lid closed to prevent dust from collecting on the head block. Turn the instrument off.

CYCLE-SEQUENCING		
DATE EFFECTIVE 07-24-2010	APPROVED BY ELI SHAPIRO	PAGE 1 OF 5

PURPOSE: Following the duplex mtDNA amplification, the samples identified as probative will be sequenced to determine the mtDNA profile. The Sanger method is used to cycle sequence the mtDNA in question using fluorescent dideoxynucleoside triphosphate bases chain terminators. The Applied Biosystems Big Dye Terminator Cycle Sequencing Kit is used.

PROCEDURE:

1. Open the 3130xl Workbook that was created during the ExoSAP-IT procedure. Click on **Enable Macros**. Then click on **Don't Update**. If changes need to be made, make sure to be in the "Samples" tab.

2. Click on the "Populate Sheet" button. The spreadsheet will then transfer the screen automatically to the "3130Sheet" tab**.** Check for accuracy. Do not make any changes. If changes are needed, select all the populated cells and "clear content". Return to the "Samples" tab, make the changes and repopulate the "3130sheet" tab.

3. Click on the "Pre-Record" tab and click on the "Create Plate record" button. The spreadsheet sheet will then transfer the active screen automatically to the "Plate Record" tab. Click on the "Delete Row" button.

4. Save your 3130xl Workbook in "Excel Macro-Enabled" format.

5. Create and save this worksheet as a text file in the same Sheets Archives folder that the 3130xl Worksheet is saved in (in preparation for the 3130xl import). Perform the following steps:

   a. Make sure to be in the "Plate Record" tab.
   b. Save As "other format".
   c. The save as pop-up window will appear.
   d. Check that the File name is correct in the "File name" field.
   e. Select "Text (tab delimited)" in the drop down menu of the "save as type" field.
   f. Click "Save"; "OK"; and "Yes".

6. At this point, your "Plate Record" tab will change its name to indicate your file name (e.g. B08-015). Review the plate record for accuracy. If a change is needed, right click on the renamed "Plate Record" tab, and rename it "Plate Record." Then repeat steps 1-6.

7. Click on the "3130sheet" tab and check **Print Preview**. Select "Set Up"and set to Portrait and 90%, then **Print**. **This will be your Cycle Sequencing Run Sheet.**

8. Click on the "Master Mixes" tab and print (Portrait and 75%). This sheet contains final volume amounts needed for premixes as described later in this section.

9. Close the 3130xl workbook. Click "no" after the next prompt.

10. Copy both the excel and text files onto an external USB drive or into appropriate network folder. You will need both files on the 3130xl instrument.

11. The cycle sequencing reactions are done in a 96-well plate. Prepare the samples and reagents needed for cycle sequencing and be witnessed according to the sample names and order listed on the Cycle Sequencing worksheets that were previously printed out.

12. The amount of template DNA and water needed for each sample is calculated by the spreadsheet and filled in on the last two columns for each sample. This calculation takes into account the total volume and concentration of amplified product present in the sample tube following the addition of ExoSAP-IT necessary for each sample (see the ExoSAP-IT section). The concentration of the DNA is then adjusted based on the new volume of the sample plus the ExoSAP-IT and the volume of template DNA that is to be added to each sample is calculated.

    The target amount for cycle sequencing is 5 ng of amplified product.

    The following formula is then used to create each sample for cycle sequencing

    4 µl of Big Dye Terminator Ready Reaction Mix + 2 µl of Sequencing Buffer + 3.2 µl Primer (1 µM concentration) + mtDNA template + Water = 20 µl total volume.

    Samples with less than 5 ng of amplified product in 3*µl* may be cycle-sequenced using 3µl of the sample using "3130xl Manual Entry" worksheet.

13. If a dilution of template DNA is necessary, it will be indicated on the cycle sequencing worksheet in the comment column as "x @ 1/10th" where x is the input volume. If no dilution is necessary, the comment column will contain the notation "neat." The amount of water sufficient to make 20μl reaction volume is then calculated. If a sample is a negative control sample, the gel concentration of zero will result in a default template input of 3μl, with the notation of "control" in the comment column. The spreadsheet cannot indicate dilution factors greater than 1/10. For situations where the amount of DNA indicated is less than 1μl @1/10 dilution, calculate the volume of extract required @1/100 dilution (multiplication by 10), and use this volume of a 1/100 dilution. Also calculate, by subtraction, the correct volume of water to add to the reaction. Note the correct aliquots on the worksheet.

For a detailed description of the calculations performed in this spreadsheet, refer to Appendix D- Detailed Cycle Sequencing/3130xl Spreadsheet Calculations.

14. A ***master mix for each primer*** can be made with the following formula:

a. For (N+2) samples, add:
- 4 µl x (N+2) Big Dye Terminator Ready Reaction Mix
- 2 µl x (N+2) Sequencing Buffer
- 3.2 µl x (N+2) primer (1uM concentration)

A ***master mix for each sample DNA*** can be made with the following formula:

b. For N samples, add:
- X µl x (N) mtDNA sample DNA, where X is the amount of mtDNA needed as calculated by the spreadsheet
- Y µl x (N) Water, where Y is the amount of water needed as calculated by the spreadsheet

Note: The calculations for the two master mixes mentioned above are done by the 3130xl workbook. They are located by clicking on the "Master Mixes" tab and were previously printed for reference as mentioned above.

c. Include all controls for each primer that is used for a sample. If a sample is repeated starting at the cycle sequencing step the original negative controls do not have to be repeated if the first test was successful.

d. **The re-cycle sequencing step requires the following:**

- **A new cycle sequencing amplification negative control for each primer used in re-cycle sequencing to account for the cycle sequencing reagent.**
- **A positive control, for each primer used in re-cycle sequencing to report on the integrity of the reaction.**
- Samples can be re-cycle sequenced with more (-recych) or less (-recycl) input DNA if necessary. Based on validation, up to 90ng of DNA can be used for recych. Use "manual entry" workbook. If recych sample volume would be more than 3µL, see supervisor.

15. Use the following settings to amplify the cycle sequencing samples:

9700 Thermal Cycler User: mtDNA File: BDT cycle seq	The cycle sequencing amplification file is as follows: Soak at 96°C for 1 minute 25 cycles: - Denature 96°C for 15 seconds - Anneal at 50°C for 1 seconds - Extend at 60°C for 1 minutes Storage soak at 4°C indefinitely

July 24, 2010 – Initial version of procedure.

SDS CLEANUP		
DATE EFFECTIVE 07/24/2010	APPROVED BY ELI SHAPIRO	PAGE 1 OF 2

PURPOSE: To help separate the primers from the cycle-sequenced DNA with the addition of 2% SDS to the samples, prior to Centri-Sep filtration.

PROCEDURE:

Do not refrigerate the 2% SDS tubes. This will cause the SDS to precipitate out of solution. Store the 2% SDS tubes at room temperature. Ensure that there is no precipitate in the tube before adding to samples.

1. Add 2μl of 2% SDS to each tube of cycle-sequenced DNA. Vortex and spin down the plate(s) in a centrifuge.

2. Place the tubes in a thermal cycler, using the following conditions-

9700 Thermal Cycler User: mtDNA File: SDS	The 2% SDS incubation file is as follows: Soak at 98°C for 5 minutes Storage soak at 25°C for 10 minutes

3. When the tubes are back to room temperature following the 25°C soak, proceed to the Centri-Sep purification.

Revision History:
July 24,2010 – Initial version of procedure.

SDS CLEANUP		
DATE EFFECTIVE 07/24/2010	APPROVED BY ELI SHAPIRO	PAGE 2 OF 2

CENTRI-SEP SAMPLE FILTRATION		
DATE EFFECTIVE 07-24-2010	APPROVED BY ELI SHAPIRO	PAGE 1 OF 2

PURPOSE: Prior to sample electrophoresis, sequenced products must be purified in order to remove unincorporated dye terminators.

A. Procedure for Single Columns

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 to 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 2 hours 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.

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

5. Spin the assembly at 700 x g for 2 minutes to remove interstitial fluid. Be sure to note the orientation of the columns. At this point the columns should be used as soon as possible for the loading of cycle-sequenced DNA product.

6. 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.

7. Place column into labeled sample collection tube and spin at 700 x g for 2 minutes maintaining original orientation. The purified sample will collect in the bottom of the tube.

8. Discard the column and dry the sample in a vacuum centrifuge (approximately 15-20 minutes). Do not over dry samples.

B. Procedure for Centri-Sep 8 Strips

1. Determine how many strips are necessary to filter the amplified samples. Separate the desired number of strips by cutting the foil between the strips with scissors.

2. Open the well outlets on each strip by cutting off the bottom edge with scissors. Cut at the narrowest part of the bottom of the tube.

3. Peel off the top foil and arrange the strips evenly on deep-well centrifuge plates. Spin the plates at 750 rcf for 2 minutes to remove the liquid.

4. Arrange the newly drained strips on a new 96-well plate. Add the amplified sample to each column.

5. Once all of the samples are loaded, place the 96-well plate with the Centri-Sep 8 Strips into the centrifuge, and spin at 750 rcf for 2 minutes.

6. Confirm that all of the samples passed through the strip into the wells of the 96-well plate, and discard the Centri-Sep 8 Strip.

7. Evaporate the samples in the 96-well plate at 75°C in a thermalcycler with the lid open.

8. If the samples are not going to be loaded immediately, they should be stored as dried pellets at 4°C for no longer then 14 days. When ready, proceed to 3130xl setup.

ABI 3130*xl* SEQUENCING		
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PURPOSE: The 3130*xl* 16-capillary array system is used to electrophoretically analyze samples following cycle sequencing and cleanup. The system uses 96-well plates containing the samples of interest, and can process 16 separate samples with each injection. Sequence data is generated at the end of the run for downstream sequencing analysis.

A. Setting up a 3130*xl* Run

1. Turn on the computer. Make sure computer is fully booted to the Windows desktop. To login, the User should be "ocmelims" and the password should be "passw0rd". If the instrument is not on, turn it on. The status bar light will change from solid yellow (indicates instrument is booting) to blinking yellow (indicates machine is communicating with computer) and then to solid green (indicates instrument is ready for command).

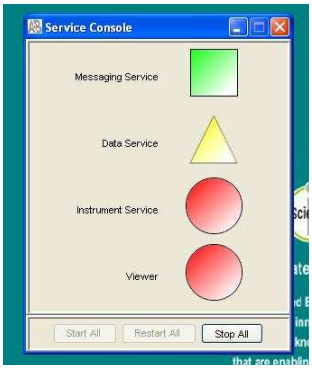
2. On the desktop, click on the shortcut for the respective instrument's data file. The main path to this data file is:

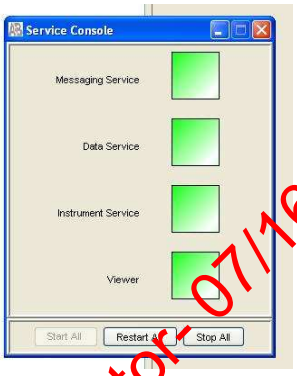
   E:\Applied Biosystems\UDC\data collection\data\ga3130xl*Instrumentname*

3. Once there, create a master file using the following format: "*InstrumentnameYear-Run Number* Files" (e.g. Batman08-015 Files) within the appropriate archive folder (e.g. Batman 2008). Move the 3130xl mtDNA worksheet excel and text files into this master file.

4. Open the 3130*xl* Data Collection v3.0 software by double clicking on the desktop Icon or select **Start > All Programs > AppliedBiosystems > Data Collection > Run 3130xl Data Collection v3.0** to display **the Service Console.**.

   By default, all applications are off indicated by the red circles. As each application activates, the red circles (off) change to yellow triangles (activating), eventually progressing to green squares (on) when they are fully functional.

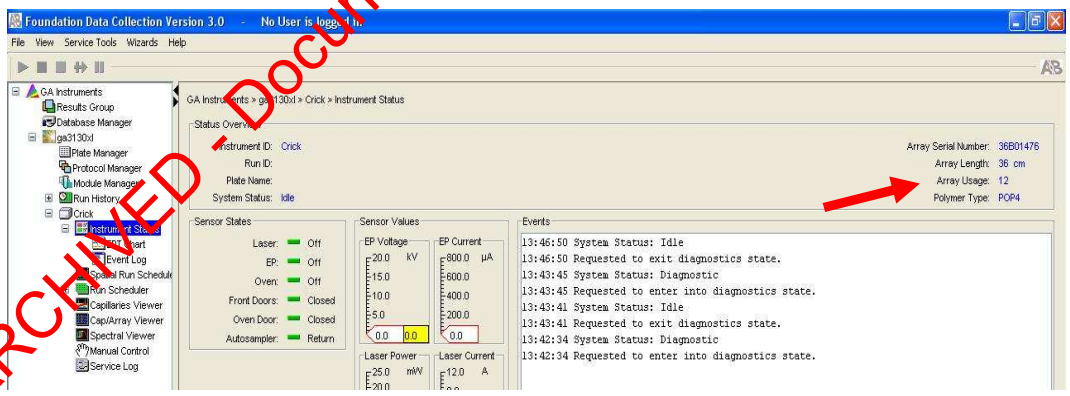




NOTE: This process could take several minutes. The Service Console must <u>not</u> be closed or it will shut down the application.

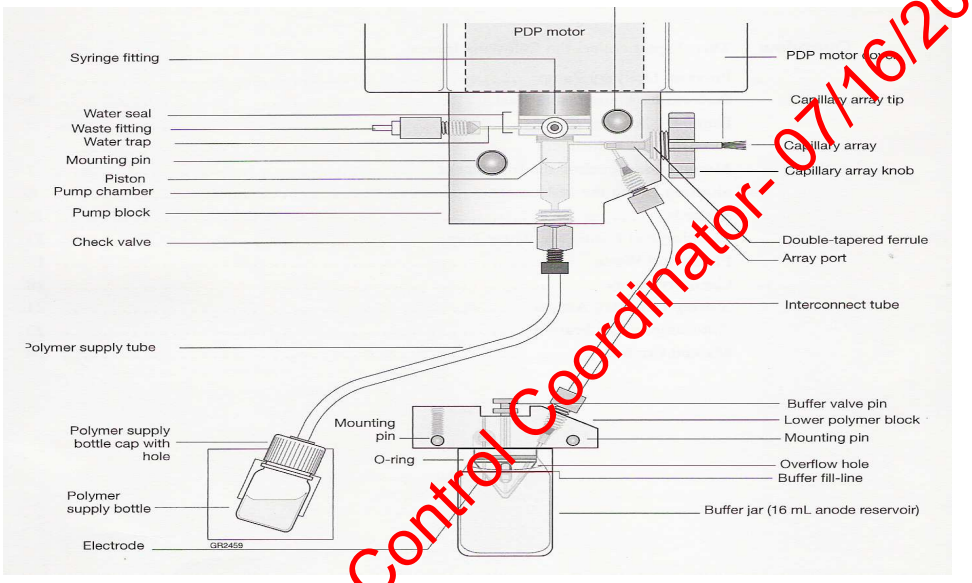
Once all applications are running, the **Foundation Data Collection** window will be displayed at which time the **Service Console** window may be minimized.

5. Check the number of injections on the capillary in the 3130xl binder and in the **Foundation Data Collection** window by clicking on the **ga3130xl > *instrument name* > Instrument Status**. If the numbers are not the same, update the binder. If the number is ≥ 140, notify QC. Proceed only if the number of injections you are running plus the usage number is ≤ 150.



6. Check the binder to see when the POP6 was last changed. If it is >7 days, proceed with POP6 change (See part F of this Section) and then return to Step 9.

7. Check the level of POP6 in the bottle to ensure there is enough for your run (approximately 600 µl is needed per injection). If there is not, proceed with POP6 change (See part F of this section) and then return to Step 9.



8. If you are the first run on the instrument of the day, proceed with steps 9 - 17. If a run has already been performed on the instrument that day, skip to "**Creating a Plate ID"**

9. Close the instrument doors and press the tray button on the outside of the instrument to bring the autosampler to the forward position.

10. Wait until the autosampler has stopped moving and then open the instrument doors.

11. Remove the three plastic reservoirs from the sample tray and anode jar from the base of the lower pump block and dispose of the fluids.

12. Rinse and fill the "water" and "waste" reservoirs to the line with Gibco® water.

13. Make a batch of 1X buffer (45 ml Gibco® water, 5 ml 10X buffer) in a 50mL conical tube. Record the lot number of the buffer, date of make, and initials on the side of the tube. Rinse and fill the "buffer" reservoir and anode jar with 1X buffer to the lines.

14. Dry the outside and inside rim of the reservoirs/septa and outside of the anode jar using a Kimwipe and replace the septa strip snugly onto each reservoir. **If these items are not dry, arcing could occur thus ruining the capillary and polymer blocks.**

15. Place the reservoirs in the instrument in their respective positions, as shown below:

Water Reservoir (waste) 2	Water Reservoir (rinse) 4
Cathode Reservoir (1X buffer) 1	Empty 3

16. Place the anode jar at the base of the lower pump block.

17. Close the instrument doors

B. Creating a Plate ID

1. Click on the **Plate Manager** line in the left window.

2. Select **Import** from the bottom of the screen. Find the text file that was previously saved in the master file for the 3130xl run data (e.g. B08-015.txt file present in the **Batman08-015 files** folder)

3. Click on **OK.**

C. Preparing the DNA Samples for Sequencing

Arrange amplified samples in a 96-well rack according to how they will be loaded into the 96- well reaction plate. Sample order is as follows: A1, B1, C1, D1... G1, H1, A2, B2, C2... G2, H2, A3, B3, C3, etc. Thus the plate is loaded in a columnar manner where the first injection corresponds to wells A1 to H2, the second injection corresponds to wells A3 to H4 and so on. Label the side of the reaction plate with the name used for the Plate ID with a sharpie.

1. Remove the Hi-Di formamide from the freezer and allow it to thaw. Add 10µl of formamide to each dried sample and mix to bring the sample into solution.

   Once formamide is thawed and aliquoted, discard the tube. Do not re-freeze opened tubes of Hi-Di formamide.

2. If single Centri-Sep columns were used, load the entire 10 µl of the resuspended samples into the 96-well tray in the appropriate wells. The injections are grouped into 16 wells starting with A1, B1, and so on moving down two columns ending with 2G, 2H, for a total of 16 wells. Fill any unused wells that are part of an injection set (eg. containing <16 samples) with 10 µl of Hi-Di formamide.

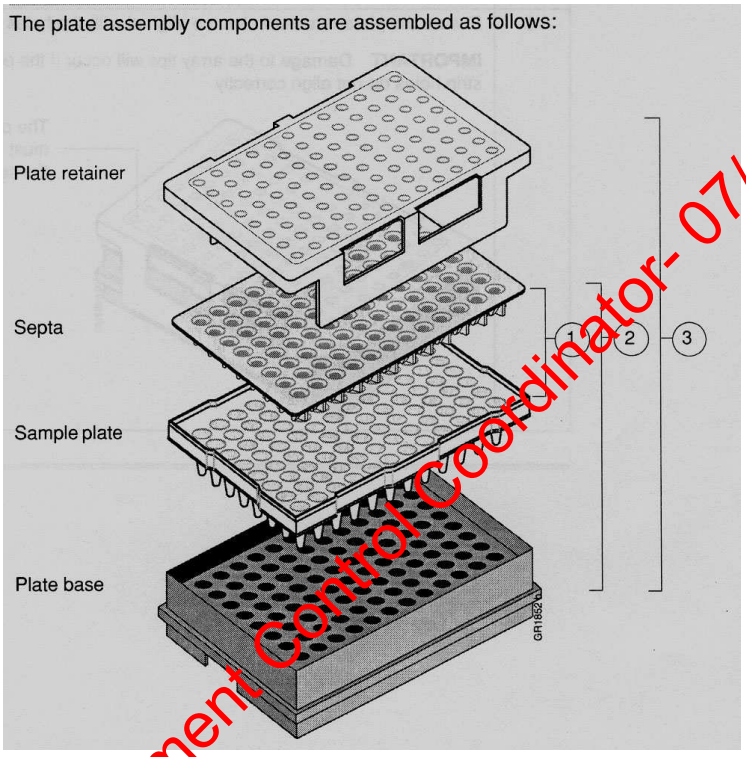
3. Once all of the samples have been added to the plate, place the 96-well septa over the reaction plate and firmly press the septa into place. Spin plate in the centrifuge for one minute.

4. Remove the reaction plate from the base and heat denature samples in the 95°C heatblock for 2 minutes followed by a quick chill in the 4°C chill block for 5 minutes. Centrifuge the tray for one minute after the heat/chill.

5. Once denatured, place the plate into the plate base. Secure the plate base and plate with the plate retainer.

   IMPORTANT: **Damage to the array tips will occur if the plate retainer and septa strip holes do not align correctly.**

   Do not write on the septa with pen, markers, sharpies, etc. Ink may cause artifacts in samples. Any unnecessary markings or debris on the septa may compromise instrument performance.



D. Placing the Plate onto the Autosampler (Linking and Unlinking Plate)

The Autosampler holds up to two, 96-well plates in tray positions A and B. To place the plate assembly on the autosampler, there is only one orientation for the plate, with the notched end of the plate base away from you.

1. In the tree pane of the Foundation Data Collection v3.0 software click on **GA Instrument > ga3130xl > *instrument name* > Run Scheduler > Plate View**

2. Push the tray button on the bottom left of the machine and wait for the autosampler to move forward and stop at the forward position.

3. Open the doors and place the tray onto the autosampler in the correct tray position, A or B. **There is only one orientation for the plate.**

4. Ensure that the plate assembly fits flat in the autosampler. Failure to do so may allow the capillary tips to lift the plate assembly off the autosampler.

   When the plate is correctly positioned, the plate position indicator on the **Plate View** page changes from gray to yellow. Close the instrument doors and allow the autosampler to move back to the home position.

   NOTE: When removing a plate from the autosampler, be careful not to hit the capillary array. Plate B is located directly under the array, so be especially careful when removing this tray.

Linking/Unlinking the Plate record to Plate

5. On the plate view screen, click on the plate ID that you are linking. If the plate ID is not available click **Find All**, and select the plate ID created for the run.

6. Click the plate position (A or B) that corresponds to the plate you are linking.

   NOTE: It may take a minute for the plate record to link to the plate depending on the size of the sample sheet.

   If two plates are being run, the order in which they are run is based on the order in which the plates were linked.

   Once the plate has been linked, the plate position indicator changes from yellow to green when linked correctly and the green run button becomes active.

7. To unlink a plate record just click the plate record you want to unlink and click "**Unlink".**

E. Viewing Run Schedule and Starting Run

1. In the tree pane of the Foundation Data Collection software, click **GA Instruments > ga3130xl > *instrument name* > Run Scheduler > Run View.**

2. The **RunID** column indicates the folder number(s) associated with each injection in your run (e.g. *Batman-2008-0114-1600-0197).* The folder number(s) should be recorded in the **3130xl Usage Log** binder along with the run control sheet name.

3. Click on the run file to see the Plate Map or grid diagram of your plate on the right. Check if the blue highlighted boxes correspond to the correct placement of the samples in the injections.

4. **NOTE: Before starting a run, check for air bubbles in the polymer blocks. If bubbles are present, click on the Wizards tool box on the top and select "Bubble Remove Wizard". Follow the wizard until all bubbles are removed.**

5. Click on the green **Run** button in the tool bar when you are ready to start the run. When the **Processing Plate** dialog box opens (You are about to start processing plates…), click **OK**.

6. To check the progress of a run, click on the **Cap/Array Viewer** or **Capillaries Viewer** in the left window. The **Cap/Array Viewer** window will show the raw data of all 16 capillaries at once. The **Capillaries Viewer** window will show you the raw data of the capillaries you select to view.

   IMPORTANT: Always exit from the **Capillary Viewer** and **Cap/Array Viewer** windows. During a run, do not leave these pages open for extended periods. This may cause unrecoverable screen update problems. Leave the **Instrument Status** window open.

   The visible setting should be:

EP voltage 12.2 kV	EP current (no set value)
Laser Power prerun 15 mW	Laser Power during run 15mW
Laser current (no set value)	Oven temperature 50°C

   Expected values are: EP current constant around 40-60 µA starting current
   EP current constant around 70-80 µA running current
   Laser current: 5.0 A ± 1.0 A

   It is good practice to monitor the initial injections in order to detect problems.

F. Water Wash and POP Change

Refer to Section A, pg. 2 for schematic of 3130*xl* while proceeding with the water wash and POP change procedure.

1. Remove a new bottle of POP6 from the refrigerator.
2. Select **Wizards** > **Water Wash Wizard**
3. Click “Close Valve”
4. Open instrument doors and remove the empty POP bottle.
5. With a dampened Kimwipe®, wipe the polymer supply tube and cap. Dry.
6. Replace POP bottle with the water bottle filled to the top with Gibco® Water.
7. Remove, empty, and replace the anode buffer jar on the lower polymer block.
8. Click “Water Wash.” This procedure is will take approximately 4 minutes.
9. When the water wash is finished click “Next”
10. Select “Same Lot” or “Different Lot”
11. Remove water bottle from the lower polymer block. Dry supply tube and cap with a Kimwipe®.
12. Replace with a new bottle of room temperature POP.
13. Click “Next.”
14. Click “Flush.” This will take approximately 2 minutes to complete.
15. Inspect the pump block, channels, and tubing for air bubbles.
16. Click “Next.”

3130xl Genetic Analyzer Troubleshooting

Instrument Startup

Observation	Possible Cause	Recommended Action
No communication between the instrument and the computer (yellow light is blinking).	Instrument not started up correctly.	Make sure the oven door is closed and locked and the front doors are closed properly. If everything is closed properly, start up in the following sequence: a. Log out of the computer. b. Turn off the instrument. c. Boot up the computer. d. After the computer has booted completely, turn the instrument on. Wait for the green status light to come on. e. Launch Data Collection software.
Red light is blinking.	Incorrect start up procedure.	Start up in the following sequence: a. Log out of the computer. b. Turn off the instrument. c. Boot up the computer. d. After the computer has booted completely, turn the instrument on. Wait for the green status light to come on. e. Launch the Data Collection Software.
Computer screen is frozen.	Communication error. This may be due to leaving the user interface in the Capillary View or Array View window.	There will be no loss of data. However, if the instrument is in the middle of a run, wait for the run to stop. Then, exit the Data Collection software and restart as described above.

Observation	Possible Cause	Recommended Action
Autosampler does not move to the forward position**.**	Possible communication error, OR Oven or instrument door is not closed.	Restart the system, and then press the Tray button. OR a. Close and lock the oven door. b. Close the instrument doors. c. Press the Tray button.
Communication within the computer is slow.	Database is full.	Old files need to be cleaned out of the database. Follow proper manual procedures described in the ABI Prism 3130xl Genetic Analyzer User's Manual.

Spatial Calibration

Observation	Possible Cause	Recommended Action
Unusual peaks or a flat line for the spatial calibration.	The instrument may need more time to reach stability. An unstable instrument can cause a flat line with no peaks in the spatial view.	Check or repeat spatial calibration.
	Improper installation of the detection window.	Reinstall the detection window and make sure it fits in the proper position.
	Broken capillary resulting in a bad polymer fill.	Check for a broken capillary, particularly in the detection window area. If necessary, replace the capillary array using the Install Array Wizard.
	Dirty detection window.	Place a drop of METHANOL onto the detection window, and dry. Use only light air force.
Persistently bad spatial calibration results.	Bad capillary array.	Replace the capillary array, and then repeat the calibration. Call Technical Support if the results do not improve.

Spectral Calibration

Observation	Possible Cause	Recommended Action
No signal.	Incorrect preparation of sample.	Replace samples with fresh samples prepared with fresh formamide.
	Air bubbles in sample tray.	Centrifuge samples to remove air bubbles.
If the spectral calibration fails, or if a message displays "No candidate spectral files found".	Clogged capillary	Refill the capillaries using manual control. Look for clogged capillaries during capillary fill on the cathode side.
	Incorrect parameter files and/or run modules selected.	Correct the files and rerun the calibration.
	Insufficient filling of array.	Check for broken capillaries and refill the capillary array.
	Expired matrix standards	Check the expiration date and storage conditions of the matrix standards. If necessary, replace with a fresh lot.
Spike in the data	Expired polymer.	Replace the polymer with fresh lot using the change Polymer Wizard.
	Air bubbles, especially in the polymer block tubing.	Refill the capillaries using manual control.
	Possible contaminant or crystal deposits in the polymer.	Properly bring the polymer to room temperature; do not heat to thaw rapidly. Swirl to dissolve any solids. Replace the polymer if it has expired.

Run Performance

Observation	Possible Cause	Recommended Action
No data in all capillaries	Bubbles in the system.	Visually inspect the polymer block and the syringes for bubbles. Remove any bubbles using the Change Polymer Wizard. If bubbles still persist, perform the following: a. Remove the capillary array. b. Clean out the polymer bottle. c. Replace polymer with fresh polymer.
No signal.	Dead space at bottom of sample tube. Bent capillary array. Failed reaction. Cracked or broken capillary	Centrifuge the sample tray. Replace the capillary array Repeat reaction. Visually inspect the capillary array including the detector window area for signs of breakage.
Low signal strength.	Poor quality formamide. Insufficient mixing. Weak amplification of DNA Instrument/Laser problem	Use a fresh lot of formamide Vortex the sample thoroughly, and then centrifuge the tube to condense the sample. Re-amplify the DNA. Run instrument diagnostics.

Observation	Possible Cause	Recommended Action
Elevated baseline	Possible contamination in the polymer path.	Wash the polymer block with hot water. Pay particular attention to the pump block, the ferrule, the ferrule screw, and the peek tubing. Dry the parts by vacuum pump before replacing them onto the instrument.
	Possible contaminant or crystal deposits in the polymer.	Bring the polymer to room temperature, swirl to dissolve any deposits. Replace polymer if expired.
	Poor spectral calibration.	Perform new spectral calibration.
	Detection cell is dirty.	Place a drop of methanol onto the detection cell window.
Loss of resolution.	Too much sample injected.	Dilute the sample and reinject.
	Poor quality water.	Use high quality, ultra pure water.
	Poor quality or dilute running buffer.	Prepare fresh running buffer.
	Poor quality or breakdown of polymer.	Use a fresh lot of polymer.
	Capillary array used for more than 150 injections.	Replace with new capillary array.
	Degraded formamide.	Use fresh formamide and ensure correct storage conditions.
	Improper injection and run conditions.	Notify QA to check default settings.

Observation	Possible Cause	Recommended Action
Poor resolution in some capillaries.	Insufficient filling of array.	Refill array and look for cracked or broken capillaries. If problem persists contact Technical Support.
No current	Poor quality water.	Use high quality, ultra pure water.
	Water placed in buffer reservoir position 1.	Replace with fresh running buffer.
	Not enough buffer in anode reservoir.	Add buffer up to fill line.
	Buffer is too dilute.	Prepare new running buffer.
	Bubbles present in the polymer block and/or the capillary and /or peek tubing.	Pause run and inspect the instrument for bubbles. They may be hidden in the peek tubing.
Elevated current.	Decomposed polymer.	Open fresh lot of polymer and store at 4°C.
	Incorrect buffer dilution.	Prepare fresh 1X running buffer.
	Arcing in the gel block.	Check for moisture in and around the septa, the reservoirs, the oven, and the autosampler.

Observation	Possible Cause	Recommended Action
Fluctuating current	Bubble in polymer block.	Pause the run, check the polymer path for bubbles, and remove them if present.
	A slow leak may be present in the system.	Check polymer blocks for leaks. Tighten all fittings.
	Incorrect buffer concentration.	Prepare fresh running buffer.
	Not enough buffer in anode.	Add buffer up to the fill line.
	Clogged capillary.	Refill capillary array and check for clogs.
	Arcing.	Check for moisture in and around the septa, the reservoirs, the oven, and the autosampler.
Poor performance of capillary array used for fewer than 150 runs.	Poor quality formamide	Prepare fresh formamide and reprep samples.
	Incorrect buffer.	Prepare new running buffer.
	Poor quality sample, possible cleanup needed.	Desalt samples using a recommended purification protocol (e.g., microcon).
Migration time becomes progressively slower.	Leak in the system.	Tighten all ferrules, screws and check valves. Replace any faulty parts.
	Improper filling of polymer block.	Check polymer pump force. If the force needs to be adjusted, make a service call.
	Expired polymer.	If necessary, change the lot of polymer.

Observation	Possible Cause	Recommended Action
Migration time becomes progressively faster.	Water in polymer bottle resulting in diluted polymer.	Replace the polymer, making sure the bottle is clean and dry.
Arcing in the anode – lower polymer block.	Moisture on the outside of the lower polymer block.	Dry the lower block. If damaged, replace lower polymer block.
Error message, "Leak detected" appears. The run aborts.	Air bubbles in the polymer path. Pump block system is loose/leaking. Lower pump block has burnt out. When there is condensation in the reservoir(s) this will cause electrophoresis problems and burn the lower block	Check for bubbles and remove if present, then check for leaks. Make sure all ferrules, screws, and tubing is tightly secure. Ferrule in capillary end of block may be positioned wrong or missing. Check for this ferrule. Replace the lower block.
Buffer jar fills very quickly with polymer.	Air bubbles in the polymer path. Lower polymer block is not correctly mounted on the pin valve.	Check for bubbles and remove if present. Then, look for leaks. Check to make sure the metal fork is in between the pin holder and not on top or below it.

Observation	Possible Cause	Recommended Action
Detection window pops out while replacing the capillary array. Replacing the window in the correct orientation is difficult.	Tightening of the array ferrule knob at the gel block causes high tension.	Loosen the array ferrule knob to allow the secure placement of the window. Re-tighten and close the detection door.
Detection window stuck. It is difficult to remove when changing the capillary array.		To loosen the detection window: a. Undo the array ferrule knob and pull the polymer block towards you to first notch. b. Remove the capillary comb from the holder in the oven. c. Hold both sides of the capillary array around the detection window area, and apply gentle pressure equally on both sides. d. Release.

Revision History:

July 24, 2010 – Initial version of procedure.

MITOCHONDRIAL DNA SEQUENCING ANALYSIS		
DATE EFFECTIVE 07-24-2010	APPROVED BY ELI SHAPIRO	PAGE 1 OF 13

PURPOSE: To compile the sequence data generated by the ABI 3130xl into a project for analysis, by editing the sequence data and compiling a consensus sequence that can be compared with the revised Cambridge Reference Sequence (rCRS) to determine the mitochondrial DNA type.

PROCEDURE:

The data following the 3130xl run will be saved on the local 3130xl computer in separate injection folders. These folders, along with the run statistics and the run sheets will be saved into a folder in the Mito_Data drive. The run data is also copied into an Analysis Folder in the Mito_Data drive for analysis. Samples run using the ABI Big Dye Terminator kit will need to be processed using ABI Sequence Analysis software for the basecalls to be assigned. Once the files have been processed with Sequence Analysis, they will be imported into the GeneCodes Sequencher software alignment program for consensus sequence analysis and interpretation of the mitochondrial DNA type.

A. Transfer of the 3130*xl* run data into the master file

1. On the desktop, click on the shortcut for the respective instrument's data file. The main path to this data file is:

   E:\Applied Biosystems\UDC\data collection\data\ga3130xl*Instrumentname*

   Once there, identify the injection folders of the runs you wish to analyze.

2. Copy these injection folders into the master file (e.g. Batman08-015 files) that was created earlier making sure that all of the run statistic files and log files are included.

3. Also copy the newly created run files into the respective archive files in the MITO DATA directory.

B. Sequence Analysis

1. Open the Sequence Analysis program by double clicking on the icon. Login using your username and password.

2. Click on the import samples icon in the upper left of the screen, or go to **Add Sample(s)** under the File menu.

3. In the new window that pops up, locate the master file of the run that you wish to analyze. Click on the individual run files within the master file, and for each one click **Add Selected Samples>>** at the bottom of the window. As this is done, a list in the right of the window will populate with the samples from the run. Click **OK** when finished importing samples.

4. The **Add Sample Status** window will indicate the progress of importing the samples. When this finishes, the samples will appear in the top window of the screen. Maximize this area by dragging the center divider bar to the bottom of the window.

5. The samples should all have the boxes under **BC** (base calling) checked. Click on the **Green Arrow** at the top of the screen to begin the analysis of the samples.

6. The **Analysis Status** window will indicate the progress of analyzing the samples. As samples are analyzed, the **BC** column will display a green, blue or yellow box around the check box indicating the quality of the base calling:
   Green: Indicates a successful base calling for that sample
   Blue : Indicates a problem in base calling the data for that sample
   Yellow: Indicates that there is no data to be analyzed for that sample

7. If the sequencing analysis is successful, click the yellow floppy-disk icon in the top left of the screen to **Save All Samples**, or click on **Save All Samples** under the File menu.

8. Print the analysis report page. Click on the **Analysis Report** button at the top of the page (white button with AR; A is in red and R in blue), or select **Analysis Report** under the Analysis menu at the top of the screen. When the report page opens, right click on the column headings for the analysis report. Deselect the following options- *PP Status, Peak 1, Base Spacing, # Low QV, # Med QV, # High QV, Sample Score, CR Start, and CR Stop*. The visible column headings will show the following- **BC Status, Well, Cap #, LOR, 'A' S/N, 'C' S/N, 'G' S/N, 'T' S/N, Avg S/N**. To ensure that all sample identification fits onto one row, deselect "Fit Columns to Window" (lower left of screen), select size "8" font, and increase the width of "Sample File Name" column.

9. Create a PDF file of the Analysis Report by clicking **Print** and send the file to **Adobe PDF**. Be sure the page set-up is set to portrait before creating the PDF file. Check the PDF file to make sure that the complete Sample Names and Sample Descriptions are present (e.g., not cut off). If necessary, make formatting adjustments, resend Adobe PDF file, and recheck. Following the successful creation of the PDF file, save it in the run folder (e.g., save in *B08-040* with run name *B08-040 Analysis Report*) that is contained within the Analyzed Archive.

10. Print a copy of the PDF Analysis Report for the run archive. Close the PDF Analysis Report and the Analysis Report screen.

11. Click **Exit** under the File menu to close out the Sequencing Analysis program.

12. The **Analysis Report** will be placed together with the run review sheet, the 3130xl run sheet, and the 3130xl cycle-sequencing worksheet for review and archiving.

C. Sequencher

Before you begin Sequencher, make sure that the USB key is fully inserted in a local USB port on the computer, and the computer fully recognizes the key. If successful, the computer will make a "Ding-Dong" sound. Starting Sequencher without this USB key will cause the program to lock out all editing capabilities.

1. Open Sequencher. Under the **Contig** menu, select **Consensus to Forensic Standards**. This only needs to be done after opening Sequencher for the first time. Once set, it will remain until the program is closed. Also, check to make sure that the Assembly parameters used to creat contigs are set to the proper settings. Click on the box in the upper left-hand corner that is marked "Assembly Parameters". The following settings should be selected:

Assembly Algorithm:	Dirty Data (radial button)
Optimize Gap Placement:	Use ReAligner (check box)
	Prefer 3' Gap Placement (check box)
Minimum Match Percentage:	85% (slide bar)
Minimum Overlap:	20 (slide bar)
Assemble By Name:	not Enabled (deselected)

2. Under the **File** menu, go to **Import** and select **Sequences**.

3. Find the sequence files that were copied to the Analyzed Archive. To simplify, under File of Type select "With Chromatogram Sequences." To select all of the files press and hold the Shift key, and click on the last file. Once the files are selected, click **Open.** Maximize the analysis window to view all of the samples and sample data.

4. Import the appropriate reference sequence into the project for every contig that needs to be built. The reference files (HVI.spf or HVII.spf) are located on the Forensic Biology network (Mito Data/Reference Seqs).

   These files can be saved to the desktop or local hard drive for easier access.

5. Holding down the Shift or the Control key, click on imported rCRS file, and the forward and reverse sequence files that will make up the contig.

6. At the top of the screen, click on the **Assemble to Reference** button. If the samples contain viable data, the contig will be formed. If the samples do not contain quality data, they will not import into the contig. Name the contig according to the sample name and hypervariable region sequenced. (e.g.- Hair 1A HVII)

7. Select the new Contig icon by clicking on it once. In the **Contig** menu at the top of the screen, select **Trim to Reference Sequence**.

8. Double click on the Contig icon. When the contig diagram window opens, click on the **Bases** button at the top left of the screen.

   A new window will open showing the individual sequence files above the rCRS reference sequence at the top of the window and the consensus sequence at the bottom of the window. Individual sequences, including the reference sequence can be moved up or down by placing the cursor on the name of the sequence in the upper left box and dragging the sequence up or down.

   Under the consensus sequence is a series of "•" and "+" symbols. The "•" symbols highlight base call disagreements from the rCRS and the "+" symbols highlight ambiguities in the consensus sequence.

9. To view the chromatogram data and the sequence data together, highlight a base in the consensus sequence and click the **Show Chromatograms** button at the top of the screen. This will open a second window showing the chromatogram data for all of the sequences in the contig. Notice that the reverse primer sequence has been reversed and compiled in the process of building the contig. Adjust the position of the two screens so that all of the sequence data is visible along with the chromatogram data, and so all of the base positions can be reviewed. **Use this display and review all sequence positions.**

10. To quickly move from one ambiguity to the next in the consensus sequence, click on the first base in the sequence and then press **Control-N** simultaneously on the keyboard. This will jump both the sequence data and chromatograms to the next ambiguous position. To find only the instances where the contig is in disagreement between the strand data, click on the sequence data and press **Control-D**.

    <u>IMPORTANT</u>- In the instances where length and/or sequence heteroplasmy is present and yields single-stranded DNA regions and/or ambiguous bases, or un-editable N calls are present, the consensus setting on the Sequencher program must be set to Plurality mode to analyze that particular sample.

11. **To edit a base call**, click on the base in question in the consensus sequence or individual sequence, and press the appropriate letter on the keyboard according to the following:

Standard Codes	IUPAC Codes *
A- Adenine	R- A or G
C- Cytosine	Y- C or T
T- Thymine	K- G or T
G- Guanine	M- A or C
N- *Ambiguous*	S- C or G
	W- A or T

*See Nomenclature section of this manual for further discussion.

12. **To delete a base**, click on the base in question and do one of the following:

    a. To have the bases fill in from the left side of the strand, press the delete key**.**
    b. To have the bases fill in from the right side of the strand, press the **backspace** key, and follow the on-screen instructions.

13. **To insert a base**, press the **Tab** key and follow the on-screen instructions.

14. **To shift the entire strand,** place the cursor over the strand in question and press and hold the **Ctrl** key. The cursor will turn into a open hand icon. Click and hold using the icon and the hand will "grab" the strand, allowing you to move the entire strand left or right.

15. **To move a single base**, place the cursor over the base in question and press and hold the **Alt** key. The cursor will turn into a lasso icon. Click and hold using the icon and the lasso will "grab" the base, allowing you to move the base.

16. **To highlight a section of the sequence**, click on the beginning base in the sequence you wish to highlight, and then move the cursor to the last base in the sequence section, press and hold the **Shift** key, and click on the final base.

17. **To review edits press and hold Ctrl E.**
    All edits must be documented on the mtDNA Sequence Analysis Editing Sheet. Editing sheet can be found in the form manual.

    - The sheet is in Excel format

- Column "Reason" has a pull down menu to document reason for editing (see Section 17).
- Column "Region "has a pull down menu to choose from "HVI", "HVII" or, "HVI and HVII".
- Column "Method" has a pull down menu to choose from "n/a", "Forensic" or, "Plurality".
- Column "Built" has a pull down menu to choose from "built" or "could not be built".
- Editing Sheets should be archived as follows:
- Save **in** (for samples): M:\MITO_DATA\Project Archive\.*corresponding folder and subfolder (As in Missing Person Cases\2008\FB10-12345)*
- Save **as** (for samples): **FBXX-12345 HVI and FBXX-12345 HVII (see guidelines).**
- Save **in** (for Positive Controls,): M:\MITO_DATA\PC archive\PC folder (as in PCmmddyy-hhmm *)*
- Save **as** (for Positive Controls): PCmmddyy-hhmm HVI, PCmmddyy-hhmm HVII
- For Negative controls: No editing sheet is needed UNLESS it can be built into a contig, then:
  - Save **in** (EN's, AN's): M:\MITO_DATA\Analyzed Archive*corresponding folder and sub folder (As in Batman2008\Batman08-013 files)*
  - Save **as** (EN's, AN's): , ENmmddyy-hhmm , ANmmddyy-hhmm …

Print a copy of the editing sheet for signature of analyst and reviewer, and for the purpose of archiving. A copy of original should be placed in associated case files.

18. When the sequencing analysis and editing are completed, the contigs that were built need to be archived in the appropriate folders. To archive contigs, hightlight contigs that are to be archived together, go to file → export → selection as subproject. Click on Browse button and select folder for contig to be archived. Make sure that the format is set to 'Sequencher Project'. Click the Export button. Enter project name, e.g. PCddmmyy-hhmm for positive control contigs and FBXX-12345 for sample contigs.
    - Positive Control contigs should be archived as follows: M:/MITO_DATA/PC Archive/*year/PCmmddyy-hhmm.*
    - Samples contigs should be archived as follows: M:/MITO_DATA/Project Archives/*sample type/FBXX-12345.* Sample type should be 'Casework' for evidence and associated exemplars and should be 'Missing Person cases' for all Missing person samples.

19. Once contigs have been exported and archived, open appropriate project, go to the **Contig** menu and click on **Compare Consensus to Reference**. If necessary, widen the contig name column to view the entire contig name . To print, select **Reports, Entire Table, Open Report, and Print**. Select **portrait** as the orientation, and print.

    Note: It may be necessary to create PDF files of the sequencing data when necessary for a case that is scheduled for trial (e.g. sequencing electropherograms for the defense attorney to view). This will be done when necessary and saved onto a CD disk that will be kept in the file. Copies of the disk will be made as necessary for court purposes.

20. Optional (as noted above): Print the **Compare Consensus to Reference** page again, but this time send the file to **Adobe PDF**. Click **Print.** Save the PDF file in the master run file**,** named as the sample name plus "diff," eg. "FB05-0005m hair 1 HVI diff." Close the Adobe window. Close the Difference Review window.

21. At the top of the sequence comparison page, click on the **Summary** button. When the **Summary View** window opens, click on **File, Print Setup**, and select "landscape." Then click on the **Ruler** button at the top of the page and adjust the margins (triangles on ruler) and adjust column spacing as needed to print entire sample ID.

22. Print the **Summary** page. To do this, select the **File** menu and select **Print**.

23. Optional (as noted above): Print the **Summary** page again, but this time send the file to **Adobe PDF**. Click **Print.** Save the PDF file in the master run file**,** named as the sample name plus "sum," eg. "FB05-0005m hair 1 HVI sum." Close the Adobe window.

    NOTE: Printing of the chromatograms is usually not necessary, and should only be done when needed for the casefile. If the chromatograms do not need to be printed, skip to step 26.

24. Click on the **Overview** button. In the Contig Diagram, double click on the individual sequence files. The sequence window of the file will open. Click on the **Show Chromatogram** button at the top of the screen. A new window will open. In the upper left corner is a slider bar, the four-color bases and two buttons: a dot and a vertical bar. **DO NOT CLICK THE VERTICAL BAR, AS THIS WILL ERASE THE HEADER FROM THE PRINTED PAGE.**

Print this chromatogram as edited data, trimmed to the hypervariable region of interest. To do this, select the **File** menu and select **Print Setup**. Select **landscape** as the orientation, and print.

a. If you are printing the forward strand, under **Page Range** select **the FIRST four (4) pages** of the chromatogram.
b. If you are printing the reverse strand, under **Page Range** select **the LAST four (4) pages** of the chromatogram.

Repeat step 24 for every separate chromatogram file.

25. Optional (as noted above): Create an Adobe PDF file for this chromatogram as edited data, trimmed to the hypervariable region of interest. To do this, select the **File** menu and select **Print Setup**. Select **landscape** as the orientation, and print to **Adobe DPF** from the drop-down list. Click **OK**. Under the **File** menu, select **Print**.

a. If you are printing the forward strand, under **Page Range** select **the FIRST four (4) pages** of the chromatogram.

b. If you are printing the reverse strand, under **Page Range** select **the LAST four (4) pages** of the chromatogram.

Confirm that the **Adobe PDF** is selected, and click **Print.** Save the PDF file in the master run file**,** named as the sample name plus the primer, e.g. "FB05-0005m hair 1 HVI A4." Close the Adobe window.

Repeat step 25 for every separate chromatogram file.

26. Click on the **Overview** button.

27. The lines at the top of the diagram indicate the forward and reverse strands in relation to the rCRS sequence, and the green bar below the lines indicates which areas of the rCRS are covered by the available sequence data. If the sequence is present in both the forward and reverse strand, the bar will be green with thin white strips on the top and bottom. If there is a partial coverage in only one direction, the bar will contain a light blue pattern.

28. Print out the **Contig Diagram**. Under the **File** menu, select **Print Setup**. Select **landscape** as the orientation, and print (select printer from the drop-down list). Click **OK**. Under the **File** menu, select **Print**. Confirm that the appropriate printer is selected and click **Print.**

29. Optional (as described above): Print the **Contig Diagram** page again, but this time send the file to **Adobe PDF**. Select **landscape** as the orientation, and click **Print.** Save the PDF file in the master run file**,** named as the sample name plus "map," eg. "FB05-0005m hair 1 HVI map." Close the Adobe window.

30. After each run is analyzed, fill all the run information and rerun information on the run/rerun review sheet. The review sheet can be found in the MTDNA forms folder.

- There is a pull down menu in the third column to choose comments.
- When primers can be used for a Positive Control or a sample sequencher file, enter the sequencher file name in the last column. All the negative controls and failed or unused primers should stay in the run sequencher file (e.g., B08-013).
- Save in: M:\MITO_DATA\Analyzed Archive*corresponding folder and sub folder (e.g. in B2008\B08-013 files)*
- Save as: **RR**machinenameyy-run# **(**e.g. **RR**B08-013**)**
- After review, original goes into B(atman) or S(piderman) binders, and a copy is placed in the casefile for each associated case.

31. For Positive Controls, fill out a Positive Control Review Sheet. The Positive Control Review Sheet can be found in the MTDNA forms folder.

- After each run is analyzed, all the primers of a positive control (PC) that can be used to build a contig will be transferred to a new sequencher file for this PC
- The sequencher file will have the PC date and time as name (like PC090908-090201) and will be saved in the PC archive in the MITO_DATA directory
- The control Review sheet has to be completed and reviewed (saved in PC folder as e.g. PC090908-1000).
- The original goes into the PC binder and a copy is placed in each associated case file.

D. File Output and Construction

Arrange the paperwork in following order, from bottom to top:

a. Run/rerun review sheets
b. Control review sheets
c. Sequencher Chromatogram printouts (if necessary)- *landscape*
d. Contig Diagram- *landscape*
e. Summary View- *landscape*
f. Editing Sheet
g. Compare Consensus to Reference- *portrait*

Landscape pages should be arranged in the file so that the right side of the landscape view faces the top edge of the file, and the left side of the landscape view faces the bottom side of the file.

E. Data Review

1. Once all of the Sequencher data has been completed and the file have been archived, pass the entire set of sample printouts for one 3130 run to another IA for data review.

2. For the IA performing the review, the following steps must be performed.

   a. Open the respective file from the appropriate archive located in the MITO_DATA directory. Review all sequencher files.

   b. For each sample, including Positive Control(s), ensure that all edits reflected on the editing sheet are valid and accounted for within the data set.

   c. For positive controls, ensure that the proper type is displayed on the Compare Consensus to reference page.

   d. For negative controls, open the bases and chromatogram windows for each primer for each sample to ensure that no base calling data is present. If necessary, attempt to re-build a contig using questionable negative controls.

e. Ensure for every sample that the paperwork is assembled in order and reflects the entire project and sample names.

3. If a problem is found, mark the occurrence within the paperwork and return the paperwork to the original analyst.

F. Archiving the Sequencher Data

Data will be archived in **BINDERS, CASEFILES, and ELECTRONIC FILES**.

ARCHIVED IN **BINDERS**:

Instrument binders (e.g. Batman)
3130xl worksheet, original
Analysis report, original
Editing sheets for Neg controls that could build into a contig, original
Run review sheets, original

PC Binder

For each positive control:
Positive control review sheet, original
Contig diagrams, original
Sequence summaries, original
Editing sheets, original
Difference review sheets, original

FB **CASEFILES**:

3130xl worksheet(s), copy
Run review sheet(s), copy
Positive control review sheet, copy
Positive control editing sheet, copy
FB sample contigs, originals
Contig diagrams
Sequence summaries
Editing sheets
Difference review sheets

ARCHIVED IN **ELECTRONIC FILES**:

Analyzed run files (e.g. B08-015)
- Run files
- 3130 worksheet
- Run review sheet
- Analyzed run sequence files
- Neg controls that could build into a contig

PC archives (e.g. PC_053108-1306)
- PC Sequencher file
- PC editing sheets
- PC review sheet

FB project archive (e.g. FB08-12345)
- FB Sequencher file
- FB editing sheets

NOTE: If a positive CTR is sequenced twice (e.g., during QC tests), the name will stay the same as in PCmmddyy-hhmm but a lettered suffix will be added after each new sequencing as in PC-mmddyy-hhmm-A, PC-mmddyy-hhmm-B….
For Editing sheets: if after review, changes on the electronic file are necessary, the electronic edit sheet will be saved with the "-reviewerinitials" suffix.

A backup of all of the sequencing data contained in the MITO_DATA directory will be archived by DOITT.

Revision History:
July 24, 2010 – Initial version of procedure.

SEQUENCE NOMENCLATURE AND ALIGNMENT		
DATE EFFECTIVE 07-24-2010	APPROVED BY ELI SHAPIRO	PAGE 1 OF 5

Nucleotide positions are designated according to the standard one-letter code based on the nomenclature system adopted by the International Union of Pure and Applied Chemistry (IUPAC; see table below). Note that an "N" is used to denote unresolved sequence ambiguities where N can be any one of the four bases. **IUPAC codes that designate two possible bases should only be used in instances of sequence heteroplasmy.**

IUPAC code	Base designation	IUPAC code	Base designation
G	Guanine	R	A or G
A	Adenine	Y	C or T
T	Thymine	K	G or T
C	Cytosine	M	A or C
N	G, A, T, or C	S	C or G
		W	A or T

A. Using Sequencher 4.9

1. Sequence differences between the questioned sample and the revised Cambridge Reference Sequence (rCRS) are generated and printed out from the Comparison Report file in Sequencher. These differences are organized by hypervariable region (eg., one difference review file is generated for each HVI and HVII region). The differences are listed in order of occurrence on the mtDNA molecule.

2. In most cases, the alignment of a given mtDNA sequence with that of rCRS is straightforward. However, care must be taken in the placement of insertions and deletions in reference to that of rCRS according to the following standard nomenclature:

   a. Characterize profiles using the least number of differences from the reference sequence. Align the 310 T base in the rCRS with a T whenever possible.

   b. If there is more than one way to maintain the same number of differences with respect to the reference sequence, differences should be prioritized in the following order: (i) substitutions: transitions are favored over transversions, (ii) insertions/deletions (indels).

c. Insertions and deletions should be placed furthermost 3' to a homopolymeric region, with respect to the light strand of rCRS. Insertions and deletions should be combined in situations where the same number of differences to the reference sequence is maintained. In situations involving the "AC" motif, treat this motif as a homopolymeric region with respect to indels in the AC repeat region. Alignment rules a, b, and c are described in Budowle, et al, 2007. For casework samples where alternative alignment following the hierarchy of Wilson, et al, 2002a,b, is also possible, the alternative alignment does not need to be included in the case file.

3. Insertions (INS) should be listed to the right of a particular nucleotide position. Insertions are documented by first noting the site immediately 5' to the insertion followed by a point and a "1" for the first insertion, a "2" if there is a second insertion, and so on.

4. Deletions (DEL) should be listed exactly where the known base in the reference sequence is missing in the sample sequence to minimize the number of differences between the questioned sample and the rCRS reference sequence. Deletions are noted by a ":" on the Sequencher printout in the consensus sequence.

5. **Sequence heteroplasmy** (also known as point or site heteroplasmy) occurs when a single sample contains at least two mtDNA sequences that differ at one or two nucleotide positions. The appropriate one-letter IUPAC code will be used during the editing of a given site that shows sequence heteroplasmy. This designation will be reflected in the Sequencher Comparison Report. In addition, the presence of sequence heteroplasmy at the given nucleotide position for the respective heteroplasmic bases will be documented on the editing sheet.

6. **Length heteroplasmy** occurs in regions that contain many tandem C nucleotides. These regions are commonly referred to as polycytosine or C-stretch regions. Length heteroplasmy refers to a sample that has at least two types, each one differing by the total number of C nucleotides at a given C-stretch.

   a. **It will be noted if a given casework sample has length heteroplasmy in HVI.** The number of C residues, however, in the area with HVI length heteroplasmy will not be recorded. Length heteroplasmy in HVI most commonly arises when there is a substitution of a C for a T at position 16,189. The reference type in HVI is C_5TC_4. Sequences showing length heteroplasmy in HVI will be truncated to fit the C_5TC_4 format including the T to C change at position 16,189.

   b. **It will be noted if a given casework sample has length heteroplasmy in HVII.** Length variants in HVII are commonly observed in the number of C residues preceding a T residue at position 310. It is often possible to determine unambiguously the dominant length variant in this region. The profile used for further analysis in Sequencher should be composed of only the major type as determined by the analyst.

B. Using Sequencher 4.1.4Fb14

1. Sequence differences between the questioned sample and the revised Cambridge Reference Sequence (rCRS) are generated and printed out from the Difference Review file in Sequencher. These differences are organized by hypervariable region (eg., one difference review file is generated for each HVI and HVII region). The differences are listed in order of occurrence on the mtDNA molecule. "Ref" (reference) and "Con" (consensus) indicate what bases are present in the rCRS and the questioned sample, respectively, at the designated mtDNA sequence positions.

2. In most cases, the alignment of a given mtDNA sequence with that of rCRS is straightforward. However, care must be taken in the placement of insertions and deletions in reference to that of rCRS according to the following standard nomenclature:

   a. Characterize profiles using the least number of differences from the reference sequence. Align the 310 T base in the rCRS with a T whenever possible.

   b. If there is more than one way to maintain the same number of differences with respect to the reference sequence, differences should be prioritized in the following order: (i) substitutions: transitions are favored over transversions, (ii) insertions/deletions (indels).

   c. Insertions and deletions should be placed furthermost 3' to a homopolymeric region, with respect to the light strand of rCRS. Insertions and deletions should be combined in situations where the same number of differences to the reference sequence is maintained. In situations involving the "AC" motif, treat this motif as a homopolymeric region with respect to indels in the AC repeat region. Alignment rules a, b, and c are described in Budowle, et al, 2007. For casework samples where alternative alignment following the hierarchy of Wilson, et al, 2002a,b, is also possible, the alternative alignment does not need to be included in the case file.

3. Insertions (INS) should be listed to the right of a particular nucleotide position. Insertions are documented by first noting the site immediately 5' to the insertion followed by a point and a "1" for the first insertion, a "2" if there is a second insertion, and so on.

4. Deletions (DEL) should be listed exactly where the known base in the reference sequence is missing in the sample sequence to minimize the number of differences between the questioned sample and the rCRS reference sequence. Deletions are noted by a ":" on the Sequencher printout in the consensus sequence.

5. **Sequence heteroplasmy** (also known as point or site heteroplasmy) occurs when a single sample contains at least two mtDNA sequences that differ at one or two nucleotide positions. The appropriate one-letter IUPAC code will be used during the editing of a given site that shows sequence heteroplasmy. This designation will be reflected in the Difference Review. In addition, the presence of sequence heteroplasmy at the given nucleotide position for the respective heteroplasmic bases will be documented on the editing sheet.

6. **Length heteroplasmy** occurs in regions that contain many tandem C nucleotides. These regions are commonly referred to as polycytosine or C-stretch regions. Length heteroplasmy refers to a sample that has at least two types, each one differing by the total number of C nucleotides at a given C-stretch.

   a. **It will be noted if a given casework sample has length heteroplasmy in HVI.** The number of C residues, however, in the area with HVI length heteroplasmy will not be recorded. Length heteroplasmy in HVI most commonly arises when there is a substitution of a C for a T at position 16,189. The reference type in HVI is C_5TC_4. Sequences showing length heteroplasmy in HVI will be truncated to fit the C_5TC_4 format including the T to C change at position 16,189.

   b. **It will be noted if a given casework sample has length heteroplasmy in HVII.** Length variants in HVII are commonly observed in the number of C residues preceding a T residue at position 310. It is often possible to determine unambiguously the dominant length variant in this region. The profile used for further analysis in Sequencher should be composed of only the major type as determined by the analyst.

EDITING GUIDELINES		
DATE EFFECTIVE 07-24-2010	APPROVED BY ELI SHAPIRO	PAGE 1 OF 1

Sequencher base calls can be modified if the underlying data support it. The analyst can change an "N" call into a base determination, insert an additional base, remove a position, or trim a sequence. A base call must not be edited without proper justification.

Reasons for base removal are:

- Extra base inserted due to broad peak, peak artifact, or analysis default spacing

Reasons for base insertion are:

- Base omitted however authentic peak is present
- To maintain proper spacing

Reasons for changing a base to an "N" or to a degenerate IUPAC code are:

- Ambiguous bases are detected
- Dye or electrophoretic artifact interference
- Due to sequence or length heteroplasmy

Reasons for changing an "N" call to a base is:

- Base omitted or called "N", however authentic peak is present
- Dye artifact or electrophoretic interference
- Neighboring peak interference

Reasons for trimming a sequence:

- Trimmed to remove end sequence (sequence tail removal)
- Trimmed rCRS and sequences to other (shorter) sequence position for duplication

Editing for other reasons should be documented with a comment explaining the edit.

Many software calls can be easily resolved and corrected by the analyst. However, ambiguous situations should not be edited. If an electrophoresis problem is suspected, this sample should be re-injected. Sequence information at each base position should be confirmed by data from both DNA strands when possible. Single-stranded regions present due to length heteroplasmy, must be confirmed by confirmatory sequencing of the same strand in the same direction. The Sequencher complementary strand alignment will flag conflicts between the two sequencing directions for all strands imported into the contig.

Revision History:

July 24, 2010 – Initial version of procedure.

GUIDELINES FOR CONTROLS

A. Negative controls

Negative controls are considered negative if there is no detectable DNA based on the quantitation procedure and no signal is seen after 3130xl electrophoresis. For DNA sequencing analysis, the controls are also considered negative if signals were obtained, but the resulting base calls fail to form a contig in Sequencher analysis: that is to say there is either no "readable" sequence present or any sequence that is there cannot align to the reference sequence.

A "readable" sequence from a negative control run is a sequence that can be aligned to the rCRS for >90 consecutive bases with no more than 3 "N" calls within any 10 consecutive bases.

Two negative controls are associated with each sample: the extraction negative (ext neg or e neg) and the amplification negative (amp neg) controls. The former tests for potential DNA introduced during extraction through amplification, while the latter tests for the presence of any background DNA that was introduced during the amplification, or present in the amplification reagents. Both of these controls need to be processed for all sequencing primer sets.

Decision matrix for passing, failing or retesting negative controls is as follows:

1. Product gel or Agilent

Control	Result	Action required
Amplification negative and Extraction negative	No band/Agilent value <0.5 ng/µl No band/Agilent value <0.5 ng/µl	Controls pass this stage; proceed with linear array and/or sequencing analysis.

Control	Result	Action required
Amplification negative and Extraction negative	No band/Agilent value <0.5 ng/µl Band/peak seen	Requantify extraction negative to confirm result. Re-amplify extraction negative to confirm presence of DNA, samples can proceed if re-amplification is clean. If the extraction negative control still yields a band/peak following re-amplification, it is preferable to re-extract this sample if more sample is available. If sample amount is limiting, analyst may proceed with caution (see comments listed for this scenario in **Sequencing results** section). However, if the amount of DNA present in the extraction negative sample exceeds 10% of any associated sample (DNA amounts determined by Agilent), that sample is invalid. The failed extraction negative may be sequenced for quality control purposes.

Control	Result	Action required
Amplification negative and Extraction negative	Band/peak seen No band/Agilent value <0.5 ng/µl	Requantify amplification negative to confirm results. This sample set should be re-amplified. This is preferable. Since this result indicates that the background DNA is limited to the amplification control tube rather than being ubiquitous in all samples, it is left to the analyst's discretion to proceed with this amplification set if no additional sample is available. If the amount of DNA present in the amp neg exceeds 10% of any associated sample, that sample is invalid. In addition, the results are only valid if the sequence detected for the amp neg does not match any of the associated samples or any of the samples in the case
Amplification negative and Extraction negative	Band/peak seen Band/peak seen	Requantify amplification and extraction negative to confirm results. Amplification fails and samples must be re-amplified. The negative controls may be sequenced for quality control purposes.

2. Linear array

Control	Result	Action(s) suggested
Amplification Negative and Extraction Negative	no signal no signal	Controls pass this stage; proceed with interpretation and sequencing analysis if desired.
Amplification Negative and Extraction Negative	no signal bands seen	Re-hybridize if spill-over is suspected. Re-amplify and re-hybridize extraction negative to confirm presence of DNA. Samples can be interpreted and sequenced if re-amplification does not yield linear array signals. If amount of extract available is limited, proceed to sequencing and do not interpret linear array.

Control	Result	Action(s) suggested
Amplification Negative And Extraction Negative	bands seen no signal	Re-hybridize if spill-over is suspected. This sample set should be re-amplified. This is preferable. Since this result indicates that the background DNA is limited to the amplification control tube rather than being ubiquitous in all samples, it is left to the analyst's discretion to use the data of this amplification set if no additional samples or extraction negative extract are available. The results are only valid if the linear array type detected for the amplification negative does not match any of the associated samples and in addition does not match any of the samples in the case

Control	Result	Action(s) suggested
Amplification Negative and Extraction Negative	bands seen no signal	Re-hybridize if spill-over is suspected. This sample set should be re-amplified. This is preferable. Since this result indicates that the background DNA is limited to the amplification control tube rather than being ubiquitous in all samples, it is left to the analyst's discretion to use the data of this amplification set if no additional samples or extraction negative extract are available. The results are only valid if the linear array type detected for the amplification negative does not match any of the associated samples and in addition does not match any of the samples in the case.
Amplification Negative and Extraction Negative	bands seen bands seen	Re-hybridize if spill-over is suspected. If this does not resolve the issue, the amplification fails and samples must be re-amplified and re-hybridized.

3. Sequencing results

If an extraction or amplification negative control contains a sequence that matches a case sample, this result should be confirmed by recycle-sequencing. If confirmed, then the test fails and retesting must start at the point of sample re-extraction or amplification, respectively. The determined sequence for the extraction or amplification negative control must contain at least 90 bases or more of readable sequence in order to be used in sequence comparisons with case samples.

Control	Result	Action(s) suggested
Amplification negative and Extraction negative	Negative Negative	Controls pass this stage; proceed with interpretation and/or sequencing analysis.
Amplification negative and Extraction negative	Negative Readable sequence for some but not all primers	Recycle-sequence the extraction negative to confirm results. The fact that some of the sequencing primers did not yield a result indicates that the level of PCR product contamination is very low. Reamplification of the extraction negative may be performed to ensure that the contamination is present. Depending on the amount of original sample present, analyst may choose to re-extract the sample. Sample results for the affected primer set, however, can be interpreted and reported if the sequence is different from all case samples.

Control	Result	Action(s) suggested
Amplification negative and Extraction negative	Readable sequence for some but not all primers Negative	Recycle-sequence the amplification negative to confirm results. The fact that some of the sequencing primers did not yield a result indicates that the level of PCR product contamination in the amp neg is very low. If more DNA extract is available, it is preferable to re-amplify the sample. If the DNA extract is limiting or re-amplification yields the same results, then sample results for the affected primer set can be interpreted and reported if the sequence is different from all associated samples, in additional to all samples in the case.
Amplification negative and Extraction negative	Negative Readable sequence for all primers	Recycle-sequence the extraction negative to confirm results. If possible, repeat extraction and testing of samples. Reamplification of the questionable extraction negative may be performed to ensure that the contamination is indeed present. If no sample is available for retesting, the results can be interpreted and reported if the sequence is different from all associated samples, in additional to all samples in the case.

Control	Result	Action(s) suggested
Amplification negative and Extraction negative	Readable sequence all primers Negative	Recycle-sequence the amplification negative to confirm results. If possible, repeat amplification and testing of samples. If no sample and Extraction negative extract is available for retesting, the results can be interpreted and reported if the sequence is different from all associated samples, in additional to all samples in the case.
Amplification negative and Extraction negative	Readable sequence for some or all primers Readable sequence for some or all primers	Recycle-sequence the extraction and amplification negatives to confirm results. The extraction negative cannot be interpreted because the amplification may have introduced a contaminant. The test fails and all samples and the Extraction negative must be re-amplified and re-sequenced.

<u>NOTE</u>: If it is necessary to re-sequence a casework sample from the cycle sequence step, a new cycle sequencing amplification negative control (CAN) must be created for this round of cycle sequencing. This negative control must yield a negative result for the results to be valid.

B. Positive controls

The positive control (HL60) is included for each amplification and must produce sequence that is consistent with the known polymorphisms. The positive control sample must yield results for the full read length of the associated sample contig, but at a minimum, HVI, 16024-16365, HVII, 73-340, or both. In addition, the positive control serves as the run control. Therefore, in order to be valid, every run must have a positive

control that passes specification.

The known polymorphisms in comparison to the rCRS are as follows:

HVI		HVII	
16,069	T	73	G
16,193	T	150	T
16,278	T	152	C
16,362	C	263	G
		295	T
		315.1	C

This sequence will produce the following linear array type:

Probe	16093	1A	1C	1D	1E	IIA	IIB	IIC	IID	189
HL60	1	1	1	2	2	2	6	1	1	1

If the positive control fails to produce the expected result, all samples associated with this control fail. If it is suspected that the problem is not related to the amplification but could stem from a subsequent step, the positive control and all of the samples can be retested starting either at the cycle sequencing or the 3130xl injection step.

In cases of dye interference or electrophoretic artifact, some N calls in the positive control will be allowed as follows:

1. A maximum number of 4 “N” calls for any primer strand used to build the contig will be allowed for either HVI or HVII region provided that the calls on the complementary strand are unambiguous and not contradictory to the questioned nucleotide position(s).

2. Any positions that have ambiguous N calls on both complementary strands that cannot be resolved through retesting will result in the failing of the positive control and all of the associated sample runs.

GUIDELINES FOR REPORTING

A. Linear array interpretation

1. A sample will not be used for comparisons if:

   - The sample displays a suspected partial profile (generally faint signals, more than 3 weak probe signals and/or the presence of 4 or more blank regions indicate the presence of a partial profile)

   - The sample consists of a mixture of DNA (more than 2 apparent heteroplasmic types could be caused by a mixture)

2. The numeric probe signal calls are the basis for linear array interpretation. Conclusions are as follows:

Consistent (cannot exclude)	All numerical calls match and no locus is inconclusive
Inconclusive	The two types show one difference or one or more of the regions are inconclusive (see below)
Exclusion	Two or more differences that show no evidence of heteroplasmy

3. Heteroplasmy, weak probe signals or type 0 signals are treated as follows:

Weak probe signal	Weak call versus normal call; different probe signal	Difference
	Weak call versus normal call; same probe signal	Inconclusive
	Weak call versus type 0 probe signal	Inconclusive
Type 0 probe signal	Type 0 signal call versus normal call	Difference
	Type 0 signal call versus weak probe signal	Inconclusive

	Type 0 signal call versus type 0 signal call	Consistent if no evidence of a partial profile
Heteroplasmy	Both samples display heteroplasmy at the same region	Consistent
	One sample displays heteroplasmy; one of these probe signals is also present in the other sample	Consistent
	One sample displays heteroplasmy; the probe signals are not present in the other sample	Difference

B. Sequencing: Reporting of Base Calls

1. Sequence data should be determined from both complementary strands of DNA for mtDNA regions HVI and HVII. Only under special circumstances (see 2. below) can sequence be reported for confirmed data from a single-strand.

   a. All good quality data that shows concordance for both complementary DNA strands or confirmed single-strand data can be reported. A list of reported differences from the rCRS must be accompanied by the range of nucleotides of the region that was sequenced. All possible alternative alignments are not reported.

   b. For sequence where an ambiguous calling situation occurs for one strand, it must be left unresolved and called an "N". **No more than 3 un-editable N calls are acceptable within any 10 base stretch of strand sequence data.**

   c. If an "N" base call is made on one of the DNA strands (eg., due to an electrophoretic artifact), this base position can still be reported as a base in the plurality consensus sequence as long as (i) the data on both strands are not in conflict with each other, and (ii) the data generated from the complementary or confirmatory DNA strand is clean and there is no question regarding its base call.

   d. A minimum read length of 90 contiguous base pairs of double-stranded or confirmed data that forms a contig will be valid for interpretation and for generating weight assessment.

f. A minimum read length of 90 consecutive bases of single-stranded data is necessary for any strand to be used to build a contig. Only under special circumstances (see 2. below) can data be reported for a read length of less than 90 bases.

2. Special circumstances will arise (eg., length heteroplasmy) when data from only one DNA strand can be obtained or read lengths of greater than 90 bases are not possible.

   a. For samples with HVI or HVII length heteroplasmy, additional primers should be used in order to obtain as much complementary data as possible.

   b. For sequence where no data is available for one of the complementary strands, this can still be reported given that the sequencing reaction that yielded the one strand of sequence data is repeated (confirmed) for this sample with the same or different primer in the same direction. All of the data from this region (eg., results from two cycle sequencing reactions) must be concordant between the two sequencing runs. Note: This type of rerun will satisfy conditions where a difference from rCRS or sequence heteroplasmy is being reported.

   c. Situations will arise which result in severe length heteroplasmy (e.g. in HVII, 310 C resulting in a homopolymeric stretch of 13 C residues). Under these conditions, it will be not be possible to sequence through this region in either the forward or reverse direction. This could result in the trimming of a strand (e.g., C1) and/or will yield runs with sequences generated from the complementary strand (e.g., D1) primer that are less than 90 bases. In these cases, the data will be acceptable at less than 90 bases. The guidelines described in b. above for run confirmations will also apply to the confirmation runs necessary in this scenario.

3. In situations when un-editable "N" base calls are made at a given sequence position for both DNA strands, then this base will be reported as "N". **Samples with 3 or more un-editable "N" calls within a 10 base pair region of the consensus sequence in either HVI or HVII are inconclusive.**

C. Criteria for Mixture Recognition

1. More than two heteroplasmic positions in a sample are suggestive of a DNA mixture. If possible, the sample should be re-extracted or other samples in the same case should be tested.

2. Samples that contain two heteroplasmic positions might warrant further testing of additional samples depending on the circumstances of the case. This is to make sure that the sample type in question is not due to a mixture.

D. Sequence Comparisons

1. The positive control run with that sample must type correctly in order to report the sequence for that sample.

2. If either extraction or amplification negative controls contain readable sequences, the associated case sample(s) must be compared to this data before any further sequence comparisons are made. The readable extraction or amplification negative controls must differ from all case samples by at least two bases for these case samples to be interpreted and reported (see Control Tables, section A3).

3. When comparing sequences obtained from samples, only the regions in common will be considered.

4. A specimen that yields a mixture of DNA sequences is reported as inconclusive. No comparisons and no statistical evaluation will be performed using this sample.

5. The number of C nucleotides at the HVI polycytosine C-stretch will not be considered for interpretation purposes if length heteroplasmy is present. Likewise, the number of C residues exhibited in samples with HVII length heteroplasmy is highly variable and care must be taken when making comparisons. In order for sequence concordance to be declared, a common length variant must be observed in both samples being compared.

   Differences between samples due to the absence of an HVII common length variant are treated as one difference.

6. Match Criteria for Sequencing data

Concordance	When two mtDNA sequences from separate samples (e.g. from two pieces of evidence or from evidence and a maternal family reference source) are consistent with each other in the overlapping regions, the two samples cannot be excluded as originating from the same person or from having a maternal relationship, respectively.
Inconclusive	The resulting comparison will be considered inconclusive when two mtDNA sequences from separate samples differ by one difference. In these cases other reference sources and/or further testing in order to obtain more sequence data may be helpful.
Exclusion	The resulting comparison will be considered an exclusion when two mtDNA sequences from separate samples differ by two or more differences.

7. Treatment of sequence heteroplasmy

Two identical heteroplasmic bases are present at the same position in both samples.	This is not a difference (eg., C/T vs. C/T).
One heteroplasmic base is present in one sample; a common base is present at the same position in the other sample.	This is not a difference (eg., C/T vs. C; also C/T vs. T).
One heteroplasmic base is present in one sample; a different base is present at the same position in the other sample.	This is a difference (eg., C/T vs. G).

STATISTICAL ANALYSIS		
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The frequency of occurrence of evidence sample types will be reported based on the type of analysis that was performed. When both Linear Array and DNA sequencing analysis are done on a given comparison, only the DNA sequencing statistical analysis will be reported. Nevertheless, the statistics for both analyses (when performed) will be included in the case file.

The extent of the sequence data that will be used for the database search and statistical analysis will be limited to the shortest range and most conservative reporting of the sequence in common between the evidence sample(s) and reference sample(s) used in the comparison (see previously discussed sequence reporting criteria). Statistical analysis will not be performed on partial Linear Array mitotypes.

Statistics may also be presented comparing evidentiary samples, in which case the statistical analysis will be limited to the shortest range and most conservative reporting of the sequence in common between the evidence samples.

A. For Linear Array types (mitotypes), the laboratory will use a database containing population sample mitotypes from Kline et al. 2005; complete database is found at: http://www.cstl.nist.gov/biotech/strbase/NISTpopdata/NIST_mtDNA_LINEAR_ARRAY_data.xls

This population database contains mitotypes of 666 individuals and is comprised of the following population groups: African-American (252), Caucasian (286), and Hispanic (128).

1. A search of this database can be done by using the **LA Summary & Stats** excel spreadsheet located on the Forensic Biology network. After the spreadsheet is opened, make sure the **Sequence Output** tab is selected. Then enter the Linear Array mitotype at the top of the worksheet in this manner, with exceptions noted on the stats spreadsheet:

   a. When no signal is seen in the Linear Array mitotype at a given position, then a null type (“0”) is used for the database comparison at that position.

   b. When a weak signal is seen in the Linear Array mitotype, the numerical type is used for the database comparison at that position. This is a conservative search strategy.

   c. When two signal types are seen in the Linear Array mitotype at a given position, then each type is used separately for the database search(es) and the resulting counts are combined. This is a conservative search strategy.

2. Click on the **LA Stats** tab to view the calculated population statistics for your sample mitotype. The spreadsheet calculates frequency estimates for the mitotype as described below (see B.3).

B. For sequencing data, use the database and the procedure suggested by the FBI:

1. Database

The database used to obtain a frequency estimate is maintained by the FBI (Budowle et al 1999, Monson et al 2002) and is available for download at the following web address:
http://www.fbi.gov/hq/lab/fsc/backissu/april2002/miller1.htm.

A copy of the database including the search window is found on mtDNA analysts' computers. The database contains HVI (16024-16365) and HVII (73-340) sequences from a variety of unrelated individuals.

2. Searching Profiles

The base pair range of the profile to be searched is limited to the shortest range of reported sequence in common for both compared samples (see previously discussed reporting criteria).

Click on the mtDNA icon on your screen. The search window will open. Several options are pre-selected as indicated below.

Mode: - search
Database: - forensic

Under options (in edit menu):
Listing profiles: - not checked
Length variants: - consider multiple insertions as one difference
Partial profiles: - not checked
Statistics: - display up to 2 differences
Listing haplotypes: - check to list haplotypes that appear multiple times
Date: - check "all profiles"
Heteroplasmic scenarios: - not checked
Helper Apps: - not specified

Enter your profile ID.

Enter the searchable basepair range for HVI and click **Add**. Repeat for HVII. If your sample has the standard read length (see above) just double-click on the HVI or HVII icons. Enter all differences from the rCRS.

Click **search**.

Select a temporary directory and name for the results file.

The search result consists of the number of samples with 0-2 mismatches to the searched sample in the combined database and divided into different ethnic groups.

ATTENTION:

When sequence heteroplasmy is present at a given position in the mtDNA sequence, the mtDNA database will be searched with an "N" at that position.

Even though mtDNA sequence HVII polycytosine length variants are entered, multiple C-stretch length variants at the same position are considered as one difference during the database searches of concordant sequences containing this region and will not add additional rarity. In addition, the number of "C" residues in samples with HVI length heteroplasmy is not considered for comparison purposes.

3. Frequency estimate

a. Frequency estimate when the Linear Array mitotype or mtDNA sequence is observed at least once in database.

Raw frequency estimates for the occurrence of a given mtDNA profile in the general population is based on the counting method as follows:

$$p = x/N \quad \text{(Eq. 1)}$$

Where p is the frequency estimate; x is the number of times a profile has been observed in the population database, and N is the number of profiles in the population database.

A confidence interval must be calculated from the results of the database search in order to correct the counting results for sampling errors according to the following equation

$$p \pm 1.96 [(p)(1-p)/N]^{1/2} \quad \text{(Eq. 2)}$$

The upper 95% confidence interval value (upper bound = $p + 1.96 [(p)(1-p)/N]^{1/2}$) is calculated as the maximum frequency of occurrence within each population of the same mitotype or mtDNA sequence as the searched profile.

The upper bound estimate can be calculated automatically using the Popstats spreadsheet for sequencing statistics or the LA Summary and Stats spreadsheet found on the Forensic Biology network drive.

Example #1: The Linear Array mitotype or mtDNA sequence is observed 3 times in a database containing 2000 sample profiles. The frequency estimate is 3/2000 = 0.0015; the upper bound of the confidence interval is equal to $0.0015 + 1.96[(0.0015)(0.9985)/2000]^{1/2} = 0.0015 + 0.0017 = 0.0032$.

Meaning of example #1: With 95% confidence, the maximum true frequency of the mtDNA profile is 0.0032 or 0.32%, or 1 in 310. In other words, at least 99.68% of the population can be excluded as the source of the evidence.

b. Frequency estimate when the Linear Array mitotype or mtDNA sequence is not observed in the database.

The following equation is used:

$$1 - \alpha^{1/N} \quad \text{(Eq. 3)}$$

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

Example #2: The Linear Array mitotype or mtDNA sequence is observed 0 times in the database containing 2000 sample profiles. The frequency estimate is $1\text{-}0.05^{1/2000} = 1\text{-}0.999 = 0.001$.

Meaning of example #2: For a database size of 2000 mitotypes or sequence profiles, the frequency of a mtDNA profile not observed in the database is 0.001 or 0.1%; or 1 in 1000, or, with 95% confidence, 99.9% of the population can be excluded as being the source of the evidence.

c. Based on the FBI database, the mtDNA population database search software supplies separate results of the frequency estimates for four major populations (African-American, Hispanic, Caucasian, and Asian Origin). It is not the intent of the report to draw any inference as to the population origin of the contributor(s) of the evidence.

d. The Linear Array mitotype population database of Kline, et.al., 2005, supplies separate results of the frequency for three major populations (African-American, Caucasian, Hispanic). It is not the intent of the report to draw any inference as to the population origin of the contributor(s) of the evidence.

e. Reports will present the upper bound 95% confidence interval estimate for each population group, and express this as a percentage and a frequency, e.g., an upper bound 95% confidence interval estimate of 0.5% (1 in 200). Frequency estimate will be rounded down to nearest 10 or single whole number. The intent of the report is to present a conservative range of estimates of the strength of the mitochondrial DNA comparison.

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CREATION OF A CASEFILE CD

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When all of the sequencing analysis is completed for a case, and upon request from members of the court, a CD containing all of the data for that case may be created to fulfill the request.

1. Insert a blank CD into the computer, and open the program "Roxio/Creator Home." Click on the "Data," section and select "Data Disc."

2. Depending on the specific request, the levels of the disc will be created according to the following:

   CD Main Window
   - "**Evidence**" File
     - A**nalyzed 3130xl Run Files**
       - Analyzed 3130xl data files
       - Sequence Analysis Report
     - **Sequencher Evidence Project File**

   - "**Exemplars**" File
     - **All analyzed 3130xl Run Files**
       - Analyzed 3130xl data files
       - Sequence Analysis Report
     - **Sequencher Exemplar Project File**

3. Ensure that all 3130xl data is imported from the **Analyzed Archive** on the network, NOT the *superhero* archive.

   To ensure the quality of the disc, it is advisable to copy all of the necessary data to a single location on the local computer that will create the CD. Once all of the necessary files have been compiled in this file on the local hard drive, according to the tree structure above, the entire contents of the local file can be added to the CD.

4. For the "Volume Label" fill in the casefile number, (eg. FB05-0234).

5. Label the new disk with the casefile number. The disk should be delivered to the Quality Assurance Manager for transmittal.

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DNA Sequencing

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Agilent

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APPENDIX A
OLIGONUCLEOTIDE PRIMER SEQUENCES

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Region	Primer	Nucleotide (base) Sequence	Size (no. of bases)
HVI	A1[1]	5'- CAC CAT TAG CAC CCA AAG CT -3'	20
	A4	5'- CCC CAT GCT TAC AAG CAA GT -3'	20
	B1	5'- GAG GAT GGT GGT CAA GGG AC -3'	20
	B4	5'- TTT GAT GTG GAT TGG GTT T -3'	19
	HVIF[2]	5'- CTC CAC CAT TAG CAC CCA A -3'	19
	HVIR	5'- ATT TCA CGG AGG ATG GTG -3'	18
HVII	C1	5'- CTC ACG GGA GCT CTC CAT GC -3'	20
	C2	5'- TTA TTT ATC GCA CCT ACG TTC AAT -3'	24
	D1	5'- CTG TTA AAA GTG CAT ACC GCC A -3'	22
	D2	5'- GGG GTT TGG TGG AAA TTT TTT G -3'	22
	HVIIF	5'- CAC CCT ATT AAC CAC TCA CG -3'	20
	HVIIR	5'- CTG TTA AAA GTG CAT ACC GC -3'	20

1. Nucleotide sequences for primers A1, A4, B1, B4, C1, C2, D1, and D2 are from the FBI Laboratory DNA Analysis Unit II Mitochondrial DNA Analysis Protocol (mtDNA Protocol Manual, DNA Amplification - Rev. 8, Issue Date 02/01/05 for primers A1, B1, C1, C2, D1, and D2; mtDNA Protocol Manual, Cycle Sequencing - Rev. 8, Issue Date 09/10/04 for primers A4 and B4). The primer sequences in the FBI mtDNA Protocol Manual are based on those described in the following:

Wilson MR, DiZinno JA, Polanskey D, Replogle J, Budowle, B. Validation of mitochondrial DNA sequencing for forensic casework analysis. Int J of Leg Med 1995; 108:68-74.

Wilson MR, Polanskey D, Butler J, DiZinno JA, Replogle J, Budowle B. Extraction, PCR amplification, and sequencing of mitochondrial DNA from human hair shafts, BioTechniques 1995; 18(4):662-669.

2. Nucleotide sequences for oligonucleotide primers HVIF, HVIR, HVIIF, HVIIR are from the product insert for the LINEAR ARRAY Mitochondrial DNA HVI/HVII Region-Sequence Typing Kit that is available from Roche Applied Sciences (Cat. No. 03-527-967-001; product information is available at www.roche-applied-science.com). The primer sequences in the typing kit are based on those described in:

Gabriel MN, Calloway CD, Reynolds RL, Primorac D. Identification of human remains by immobilized sequence-specific oligonucleotide probe analysis of mtDNA hepervariable regions I and II. Croat Med J 2003; 44:293-298.

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APPENDIX B
MITOCHONDRIAL DNA PRIMER LOCATIONS

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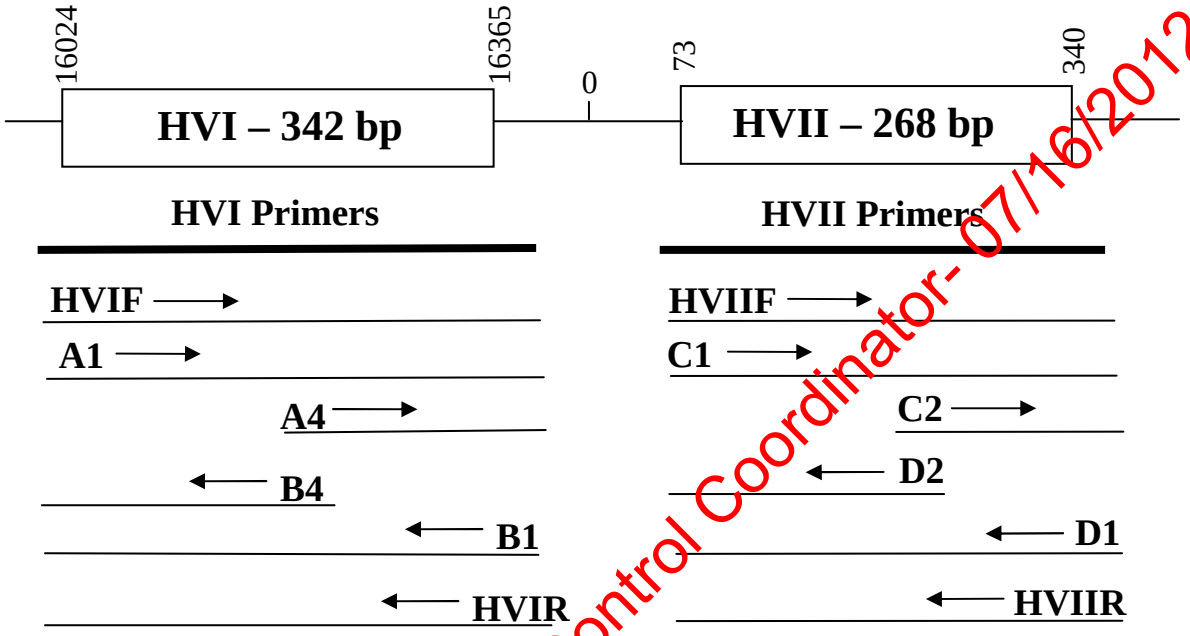

The above diagrams are not to scale. All primer positions are relative to the table below. All arrows indicate the directions (forward or reverse) that the primer amplifies along the hypervariable region.

HVI (16024 - 16365) = 342 bp		HVII (73 - 340) = 268 bp	
Primer	**Position[1]**	**Primer**	**Position[1]**
HVIF	15975	HVIIF	15
A1	15978	C1	29
A4[2]	16190	C2	154
B4[2]	16182	D2[3]	306
B1	16410	D1	429
HVIR	16418	HVIIR	429

[1] Nucleotide position is defined as the first base at the 5' end of the primer.
[2] Primers A4 and B4 are used to resolve C-stretch length polymorphisms in HVI.
[3] Primer D2 is used when necessary to resolve the reverse strand sequence when C-stretch polymorphism is present in HVII.

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APPENDIX C
REVISED CAMBRIDGE REFERENCE SEQUENCE

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Hypervariable Region I (HVI)

```
16024- TTCTTTCATG GGGAAGCAGA TTTGGGTACC ACCCAAGTAT
16064- TGACTCACCC ATCAACAACC GCTATGTATT TCGTACATTA
16104- CTGCCAGCCA CCATGAATAT TGTACGGTAC CATAAATACT
16144- TGACCACCTG TAGTACATAA AAACCCAATC CACATCAAAA
16184- CCCCCTCCCC ATGCTTACAA GCAAGTACAG CAATCAACCC
16224- TCAACTATCA CACATCAACT GCAACTCCAA AGCCACCCCT
16264- CACCCACTAG GATACCAACA AACCTACCCA CCCTTAACAG
16304- TACATAGTAC ATAAAGCCAT TTACCGTACA TAGCACATTA
16344- CAGTCAAATC CCTTCTCGTC CC -16365(end)
```

Hypervariable Region II (HVII)

```
  73- ATGCACGCGA TAGCATTGCG AGACGCTGGA GCCGGAGCAC
 113- CCTATGTCGC AGTATCTGTC TTTGATTCCT GCCTCATCCT
 153- ATTATTTATC GCACCTACGT TCAATATTAC AGGCGAACAT
 193- ACTTACTAAA GTGTGTTAAT TAATTAATGC TTGTAGGACA
 233- TAATAATAAC AATTGAATGT CTGCACAGCC ACTTTCCACA
 273- CAGACATCAT AACAAAAAAT TTCCACCAAA CCCCCCCTCC
 313- CCCGCTTCTG GCCACAGCAC TTAAACAC- 340(end)
```

Human Mitochondrial DNA Revised Cambridge Reference Sequence,

LOCUS NC_012920 16569 bp DNA circular PRI 30-APR-2010
DEFINITION Homo sapiens mitochondrion, complete genome.
ACCESSION NC_012920 AC_000021
VERSION NC_012920.1 GI: 251831106
SOURCE mitochondrion Homo sapiens (human)
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo.

REFRENCES

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Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat. Genet. 1999; 23 (2): 147. MEDLINE-99438386 PUBMED- 10508508

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APPENDIX D
DETAILED CYCSEQ/3130XL SPREADSHEET CALCULATIONS

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Example:

Sample	Quant vol. (µL)	# of LA's	LA input vol. (µl)	Misc. vol. (µl)	Quant (ng/4 ul)	Exo-Sap (ul)	DNA (µl)	Comment	H_2O (µl)
Amp. Neg	4	0	0	0	0	9.2	3.0	Control	7.8
Sample	5	1	5.4	0	56	7.9	4.3	1/10th dil.	6.5

Calculations for Amplification Negative:

1. A total of 4µL of the amplification negative was aliquotted for quantification. The quantification value was below the limit of detection (nd) for that sample, and a linear array was not run. Therefore, the analyst enters 4 into the "Quant vol." field and 0 each into the "# of LA's", "LA input vol.", "Misc vol." and "Quant value" fields.
2. The total remaining reaction volume is calculated by the spreadsheet to be 46 µL (50 µL starting volume minus 4 µl used for quantification).
3. The amount of ExoSAP-IT required is calculated by the spreadsheet according to the following guideline: 1 µl of ExoSAP-IT for every 5 µL of amplified product. Thus, the current reaction volume is divided by 5 (46/5) and the spreadsheet enters 9.2 into the "ExoSAP-IT" field.
4. The amount of template to add to the cycle sequencing reaction is calculated by the spreadsheet. In this case, a gel value of 0 instructs the program to enter the maximum volume amount of 3 µL into the template field. The spreadsheet also enters "Control" into the comments field based on the nd quantification value.
5. Finally, the amount of water is calculated based on the previously calculated sample volume to make the total volume quantity sufficient at 10.8 µL. This DNA/water mixture is now ready to be added to the cycle sequencing reaction.

Calculations for Sample:

1. In this example, 5 µL of amplified sample was aliquotted for quantification. Also, 5.4 µL of the sample was used for Linear Array analysis. The user inputs 1 into the "# of Gels" field since only 1 gel was run at the usual volume of 4 µl. The user also inputs 5 into the "Quant vol." field along with the volume of 5.4 (µL) used for the Linear Array analysis into the "LA input vol." field. The user inputs 0 into the "Misc. Vol." field. Finally, the user inputs the concentration value into the "Quant" field. This field is based on the amount of sample DNA in a volume of 4 µL.
2. The total reaction volume is calculated by the spreadsheet to be 39.6 µL, which is equal to the starting volume minus 5 µL for the quantification volume, 5.4 µL used for the Linear Array (50 - 5 - 5.4 = 39.6).
3. The amount of ExoSAP-IT required is then determined as before. The spreadsheet calculates that the reaction requires 7.9 µL of ExoSAP-IT (39.6/5).
4. In calculating the amount of template required for the cycle sequencing reaction, the spreadsheet first determines the new concentration of the DNA sample after addition of ExoSAP-IT by the dividing the original concentration [(56 ng/4 µL)(39.6 µL)] by the new volume (47.5 ul). The new concentration (11.67 ng/ µL) is then used to calculate the volume of sample needed to equal 5 ng of sample DNA [(5 ng)/(11.67 ng/ µL) = 0.428 µL]. If the final volume is less than 1 µL, the spreadsheet will indicate that a dilution is necessary in the "Comment" filed. In this example, the spreadsheet calculation indicates that 4.3 ul of a 1/10 sample dilution is required.
5. As before, the spreadsheet indicates the amount of water necessary to yield a total of 10.8 µL of sample volume for the next step of cycle sequencing.

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