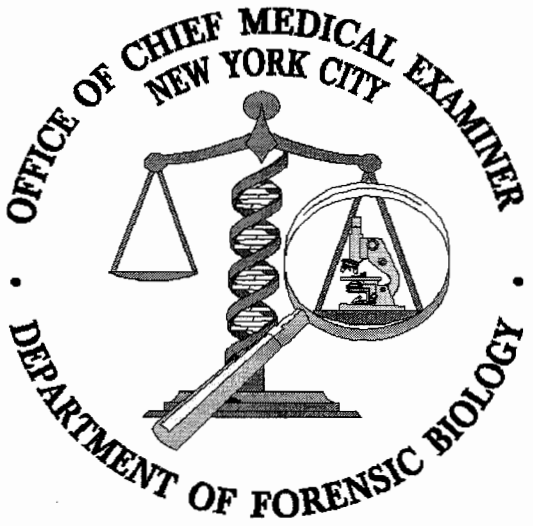
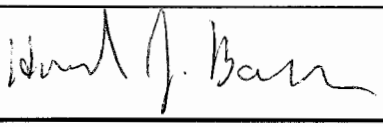


PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS VERSION 2.0

Effective date: April 8, 2005

REVIEWED/APPROVED BY			
Title	**Print Name**	**Signature**	**Date**
Deputy Director/ Technical Leader	**Howard J. Baum, Ph.D.**		**April 7, 2005**

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

TABLE OF CONTENTS		
DATE EFFECTIVE 04-08-2005	VERSION 2.0	PAGE 1 OF 3

TABLE OF CONTENTS

TABLE OF CONTENTS

1. MITOCHONDRIAL DNA PRE-AMPLIFICATION GUIDELINES

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 1

PURPOSE: To reduce the possible contamination in the pre-amplification 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.

PROCEDURES:

All gowning must be done before entering the pre-amplification room

1. **Gloves, without exception, must always be worn while in the pre-amplification room**.

2. Masks and/or face shields must be worn by visitors when an analyst is working on samples.

3. Any analyst working on samples must gown up with a full lab coat, gloves, and a mask and/or face shield. Eye protection is at the discretion of the analyst. 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 life.

4. When working in the pre-amplification laboratory, gloves must be rinsed in 10% bleach before each procedure and in-between the handling of separate samples.

5. Pipette stems must be wiped down with 10% bleach before each procedure, and in-between the pipetting of separate samples.

6. All hoods must be wiped down with 10% bleach before and after each procedure, and UV light, if available, should be applied for 30 minutes before and following each procedure.

7. 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.

8. Any 96-well tube racks and covers taken to the 9700 room in post-amp must be exposed to UV light in the Stratalinker for a minimum of 1 hour.

9. All centrifuge tubes used in the laboratory will be removed from original packaging just prior to the extraction, and only with bleached and dried gloves while fully gowned.

10. At the end of each week, the counters, sinks, refrigerator/freezer handles and door handles inside the laboratory and door handles inside the gowning room should be wiped down with 10% bleach and the UV light on top of the fridge should be turned on.

1. GUIDELINES FOR SAMPLES		
DATE EFFECTIVE 04-08-2005	VERSION 2.0	PAGE 1 OF 3

A. Nomenclature

1. All normal samples will be labeled using the standard naming conventions throughout the sample processing. At the 3100 run step, all samples must be labeled according to which primer is associated with each sample. (e.g., "Sample 1B4" for primer B4)

2. All samples re-extracted for the purposes of duplication should be labeled as "**dup**" to separately identify the re-extraction sample from the original, and this label should permanently be applied to these duplication samples (e.g., "Sample1 dupA4" for the duplicated sample cycle sequenced with primer A4)

3. All sample that are re-injected from one 3100 run to the next, with no additional cycle sequencing of that sample, should be labeled "**reinj**" for re-injection. This label will separately identify the re-injected sample from the original sample. (e.g.- "Sample 1dup-D2reinj" for the duplicated sample, cycle sequenced with primer D2 and re-injected on the 3100 run)

4. All samples that are re-cycle sequenced in order to confirm length heteroplasmy should be labeled "**conf**" to separately identify the re-cycle sequenced sample from the original. (e.g., "Sample 1A1conf" for the sample re-cycle sequenced with primer A1)

5. All samples that are reamplified or re-cycle sequenced in order to improve on the quality of the results should be labeled **"reamp"** for reamplification.

B. Repeat Analysis of Samples

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

1. Extraction stage: A fresh extraction negative control must be run.

2. Amplification stage: A new amplification negative control and HL60 positive control must be included in the re-amplification. The extraction negative control does not need to be repeated if the first test was valid and the extraction negative control was negative.

3. Cycle sequencing: A new amplification negative control must be tested for each primer used; the original extraction and amplification controls do not have to be repeated (eg., 20 ul of TE^{-4} may be used). **Note: A separate positive control and amplification negative control must be run for each primer that is being run with the sample DNA.**

4. Reinjections: A positive control must be added to any reinjection set to act as a run control. Any previously successful positive control sample may be used. No negative controls need to be repeated for reinjections of previously prepared samples, if the controls passed on the first run.

C. Batching and Duplication Guidelines

1. Evidence samples

   a. There will be no batching of evidence samples at the DNA extraction stage (eg., each sample will have its own extraction negative control). Therefore, no duplication of evidence samples will be done at the extraction level.

   b. Batching of evidence samples is allowed at all steps following DNA extraction but only within a given case (e.g., all hair samples from a single case may be amplified on one sheet, but only for that one case.)

2. Exemplar samples

   Batching of exemplar samples within a given case or from different cases will be allowed at all steps of mtDNA analysis including the DNA extraction stage.

3. General

   a. With two exceptions, evidence and exemplar samples must always be run separately in time and/or space. As with nuclear DNA samples, batching of evidence and exemplar samples is allowed during the DNA Quantitation step. Batching of evidence and exemplar samples is also allowed during product gel analysis provided that sample aliquots are done on each sample type (evidence or exemplar) at separate times.

b. Duplication of samples is only necessary when samples are batched. Situations may exist when a sample is run separately throughout all testing procedures. This sample does not need to be duplicated.

c. Duplication of a given sample is accomplished by running one informative primer for that sample in either HVI or HVII.

d. Consult the table below for how to proceed given the following test conditions:

Sample type	Inclusion (concordance)	Exclusion
Suspect exemplar	Repeat from step that sample was batched. *	No retesting necessary
Victim exemplar	No retesting necessary	Repeat from step that sample was batched. *
Evidence	Repeat from step that sample was batched unless sample matches another sample collected from the same piece of evidence.	Repeat from step that sample was batched unless sample matches another sample collected from the same piece of evidence.

*Quantiblots and product gels are excluded from the duplication process.

3. MITOCHONDRIAL DNA LABORATORY EXTRACTION PROTOCOL

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 1

Refer to the current *Protocols for Forensic STR Analysis* manual for the following procedures:

DNA Extraction
- DNA extraction guidelines
- Chelex DNA extraction from blood and buccal swabs
- Chelex DNA extraction from soft tissue
- Organic Extraction Procedure
  - Bone preparation and milling
  - Incubation for liquid and dry blood, bone marrow and tissue samples
  - Incubation for bone, teeth, and paraffin embedded tissue samples
  - Phenol Chloroform Extraction and Microcon clean-up
  - Preparation of Phase Lock Gel (PLG) tubes

Microcon 100 DNA concentration and purification

Estimation of DNA Quantity from QuantiBlot Analysis
- Sample Blotting
- Hybridization
- Color Development
- Photography
- Quality Control & Troubleshooting
- QuantiBlot Interpretation
- Modified QuantiBlot starting with extracts in microtiter plates

4. MIDEO MACRO/MICROSCOPIC DIGITAL IMAGING SYSTEM

PURPOSE: For documentation of hairs, bones, teeth, and other evidentiary items that will be altered in the process of mitochondrial DNA testing.

PROCEDURE:

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. Enter in your username and password. When the next screen appears, select the user role based on the following guidelines:

   a. **ERT (Evidence Recovery Technician)** – for logging in digital images of items for a specific case.

   b. **Scientist** – For creating a database for a case, and logging in digital images of items from that case.

   c. **Administrator** – for reviewing images from ERTs and scientists and creating new users

   NOTE: The Administrator cannot create case databases, nor can they save case images. To create a database and login images, select "Scientist."

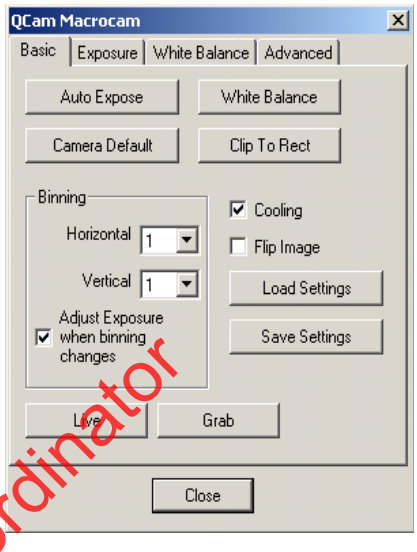
4. As a Scientist, the next screen to appear will allow you to bring up previously entered cases or create a new case. To recall a previous case, use the **Case ID** drop-down list and select the case. Then select any of the images from that case from the **Evidence ID** drop-down list. To create a new case, click the **New** button.

5. For a new case, enter the **Case ID Number** and the **Evidence ID Number** and click **OK.**

6. The next screen to appear is the main program screen. Click on the Blue Microcam button to access the **Microcam Control Panel.**

7. Make sure at this point that the stereo microscope is on, and that 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.

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

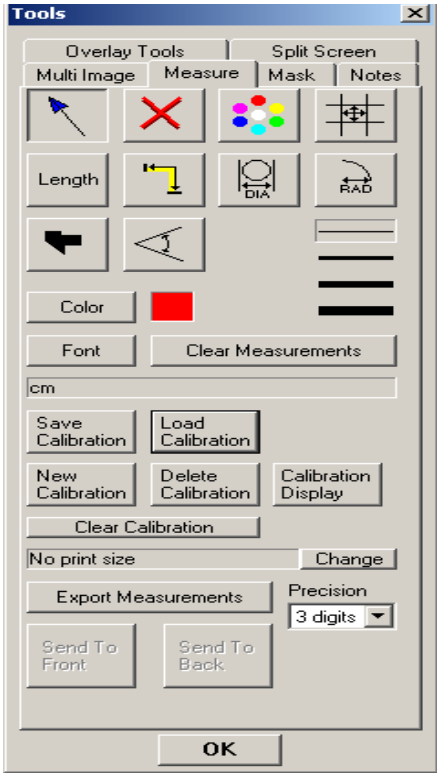


   a. Click the **Live** button.
   b. Adjust the binning so that the setting is 3 for both the horizontal and vertical.
   c. Check the **Flip Image** box.
   d. Click the **Auto Exposure** button.
   e. Adjust the intensity of the light and click **Auto Exposure** if the image is too bright or too dark.

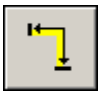
   <u>NOTE:</u> if the background color is not white or off white, place a sheet of paper in view of the lens and click on the White Balance button. Repeat step 8 once completed.

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

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

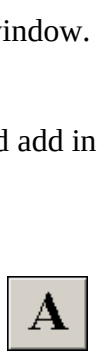


   a. Click on the **Measure** tab.
   b. Click the **Load Calibration** button.
   c. Select the calibration based on the current microscope magnification level.
   d. FOR LINEAR OBJECTS
      1) Click on the **Length** button.
      2) Select any of the length tools to measure the length of the imaged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



11. Click on the **Overlay Tools** tab on the window. Once the panel opens, perform the following:
    a. Click on the **Text** button.
    b. Click anywhere inside the image, and add in the text box for an image label.
    c. Click **OK** on the Overlay window.

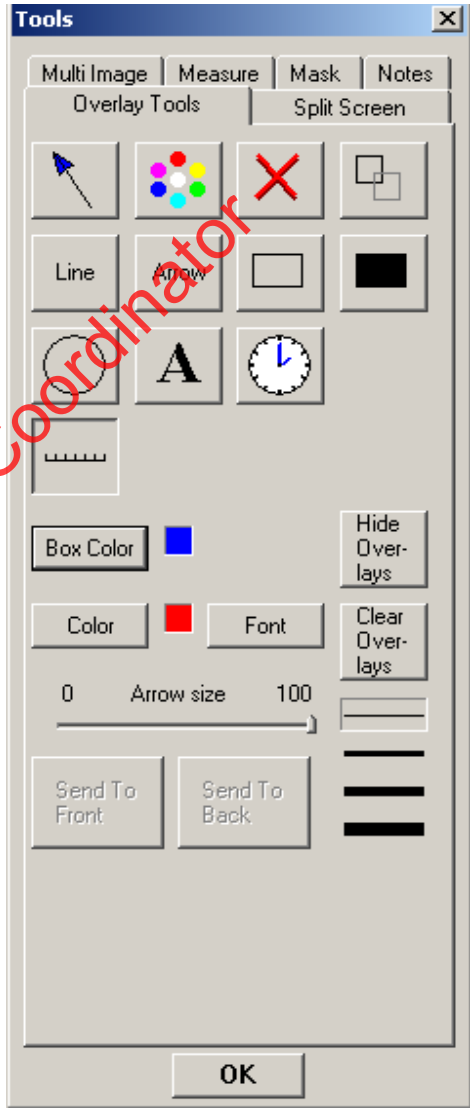
12. Save the image name by clicking on the floppy-disk icon in the main window, or by going to the **File** menu and select **Save.**



13. As a scientist, input the image name and description, and click **OK**.

14. To open a file once it's saved, go to the **File** menu and select **DB Images**. Find the case in which the image is stored, and select the image file to open.

15. To print an image once it's saved or re-opened, click on the printer icon in the main window or go to the **File** menu and select **Print**.



5. MITOCHONDRIAL DNA HAIR EXTRACTION

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 5

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

PROCEDURE:

Before the hair can be processed, the packaging must be documented, and a photomicrograph of the section of hair that will be tested should be captured. Following this, the hair will be cut, washed, and digested before the organic extraction. Since little or no genomic DNA is expected following such an extraction, a 20 µl aliquot from a total of 50μl of the hair extract will be submitted directly to Linear Array PCR amplification.

Only one hair should be processed at a time.

A. Processing the hair sample

1. Document the packaging of the hair on the mtDNA Hair Processing worksheet, and open the packaging in a Labconco Dead-Air hood.

2. Note the general features of the hair on the worksheet, including the length, color, quality, whether or not the hair is mounted, whether the hair is curly, straight, etc.

3. Identify a 2 cm region that will be cut for extraction. Carefully take the hair to the Mideo stereo-microscope and capture a detailed photomicrograph (See the Mideo section for details on instructions).

   If the hair is mounted in a slide, photograph the hair on the slide and again after it has been removed. For loose hairs, place the hairs on a kimwipe or in a weighboat and photograph using direct light.

B. Removing the hair from a mounted slide

If the hair is loose in the packaging, then skip ahead to Section C.

Process only one mounted slide at a time, the mountant softens quickly and hairs will scorch if left on the heat plate too long.

1. Turn on the heat plate and adjust the heat dial between 4-5. Place the slide on a heat plate until the mountant softens, and using forceps remove the cover slip.

2. The hair will be attached to either the coverslip or the slide. Place this piece into the xylene bath for 5 minutes or until the mountant completely dissolves.

3. Using clean forceps, carefully remove the freed hair from the xylene bath, and place in a 1.5 ml microcentrifuge tube filled with 1 ml of xylene, and place in the shaker for 5 minutes at 1400 rpm at room temperature.

4. Examine the hair under the microscope to confirm that the mountant was removed. If not, repeat the xylene wash in the microcentrifuge tube.

5. Using the Mideo stereo-microscope, capture a detailed photomicrograph of this loose hair. (See the Mideo section for details on instructions.)

C. Cutting and Washing the hair

Do not cut the root of the hair for mtDNA testing. If present, remove the root by cutting 1-2 cm up from the root and retain this segment on a labeled post-it backing and place in a secure envelope.

1. Place a clean weigh boat on top of a ruler with a metric scale. The scale will be visible through the plastic bottom of the weigh boat. Place the hair into the weigh boat and using forceps and a scalpel, **cut a 2 cm section of the hair, preferably a untreated/undyed section of the hair.** Return the unused portion of the hair to the packaging.

2. Using clean forceps, place the 2 cm section of hair into a 1.5 ml tube with 1 ml of 5% Tergazyme detergent. 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.

3. 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 dH_2O.

4. Remove the hair from the Tergazyme with clean forceps, and place the hair into the filter cup in the center of the membrane.

5. Wash the hair with 1 ml of dH_2O. Allow the liquid to pass through the filter.

6. Wash the hair with 1 ml of 0.85% saline. Allow the liquid to pass through the filter.

7. Wash the hair with 1 ml of 100% ethanol. Allow the liquid to pass through the filter.

8. 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.

D. Enzymatic digestion of the hair

1. Prepare the incubation solution using the following table:

Incubation Solution	1 hair + extraction negative
Proteinase K (20mg/ml)	30 µl
DTT (1M)	75 µl
20% SDS	7.5 µl
Organic Extraction Buffer	188 µl

2. Aliquot 150 µl of the incubation solution into a labeled 1.5ml microcentrifuge tube.

3. Using clean forceps, submerge the cleaned hair in the incubation solution.

4. Incubate the hair for 30 minutes in a 1400 rpm shaker at 56°C.

5. After 30 minutes, the hair should be dissolved. If it is not, spike the tube with 1µl of 1M DTT and incubate overnight.

6. When the hair is completely dissolved*, proceed immediately to the organic extraction.

NOTE: The hair might not completely digest even after a second input of DTT. 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 and proceed to the organic extraction by removing the supernatant from the tube, leaving the hair behind.

E. Organic Extraction of the hair

Eppendorf Phase Lock Gel (PLG) tubes make the phase separation easier but are optional. Prior to transferring incubated samples to 1.5 ml PLG tubes, these must be centrifuged at maximum speed for 30 seconds. Refer to the "Protocols for Forensic STR Analysis" manual for details on PLG preparation

CAUTION: Phenol:Chloroform:Isoamyl Alcohol is an irritant that is toxic. Its use should be confined to a hood. Nitrile or Vinyl gloves and a mask should be worn.

1. Transfer the incubation buffer containing the dissolved hair to 1.5 ml PLG tubes or microcentrifuge tubes, and add 150 µl (equal volume) of Phenol: Chloroform: Isoamyl Alcohol (25:24:1 PCIA) to the tubes. This step must be done in the fume hood.

2. Shake or vortex the tube to achieve a milky emulsion.

3. Centrifuge the tube in a microcentrifuge for 2 minutes at room temperature.

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

5. Prepare the Microcon 100 concentrator by adding 100 µl of TE^{-4} to the filter side (top) of the concentrator.

6. Transfer the aqueous phase (top layer) to the prepared Microcon 100 concentrator. Do not disturb the PLG layer. Discard the PLG tube or microcentrifuge tube containing the organic layer (if into the organic if the PLG tube is not used)into a waste bottle in the fume hood.

7. Spin the Microcon 100 concentrator for 25 minutes at 500 rcf.

8. Add 400 µl of TE^{-4} to the filter side (top) of the concentrator.

9. Spin again at 500 rcf for 25 minutes. **Most of the liquid should pass through the membrane, if not, the end volume of the sample might be greater than 50µl**.

10. Add 20 µl of TE^{-4} to the filter side (top) of the concentrator.

11. Invert the blue concentrator cup and place into a newly labeled tube. Spin at 1000 rcf for 3 minutes to collect the sample.

12. Discard the blue concentrator cup and **adjust the sample volume to 50 µl** using TE^{-4}.

13. Transfer the sample to a 1.5 ml microcentrifuge tube for storage. Store at 2 to 8°C or frozen.

F. Amplification of the hair extract

Submit 20 µl of the organic extract to PCR amplification.

6. DUPLEX MITOCHONDRIAL DNA PCR AMPLIFICATION

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 5

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.

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, and the full nDNA/mtDNA type is known. Samples that were extracted together should all be amplified together, so that every sample is run parallel to the extraction negative control.

Follow the Mito Pre-amp guidelines for handling the tubes and cleaning of the work surface. The following steps have to be preformed in the appropriate dedicated areas. Evidence samples and exemplar samples should not be handled at the same time.

A. Preparing the DNA aliquots for the amplification

1. The target amounts to be amplified are 0.1 ng of DNA for the amplification system. Follow Table I below for the amount of DNA to be added along with TE^{-4} to achieve a total of 50 µl in the reaction tube. Table II refers to the preparation of the control samples for the amplification.

2. Remember the following rules:

   If the neat and 1/10 QuantiBlot results are ≥10ng/20µl, dilute the sample 1/100 with TE^{-4} and re-quantitate. Multiply the diluted concentration by 100 to obtain the original sample concentration. Repeat the procedure if the diluted sample is still ≥10ng/20µl.

   When a dilution is made to determine DNA concentration, it is preferable to calculate the DNA concentration in the undiluted DNA extract. Then amplify the undiluted DNA extract, not the dilution. *When possible, always amplify the optimal target amount of 0.1 ng.*

Table I – Amount of DNA to be amplified.

QuantiBlot DNA Concentration (ng/20ul)	Target Volume (µl) to be amplified	TE^{-4} for target volume	Range of volumes (µl) which can be amplified
≥10	Re-quantitate		
5	Dilute 1/10 and use the dilution for amplification		
2.5	Dilute 1/10 and use the dilution for amplification		
1.25	1.6	18.4	1.0 - 20**
0.62	3.2	16.8	2.0 - 20**
0.5	4.0	16.0	2.8 - 20**
0.31	6.4	13.6	5.2 - 20**
0.25	8.0	12.0	6.8 - 20**
0.15	13.3	6.7	12.1 - 20**
<0.15	20	0	N/A

** Add TE^{-4} to a final volume of 20 µl

Table II – Control samples for amplification.

Sample	DNA	TE^{-4}
HL60 Positive Control DNA (0.1 ng/20 µl)	20 µl	---
Amplification Negative Control	---	20 µl
Extraction Negative Control		
If sample extracts are amplified neat	20 µl	---
If sample extracts require 1/10 dilution or greater	1 µl	19 µl

B. Amplification Setup[1]

1. Always use fresh 0.5 ml amplification tubes directly from packaging. Ensure that the 96 well rack is clean by exposing it to UV light in the stratalinker for a minimum of 30 minutes.

2. For each amplification set, fill out the worksheet (G:\Users\Fbiology\mtDNA\Forms\Casework\laamp) and record the appropriate lot numbers.

3. Determine the number of samples to be amplified, including controls and label a 0.5 ml PCR reaction tube for each sample.

4. Prepare a master mix tube of the Reaction Mix and Primer Mix, using the following calculation: **For <10 samples, use N+1, for >10 samples, use N+2**
   - Reaction Mix: _____ samples x 20 µl Reaction Mix = _____ µl
   - Primer Mix: _____ samples x 10 µl Primer Mix = _____ µl

5. Vortex the Master Mix and bring to the bottom of the tube. Aliquot 30 µl of the Master Mix into the bottom of each labeled 0.5 ml reaction tube.

6. Close all of the tubes. At this stage have another analyst witness the tube setup.

7. **NOTE: 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 0.1 ng of target DNA to each respective sample tube in accordance with Table I. After the addition of the DNA, cap each sample before proceeding to the next tube. **Do not vortex or mix.**

8. When finished, cover the rack and place the samples in the amplification room airlock.

[1]The Reaction and Primer Mixes used here are from the Roche Linear Array Mitochondrial DNA HVI/HVII Region-Sequence Typing Kit. See Appendix A and B for primer sequences HVIF, HVIR, HVIIF, HVIIR, used in this kit and their mtDNA sequence locations, respectively.

C. Thermal Cycling

Turn on the Perkin Elmer 9700 Thermal Cycler. (See manufacturer's instructions).

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

1. 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.

2. 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.

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.

IMPORTANT:
For all amplifications, return the microtube rack used to set up the samples for PCR to the Pre-Amp room through the Pass-Through airlock.

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 present in the amplification room will power the thermalcyclers for 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.

After the amplification process, the samples are ready to be used for analysis. They may be stored in the appropriate refrigerator at 2-8°C for a period of up to 6 months.

7. MITOCHONDRIAL DNA PRODUCT GEL ANALYSIS

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 9

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."

Thirteen samples and three concentrations of ladders can be run on a 16-well gel; on a 20-well gel, 17 samples and 3 ladders can be run. A ten-well gel (7 samples and three ladders) is also available for small batch amounts.

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 (or TAE)	50 ml	100 ml	200 ml

2. Heat the agarose solution to boiling in a microwave for 2-3 minutes, or on a hot plate with a stir bar, until the agarose is completely dissolved. Bring the volume back up to the original volume with 1X TBE. Allow the solution to cool.

3. Assemble the gel apparatus and pour the liquid agarose into the tray. The gel should be 1/4" thick or less. Position the comb and let gel solidify. For the 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 just to cover the gel.

B. Preparing the samples

1. With the exception of the positive control, mix 4 µl of each amplified sample with 1 µl of the Orange Dye Lane Marker.

2. Add the appropriate amount of the amplified HL60 positive control sample according to the current QC specification and quantity sufficient to 4 µl with 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 all 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 or the large gel at 150 volts for 1 hour (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 and plugged in such 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 and discard all EtBr waste in accordance to departmental policy. Remove, rinse with deionized water and place on the Bio-Doc-It curved side tray.

D. Bio-Doc-It Photography

1. Place the Bio-Doc-It curved-side tray in the Bio-Doc-It chamber and close the door. Turn on the Power and UV switches. **The UV selector must be at 302nm!**

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, the [+] and [-] buttons on the Bio-Doc-It can be used to digitally enhance the image. Press [LIVE] to return to a live (non-enhanced) image.

4. Once the desired image is obtained in 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 properly contrasted, press "Capture" on the control panel and then press save. The image will save as a JPEG file on the disk, and this will take about 30-40 seconds. Once done, remove the disk, open the file on any computer and print out.

5. When finished, turn off the UV switch and the Bio-Doc-It power. Remove the gel from the chamber, and label the printed photograph with your initials and date.

E. Polaroid MP4 Photography

CAUTION- exposure to UV light can be harmful to the skin and eyes. A UV-blocking face shield or UV-blocking glasses are recommended.

1. To begin, place the Fotodyne UV illuminator on the MP4 stage, add the UVP focusing template (the flourescent plastic square with the clock, calibrators, and gradients) to the UV bed. Cover with the black-handled Fotodyne UV blocker. Turn on the UV illuminator. Focus the MP4 camera on the focusing template. Once achieved, switch off the UV light and remove the template.

2. Using the Bio-Doc-It curved side tray, place the tray onto the UV bed and cover with the black-handled UV blocker. Turn on the UV illuminator.

3. Using the viewfinder, adjust the height and focus of the MP4 camera so all of the gel is visible and clearly focused. Once focused, move the orange filter over the lens to take the photograph.

4. Photograph the gel at the widest (most open) aperture for 0.5-1 seconds

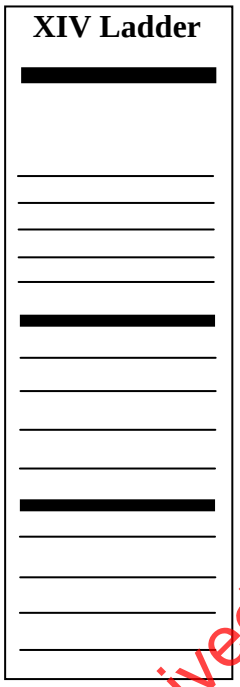
   Aperture: 4.5
   Shutter speed: Set dial to either 2 (½ second) or 1 (1 second)
   Film type: Polaroid 667

5. Remove the film and let develop for 60 seconds. If the photograph is out of focus, not exposed properly, or does not accurately record the sample or ladder bands, vary the exposure conditions and re-photograph.

6. When finished, turn off the UV bench and label the photograph with your initials and date.

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 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.

D. Proceed with sequencing.

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 add 100 pg DNA; repeat amplification.
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 emulation mode is selected.
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 (Electrophoresis problem)	**Recommended Action**
Ethidium Bromide concentration is too low in bath.	Add more Ethidium Bromide to bath, re-soak gel.

PROBLEM: **Misshaped bands for DNA samples, positive controls or Molecular weight Marker.**	
Possible Cause (Electrophoresis 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 set up 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.
Re-extract the sample using a larger piece of hair or more stain material to ensure sufficient high molecular DNA is present.	Concentrate the extracted DNA using a Microcon 100 ultrafiltration device as described in the Microcon section.

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. 4) Amplify using an additional 10 units of Taq Gold Polymerase.

8. ExoSAP-IT SAMPLE CLEANUP

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 2

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

PROCEDURE:

1. Fill out the cycle sequencing spreadsheet for each sample that will be cycle sequenced. (Microsoft Excel format. Filename: "CYCSEQ.XLS". To be found on the Forensic Biology network directory.)

   Fill in the proper columns with the number of product gels and any miscellaneous volume used from each sample, as well as the original concentration as determined by the product gel.

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

2. Based on the spreadsheet calculations, the ExoSAP-IT column will display the volume of ExoSAP-IT that is to be added to that sample.

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

3. 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

4. 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.

5. 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.

9. CYCLE SEQUENCING

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 2

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, incorporated into the Big Dye Terminator v3.1 Cycle Sequencing Kit.

PROCEDURE:

1. Begin by filling out the Cycle Sequencing worksheet. (Microsoft Excel format. Filename: "CYCSEQ.XLS". To be found on the Forensic Biology network directory.)

   Fill in the tube label, sample description, IA, and primer set for each sample.

2. Based on each sample's previous runs, fill in the number of product gels, and any miscellaneous volume used into the respective columns, as well as the concentration of the amplified sample as determined by product gel analysis.

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

3. The amount of template DNA and water needed for each sample is then calculated and filled in on last two columns for each sample. This calculation takes into account the total volume of amplified product left in the sample tube along with the concentration, and first calculates the amount 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.

4. **If the cycle sequencing reaction is to be performed in a 96-well plate, at this time you must fill out the 3100 run sheet. Refer to the ABI 3100 Sequencing Setup, Section E, Creating a Plate ID, for details. This 3100 run sheet will detail the positions of the samples on the 96-well plate, and must be witnessed along with the CycSeq worksheet.**

5. 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

If a dilution of template DNA is necessary, it will be indicated on the cycle sequencing worksheet in the comment column as " x @ 1/10" where x is the input volume. If no dilution is necessary, the comment column will contain the notation "neat." The amount of water that will be quantity sufficient to 20μl 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.

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

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

b. Aliquot 9.2 µl of master mix to each amp tube for that primer, add the mtDNA and water based on the CycSeq worksheet.

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.**

6. 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

10. SDS CLEANUP

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 1

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. Spin down the tubes in a centrifuge.

2. Place the tubes in a thermalcycler, 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.

11. CENTRI-SEP SAMPLE FILTRATION

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 3

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.

9. If the samples are not going to be loaded immediately, they should be stored as a dried pellet at 4°C for no longer then 14 days. When ready, proceed to 3100 setup.

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 x g 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 x g 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.

C. Procedure for the Centri-Sep 96 plate

1. Allow the Centri-Sep 96 plate to come to room temperature.

2. Remove the adhesive foil from the **bottom** of the plate first. Then, remove the foil from the top of the plate. Place on a deep-well centrifuge plate.

3. Spin the Centri-Sep 96 plate at 1500 x g for 2 minutes to remove the excess liquid.

4. Place the Centri-Sep 96 plate on a new 96-well plate and load the samples into the individual columns.

5. Once all of the samples are loaded, place the Centri-Sep 96 plate/96-well plate combination in the centrifuge and spin at 1500 x g 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 96 plate.

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

12. ABI 3100 SEQUENCING

PURPOSE: The 3100 16-capillary array system is used to electrophoretically analyze samples following cycle sequencing and Centri-Sep cleanup. The system uses 96-well plates containing the samples of interest, and processes 16 separate samples with each injection. Sequence data is generated at the end of the run for downstream sequencing analysis.

OVERVIEW

Turn on the computer. Make sure computer is fully booted to the Windows desktop. 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).

NOTE: Collection cannot be launched unless the light on the instrument is green! Important phrase to remember, "If the light isn't green, the machine becomes mean!"

The program that is used to operate the run and collect the data is the ABI Prism 3100 Collection Software. To begin collection, double click on the **Run 3100 Data Collection v2.0** icon. A window will open and four icons will turn from red circles to yellow triangles to green boxes, indicating that the system is operating properly. The final window to open will be the **Foundation Data Collection** window. If the Foundation Data Collection window is closed, this will appear as a red circle in the 3100 Data Collection window. Right-click on this red circle and select "Start" from the pop-up menu. Foundation Data Collection will begin again.

To decide at which point of the manual to proceed:

1. If the number of injections you are running plus the capillary usage number will exceed 150 injections, proceed with **Section A "Changing and Installing the Capillary".**

2. If the amount of POP6 in the installed syringe is low (<600 µL/tray) or greater than one week old, you will have to change it. Start with **Section C "Changing/Filling Syringes with POP6".**

3. If the capillary and POP6 are fine at the first run of the day, start with **Section D "Changing the Buffer"** to change the buffer and water.

4. If the instrument has been run prior today and buffer and water had been changed then proceed to **Section E "Creating a Plate ID".**

A. Changing and Installing the Capillary

The 3100 capillary array is a replaceable unit composed of 16 silica capillaries. The capillary usage number refers to the number of times the array has injected a set of 16 samples. Check the capillary array usage number by clicking on the instrument (either HVI or HVII) in the tree view in the left window. The number of current injections is on the main page of the collection window, under "Array usage." If this number, plus the number of your injection sets (6 for a 96 well plate) exceeds 150, the capillary array should be changed.

To replace a capillary array or to install a capillary array on an instrument, close the oven and instrument doors. From the **Wizards** menu, select **Install Capillary Wizard**. Follow the directions given in the wizard to replace or install an array.

The steps involved in the **Install Capillary Wizard** are:

1. From the Capillary Array Wizard window, select the **Remove and Discard the Array** option. Click **Next**.

2. Push the tray button. Wait for the tray to stop moving before opening the instrument doors. The Capillary Array Wizard will then instruct you to "open instrument door, oven door, and detection block door".

3. Unclip the leading-end head combs of the array from the oven by pulling on the pins to the left and right of the comb.

4. To loosen the screw holding the end of the capillary inside the upper polymer block, unscrew towards you. Remove the syringe guard and pull the upper polymer block out towards you, gently but with a bit of force, until you feel the block reach a stop point. The capillary detection cell will pop out of position. Remove the capillary from the oven slots holding it in place.

5. Completely remove the screw and ferrule holding the end of the capillary inside the upper polymer pump block. Remove the capillary assembly and discard it.

6. Completely unscrew and remove syringes and tubing from upper and lower polymer blocks. Remove upper and lower polymer blocks from instrument. Clean the upper polymer block with hot, NOT BOILING, water using the cleaning syringe. Also clean the ferrule and screw. A final rinse of deionized water should be done. Vacuum or air dry the block and put the block back in the instrument without sliding either block all the way in. Click **Next**.

7. Select **Install a New Capillary Array** from the options. Enter the **Serial Number** of the capillary array located on the capillary array box. Also record the serial number of the capillary and the date in the 3100 Capillary Electrophoresis Log binder. Keep the capillary box for future use. Click **Next**.

8. Slide the capillary leading-end head comb holder into the oven and snap the loading end of the array in place. To secure the capillary comb in place, push both buttons until they snap into place.

9. Place the capillary placement slots into their respective positions in the oven.

10. Remove the detection window protection clip from the capillary window. Make sure the window is clean (no dust or smudges). Slide the capillary assembly into the upper polymer block with the screw and ferrule and place the capillary detection cell in place. Push the polymer block back into place. Tightly screw the array assembly into the polymer block. Click **Next**.

11. Close the laser detection door and screw it shut. Close the oven door.

12. Fill the clean syringes with polymer and install them on the polymer block. Return the syringe guard. Click **Next**.

13. Push the pistons on both syringes slowly to remove any air bubbles. Continue to push the piston of the large syringe to fill both the upper and lower block with polymer.

    <u>IMPORTANT</u>: Pay close attention to any air bubbles trapped next to the end of the capillary assembly and remove them. Also, make sure that all air bubbles are removed from the assembly tubing connecting the upper and lower polymer block. This is a non-transparent tube and bubbles are not visible through it. Any bubbles left in it can cause electrophoresis problems.

14. Close the instrument doors and wait for the autosampler to stop moving. Click **No** if there are no air bubbles remaining. Click **Next**.

15. Click **Fill** to fill the new capillary array. This will take a few minutes. Wait for the **Finish** button to become active, then click it.

16. Push the **Tray** button to present the tray. Once the autosampler has stopped moving, open the doors and change the buffer in the anode jar and the water and buffer in the reservoirs in the autosampler. Close the doors and Click **Finish.** The software will not accept the new information unless the Finish button is clicked.

17. Once the capillary array wizard is finished, a spatial calibration must be done as described in the following section.

    ATTENTION: Since pump block was removed and the capillary window was disturbed, you need to perform a spatial calibration (see B.) before running samples.

18. Monitor the EP Current closely during the first injection. Make sure the EP Current is not fluctuating too much (ex. going from 20 to 160 then down again, etc.) Fluctuation of the EP Current is a good indicator that bubbles may be present and if the run is stopped immediately, the lower block can be saved. If, after 20 minute of the first run, the EP Current is constantly around 70 or 80 μA, it's good.

B. Performing a Spatial Calibration

A spatial calibration provides information about the position of the fluorescence from each capillary on the CCD. It must be performed after each time a capillary array is replaced or temporarily moved from the detection block.

1. Click on the **Spacial Run Scheduler** in the left window. If the capillary was just filled due to changing the POP6 or capillary, select "3100SpacialNoFill_1" from the drop-down list. If you have not filled the capillary recently, select "3100SpacialFill_1".

2. Click **Start.** The calibration will take 2 or 6 minutes, depending on whether, or not, the capillaries are being filled.

3. Upon completion, a dialog box will indicate if the spatial calibration was successful. Successful results yield 16 sharp peaks with similar heights having position (pixel) values of 13-16 units (15 is optimal) higher for each subsequent capillary. A good spatial should be no less than 8000 units high.

4. If the peaks do not fit these specifications, click **Reject**, and press **Start** again to perform another test. Follow step 3. If the spatial still does not fit the requirements, re-run the spacial with the "3100SpacailFill_1" option.

5. If the peaks fit the specifications in step 3, click **Accept**.

6. If the spatial calibration is continuously unsuccessful, see the 3100 troubleshooting section.

7. **Proceed with section E**

C. Changing/Filling Syringes with POP6

Check the amount of POP6 that remains in the reservoir syringe (the large 5.0 mL syringe on the left). For a full tray (i.e. 96 samples or 6 injections), there should be at least 600 µL of POP6 in the reservoir syringe or the run will not start. The array fill syringe (smaller 250 µL syringe on the right) will be filled automatically by the reservoir syringe when it comes time to fill the capillary array. **Never add fresh polymer to old polymer.**

Check the 3100 Capillary Electrophoresis Log binder to see when the polymer was last changed. The polymer should be changed once a week. Document the change of polymer and its lot number in the 3100 Capillary Electrophoresis Log binder.

Completely unscrew and remove the large syringe from the upper polymer block. Clean out the large syringe with deionized water. Do not overdraw the syringe plunger, as this will damage the syringe. Once clean, gently tap dry. In the large syringe, draw 1/3-1/2 polymer into the syringe. Reattach the large syringe to the pump block by firmly screwing it into place.

From the **Wizards** menu under **Instrument Status**, select **Change Polymer Wizard**. Follow the directions given in the wizard to put fresh polymer on the instrument.

The steps involved in the **Change Polymer Wizard** are:

1. From the Polymer Wizard window, click **Close** to close the anode buffer pin valve and **Home** to home the syringes. Click **Same Polymer Type**, the instrument uses only POP6 polymer at this time. Click **Next**.

2. Fill the large syringe with new polymer (see above). Make sure the syringe is free of any air bubbles and install it back onto the polymer block. Click **Next**.

3. Click **Open** to open the anode buffer. Enter the **polymer lot number**, **polymer type**, and **expiration date**. Click **Next.**

4. Select whether this is a new lot of polymer or the same as before, and click **Next**.

5. Push the pistons on both syringes slowly to remove any air bubbles. Use the small syringe to remove any bubbles from the corner of the block near the capillary. Continue to push the piston of the large syringe to fill both the upper and lower block with polymer and remove the remaining air bubbles.

   <u>IMPORTANT</u>: Pay close attention to any air bubbles trapped next to the end of the capillary assembly and remove them. Also, make sure that all air bubbles are removed from the assembly tubing connecting the upper and lower polymer block. This is a non-transparent tube and bubbles are not visible through it. Any bubbles left in it can cause electrophoresis problems.

6. Click **No** when all of the air bubbles are gone. Click **Next.**

7. Replace the buffer in the anode jar and Click **Finish.**

8. The capillary and small syringe must be flushed of old POP6 with new POP6. From **Wizards** in the main menu, select **Fill Capillary Wizard.** Select **50cm/POP6** in the capillary length/polymer dropdown list, and click **Fill**. This will take a few minutes to complete. When finished, click **Finished**.

9. Monitor the EP Current closely during the first injection. Make sure the EP Current is not fluctuating too much (ex. going from 20 to 160 then down again, etc.) Fluctuation of the EP Current is a good indicator that bubbles may be present and if the run is stopped immediately, the lower block can be saved. If, after 20 minute of the first run, the EP Current is constantly around 70 or 80, it's good.

NOTE: You must change the polymer weekly. The polymer is good at 25°C for about 7 days.

D. Changing the Buffer

The cathode buffer and water reservoirs located on the autosampler tray and anode buffer jar located below the lower pump block should be changed at the beginning of each day the instrument is in use.

1. To fill the water and cathode buffer reservoirs on the autosampler, close the instrument doors and press the tray button on the outside of the instrument to bring the autosampler to the forward position. **Wait until the autosampler has stopped moving and then open the instrument doors.**

2. Remove all reservoirs and anode jar and dispose of the remaining fluid. Rinse and fill the water reservoirs to the line with Ultra-Pure water and the cathode reservoir and anode with 1X running buffer, about 16 mL each. Dry the outside of the reservoirs using a Kimwipe and place a clean septa strip on each reservoir, as needed (it is OK to reuse septa).

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

3. Close the instrument doors.

   NOTE: When using the 3100, it is not necessary to fill the water reservoir in position #3.

Position of reservoirs in autosampler:

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

NOTE: Be sure that the septa fit snugly and flush on the tops of the reservoirs in order to prevent damage of the capillary tip. Also, it is important that there is no condensation on the inside of the reservoir. The septa strips must be dry!

E. Creating a Plate ID

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

2. Select "New" from the bottom of the screen. Fill in the info in the new window to name the new plate. Make sure there are no spaces in the plate name. Add a **Run Description** if necessary, the **Run Description** must only be **one line.** In the Application drop-down list, select **Seqencing Analysis**. Make sure the plate type is **96-well.** Add the owner name (eg. NYC OCME) and operator name (analyst initials). Click on "OK."

3. The Sequencing Analysis Plate Editor window will now open. Fill in the **sample name in the "sample name" column** (with no spaces; also do not use any of the following characters: \ / : * " > < | ? .), and the **tube label in the "comment" column**. The priority should be 100 for each sample. The Results Group, Instrument Protocol and Analysis Protocol are populated as the following:

Dye Set System	Results Group	Instrument Protocol	Analysis Protocol
Big Dye Terminator v3.1	mtDNA_Results	Big_Dye_v3.1	3100POP6_BDTv3-KB-DeNovo_v5.1

You only need to select the first sample in each column. Then click on the column header, and under the Edit menu, select "Fill Down" or use the keyboard shortcut Ctrl+D. The rest of the samples should populate with the same data.

4. If samples are to be re-injected, select **Add Sample Run** from the **Edit** menu. This will create a second set of Results Group, Instrument Protocol, and Analysis Protocol columns. Fill these columns out the same way as before, and they will allow the samples to be re-injected.

5. Click **OK** to save the run sheet.

6. On the main Plate Manager screen select the plate ID you just created, and click "**export**" on the bottom of the screen, and save the run sheet as a **text file** into the Run Sheets file at

   E:\Applied Biosystems\UDC\data collection\data\ga3100*Instrumentname*\Run Sheets

   Using the following format:
   InstrumentnameYear-Run # (example- Spiderman04-007.txt)

   Click **Save**.

7. On the desktop, open the Microsoft Excel file **3100run**. Click on the "Import" tab on the bottom of the pages. Click the box 1A.

8. In the Data menu at the top of the screen, click **Refresh Data**. When the menu opens, find the text file for the plate ID saved in:

   E:\Applied Biosystems\UDC\data collection\data\ga3100*Instrumentname* \Run Sheets

   Click **Import.**

9. The fields on the **Import** page should populate with the run data from the 3100 Data Collection software. Click on the **Run Sheet** tab on the bottom of the page.

10. Fill in the name of the 3100 run in the space to the right of the "Run Sheet Name." Fill in the analyst's initials next to the respective samples.

11. Save the sheet as an Excel Workbook into the Run Sheets file at:

    E:\Applied Biosystems\UDC\data collection\data\ga3100*Instrumentname* \Run Sheets

    Using the following format:
    InstrumentnameYear-Run # (example- Spiderman04-007)

    Click **Save**.

12. Print a hard copy of the run sheet and close Excel.

F. 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 10 µl of formamide to each dried sample, and mix to bring the sample into solution. Briefly spin down the samples to bring the liquid to the bottom of the tubes.

   Once formamide is thawed and aliquoted, discard of 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 10μl of Hi-Di formamide.

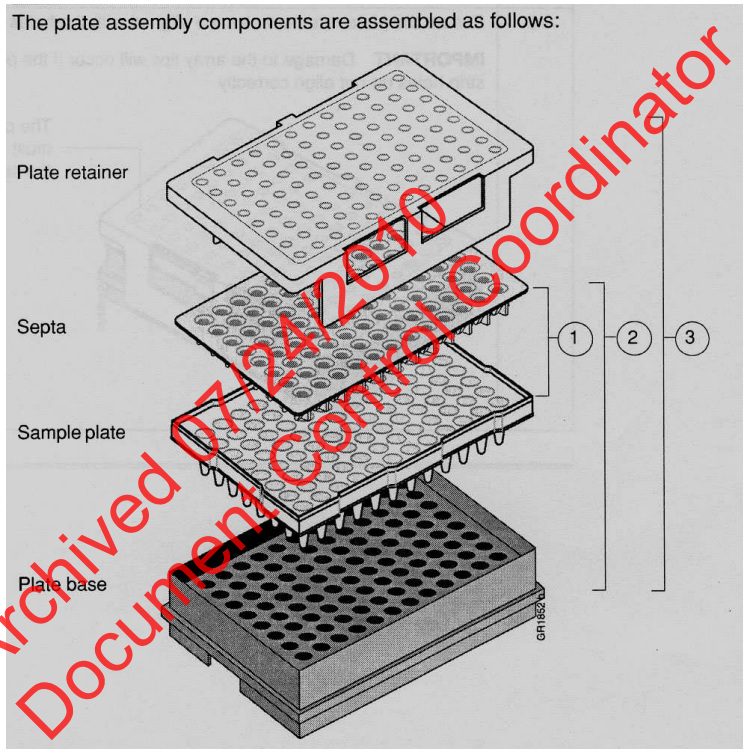
3. Once all of the samples have been added to the plate, place the 96-well Septa over the reaction plate and firmly press the septa into place. Spin plate in centrifuge for one minute.

4. Remove the reaction plate from the base and heat denature samples at 95^{o}C for 2 minutes followed by a quick chill at 4^{o}C for 5 minutes, either using two ABI 9700 thermal cyclers that are set at the two temperatures, or two 96-well thermomixers set at the two temperatures. Make sure to keep the thermal cycler lid off of the sample tray to prevent the septa from heating up. After removing tray from the heat block, check the wells for air bubbles. If there are any, centrifuge the tray to remove any air bubbles.

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

IMPORTANT: 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.



G. Placing the Plate onto the Autosampler

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. 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.

2. Open the doors and place the tray onto the autosampler in the correct tray position, A or B.

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

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

   <u>NOTE</u>: 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.

H. Linking the Plate Record to a Plate

1. On the **Plate View** screen (under **Run Scheduler** in the left window), 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. Then click the plate position (A or B) that corresponds to the plate you are linking.

2. Once the plate has been linked, the:
   - **Run Instrument** button on the toolbar becomes highlighted, meaning that the instrument is ready to run.
   - Plate position indicator for the linked plate changes from yellow to green

   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.

3. Click the **Run View** (under **Run Scheduler** in the left window) to view the run schedule. The **RunID** column indicates the folder number(s) associated with each injection in your run. These folder number(s) should be recorded in the **3100 Log** binder along with the run control sheet name. Click **Run** to start the run. NOTE: Before starting a run, check for air bubbles in the polymer blocks.

   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 **Cap/Array Viewer** and the **Capillaries 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 settings should be:

EP voltage 10.7 kV	EP current (no set value)
Laser Power Prerun 7.5 mW	Laser Power During run 15 mW
Laser Current (no set value)	Oven temperature 50°C

   Expected values are: EP current constant around 40 to 60 µA starting current;
   EP current constant around 70 to 80 µA running current
   Laser current: 5-6 A

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

I. To Unlink a Plate

1. In the **Linked Plate Records** table of the **Plate View** page, select the plate record that you want to unlink.

2. Click **Unlink**. The plate record will return to the Pending Plate Record table and the plate position indicator will return to yellow.

3100 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. OrbixWeb Daemon should be launched, if not already. f. 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. OrbixWeb Daemon should be launched, if not already. f. Launch the Data Collection Software.

Observation	Possible Cause	Recommended Action
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.
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 3100 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 block and syringes. c. Replace polymer with fresh polymer. Make sure to draw the polymer into the syringe very slowly.
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 upper polymer 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 and syringes 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 syringe resulting in diluted polymer.	Clean the syringe, make sure it is completely dry OR replace syringe.
Purging of polymer from the polymer reserve syringe.	Arcing in the anode gel block. Bubbles in syringes.	Replace lower polymer block. Remove bubbles.
Leaking polymer at the top of either syringe.	Insufficient seal around the tip of the syringe plunger.	Do not move the syringe plunger when it is dry. Make sure to wet the plunger before filling the syringe with polymer. Do not mix and match barrels and plungers.
Leaking polymer at the bottom of the polymer-reserve syringe.	Improper tightening of the array ferrule knob to the syringe and/or to the polymer block.	Ensure the array ferrule knob is tightened.
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 syringes, 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.

Observation	Possible Cause	Recommended Action
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.
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.

13. MITOCHONDRIAL DNA SEQUENCING ANALYSIS

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 12

PURPOSE: To compile the sequence data generated by the ABI 3100 into a project for analysis, by editing the sequence data and compiling consensus sequences that can be compared against the revised Cambridge Reference Sequence to determine the mitochondrial DNA type.

PROCEDURE:

The data following the 3100 run will be saved on the local 3100 computer in separate injection folders. These folders, along with the run statistics and the exported run sheet will be saved into a master file, which will be transported by CD-ROM or USB flash drive to analysis stations. Samples run in the **Big Dye Terminator v3.1** will need to be processed in **Sequence Analysis 5.1** for the basecalls to be assigned. Once the files have been processed in **Sequence Analysis 5.1**, they will be imported into **Sequencher** for consensus sequence analysis and interpretation of the mitochondrial DNA type.

A. Creating a master file for the 3100 run data

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\ga3100*Instrumentname*

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

2. Create a new file using the following format:
   "*InstrumentnameYear-Run Number* Files" (Example: Spiderman04-007 Files)

3. Move the injection folders into this new file, making sure that all of the run statistic files and log files are included.

4. Open the **Run Sheets** file, select both the .txt and the Excel files for the run and copy them into the new master file.

5. Attach the USB memory stick to the USB port of the computer. Once the computer recognizes the memory stick, copy the newly created master file to the stick. In the lower right corner, click on the **safely remove hardware** icon in the toolbar, select the USB memory stick, and click **stop**. The computer will tell you when it is safe to remove the USB memory stick.

6. Insert the USB memory stick into the Mitochondrial File-Sharing computer. Drag the newly created run files into the respective archive files on the desktop. Once a week, the data from this File-Sharing computer will be backed up.

5. To access the data on the Mitochondrial File-Sharing computer, open "My Network Places" on any desktop computer. Open the respective archive file containing the data to be analyzed, and copy the 3100 run file to the local hard drive in a designated "sequencing data" file.

B. Archiving of 3100 Run Data

1. After the run in completed on the 3100 instrument, the data from that run must be archived on the pre-formatted CD-R associated with the instrument. To do this, insert the CD-R into the CD drive and wait for the 3100 computer to recognize the disc.

2. Drag and drop the newly created master run files onto the CD-R. Once all of the files have been successfully moved, close the CD-R window.

3. Open the "My Computer" icon. Right click on the CD-ROM drive, and select "Eject" from the drop down list. The computer will finalize the data and eject the disc.

C. Sequence Analysis 5.1

1. Open the Sequence Analysis 5.1 program by double clicking on the icon. Login using your username and password.

   Note- If the same copy of Sequence Analysis is open on two different computers connected to the same network, the second computer will not allow the user to access the program. If necessary, log off the network and work locally on the computer.

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, yellow, or red box around the check box indicating the quality of the base calling:
   Green: Indicates a successful base calling for that sample
   Yellow: Indicates a problem in base calling the data for that sample
   Red: Indicates that there is no data to be analyzed for that sample

   All **Positive Control** samples and **Casework** samples should be green if the cycle sequencing was successful.

   All **Amplification Negative Controls** and **Extraction Negative Controls** should be yellow or red.

7. At the right side of the screen are columns of numbers, indicating **spacing, peak 1, start,** and **stop.** The numbers under spacing should be black if the spacing is acceptable (indicated by a green box in the BC column). If the number is red, the sample should also have a yellow or red box in the BC column. The red numbers under the spacing should be present for negative control samples.

   If a casework sample or positive control sample has a yellow/red box in the BC column and red numbers in the spacing column, re-analyze the sample by re-checking the BC column and clicking on the Green Arrow. See Troubleshooting for further information.

8. 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.

9. Print the analysis report page. Click on the **Analysis Report** button at the top of the page (white button with $^{A}_{R}$; 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, and Sample Score*. 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**.

10. **C**lick the **Print** icon**,** and verify that the print settings are on "Landscape." Click "Print." Close the Analysis Report screen.

11. Repeat the printing of the analysis report page, but this time send the file to **Adobe PDF.** Verify that the print settings are on "Landscape." Click "Print." Save the PDF file to the master analysis file for the samples being analyzed, named for the run itself (eg. Spiderman05-005). Close the Adobe PDF window.

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

13. The **Analysis Report**, **3100 Run sheet,** and **CycSeq worksheet** must be copied for all cases contained in the paperwork, and distributed to the respective analysts. The original copies will be placed together in the proper 3100 binder for archiving.

14. After the Sequencing Analysis is complete, the processed samples files must be archived back onto the Mitochondrial File-Sharing computer. To do this, ensure that the Master Run File on the local computer contains the following information:
    - All processed samples
    - 3100 Excel Run sheet for that respective run
    - Newly created PDF Sequencing Analysis Report

15. Open the "My Network Places" on the local computer and select the **Analyzed Archive** on the Mitochondrial File-Sharing computer. Find and select the Master Run file on the local computer, and copy it to the **Analyzed Archive** file on the Mitochondrial File-Sharing Computer. Once a week, the data from this File-Sharing computer will be backed up.

D. 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)

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

3. Find the sequence files that were copied to the local hard drive. To simplify, under File of Type select "ABI Files (*.ABI, *.ABD, *AB1)." To select all of the files, click and drag to highlight the files, or select the first file, 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.

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

5. Holding down the Shift 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**.

    IMPORTANT- In the instances where length heteroplasmy is present and yields single-stranded DNA regions and/or ambiguous, 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.

    All edits must be documented on the mtDNA Sequence Analysis Editing Sheet.

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. When the sequence review and the editing are completed, go to the **Contig** menu and click on **Difference Review**. Print this sheet out in portrait orientation. To do this, select the **File** menu and select **Print Setup**. Select **portrait** as the orientation, and print to the **Lexmark C720** printer from the drop-down list. Click **OK**. Under the **File** menu, select **Print**. Confirm that the **Lexmark C720** printer is selected and click **Print.**

18. Print the **Difference Review** 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.

19. At the top of the sequence comparison page, click on the **Summary** button. When the **Summary View** window opens, click on the **Ruler** button at the top of the page and adjust the right-margin triangle (◂) so that four columns are visible (drag the triangle to the extreme right edge of the ruler under the number 8). Click the **Ruler** button again to hide the ruler.

20. Print the **Summary** page. To do this, select the **File** menu and select **Print Setup**. Select **portrait** as the orientation, and print to the **Lexmark C720** printer from the drop-down list. Click **OK**. Under the **File** menu, select **Print**. Confirm that the **Lexmark C720** printer is selected and click **Print.**

21. 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.

22. 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.**

    NOTE: Printing of the chromatograms is not always necessary, and should only be done when needed for the casefile. If the chromatograms do not need to be printed, skip step 23.

23. 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 to the **Lexmark C720** printer 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 **Lexmark C720** printer is selected. Click **Print.**

    Repeat step 23 for every separate chromatogram file.

24. 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, eg. "FB05-0005m hair 1 HVI A4." Close the Adobe window.

Repeat step 24 for every separate chromatogram file.

25. Return to the Contig Diagram window.

26. 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.

27. Print out the **Contig Diagram**. Under the **File** menu, select **Print Setup**. Select **landscape** as the orientation, and print to the **Lexmark C720** printer from the drop-down list. Click **OK**. Under the **File** menu, select **Print**. Confirm that the **Lexmark C720** printer is selected and click **Print.**

28. 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.

29. Save the project file, and close and exit the Sequencher program.

E. File Output and Construction

Arrange the paperwork in following order, from bottom to top:
a. Sequencher Chromatogram printouts (if necessary)- *landscape*
b. Contig Diagram- *landscape*
c. Summary View- *landscape*
d. Editing Sheet
e. Difference Review- *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. Staple the top left corner.

F. Archiving the Sequencher Data

1. After the Sequencher analysis is complete, the project and all of the original 3100 run data must be archived back onto the Mitochondrial File-Sharing computer. To do this, create a new file in "Sequencing Data" file on the local analysis computer for the case that is to be archived (e.g.- FB05-0005).

2. Drag and drop the Sequencher project and all associated 3100 run data files into the newly created file on the local computer. Once all of the files have been successfully moved, close the file.

3. Open the "My Network Places" on the local computer, and select the **Project Archive** on the Mitochondrial File-Sharing computer. Select the newly created file, and copy it to the **Project Archive** file on the Mitochondrial File-Sharing Computer. Once a week, the data from this File-Sharing computer will be backed up.

G. 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 3100 run to another IA for data review.

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

   a. On the Mitochondrial File-Sharing computer, open the respective file from the **Project Archive** by copying the file to your local hard-drive. Review the Sequencher contigs.

   b. For each sample, 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 Difference Review 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.

4. Delete the copy of the imported project file from the local computer.

DO NOT SAVE THE PROJECT FILE BACK TO THE MITOCHONDRIAL FILE SHARING COMPUTER.

14. SEQUENCE NOMENCLATURE

1. 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

2. 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 as shown below. "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 differences between rCRS 00073-00340 and *(sample name)* HVII

Pos	Ref	Con	Required Edit
263	A	G	Change base
301	A	:	Delete base
315.1	:	G	Insert base

3. 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.

   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) insertions/deletions, (ii) transitions, and (iii) transversions.

   c. Insertions and deletions should be placed furthermost 3', 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.

4. Insertions should be listed to the right of a particular nucleotide position. Insertions are notated 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.

5. Deletions 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 ":" in the consensus sequence.

6. **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.

7. **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.

15. EDITING GUIDELINES

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, or remove a position. 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
- Trimmed to remove end sequence (sequence tail removal)

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

Many software ambiguities are easily resolved 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 sequencing the same strand in the same direction in an independent cycle sequencing reaction. The Sequencher complementary strand alignment will flag conflicts between the two sequencing directions for all strands imported into the contig.

16. CREATION OF CASE FILE CD-R

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 2

When all of the sequencing analysis is completed for a case, and the case file is submitted for review, a CD-R containing all of the data for that case must be created and stored within the case file.

1. Insert a blank CD-R into the computer, and open the program "Nero Smart Start." Click on the page-shaped icon for "Data," and select "Make Data CD."

2. The levels of the disc will be created according to the following:

CD-R Main Window
- **All Sequencher Project Files** (identified "Evidence" or "Exemplars")
- "**Reports**" File
  - Mitochondrial Laboratory Report
  - Population Statistics files
- "**Evidence**" File
  - **All analyzed 3100 Run Files**
    - Analyzed 3100 data files
    - 3100 Excel sheet & Text File
    - Sequence Analysis Report
  - "**PDF Evid**" file
    - All PDF forms from analysis
  - "**Forms Evid**" file
    - Any worksheet that was generated electronically in its entirety
- "**Exemplars**" File
  - **All analyzed 3100 Run Files**
    - Analyzed 3100 data files
    - 3100 Excel sheet & Text File
    - Sequence Analysis Report
  - "**PDF Exemp**" file
    - All PDF forms from analysis
  - "**Forms Exemp**" file
    - Any worksheet that was generated electronically in its entirety

3. Click on the "Add" button on the right of the screen, and import the necessary files according to the tree structure above. Ensure that all 3100 data is imported from the **Analyzed Archive** on the Mitochondrial File Sharing computer, NOT the 3100 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-R. 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 Nero Express window by Drag-and-Drop.

4. Once all of the necessary files have been compiled, click the "Next" button at the lower right of the screen. For the Disc Name fill in the casefile number, (eg. FB05-0234). Make sure that the boxes are checked for the "Allow files to be added later (multisession disk)" and for the "Verify data on disc after burning." Click "Burn."

5. When the message "Data verification completed successfully" is displayed, click "OK." Click "Next."

6. When finished, the program will ask the user if the format for the new disc is to be saved. Click "No." Click "Exit" to close the program.

7. Label the new disk with the casefile number, and initial and date. Secure the CD-R inside the respective casefile.

8. Any archived data copied to the local computer should be deleted, and **SHOULD NOT** be copied back to the archive file.

17. INTERPRETATION GUIDELINES		
DATE EFFECTIVE 04-08-2005	VERSION 2.0	PAGE 1 OF 9

GUIDELINES FOR CONTROLS

A. Negative controls

Negative controls are considered negative if there is no detectable band on the product gel and no signal is seen after 3100 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.

Two negative controls are associated with each sample: the extraction negative (e neg) and the amplification negative (amp neg) controls. The former tests for potential DNA introduced during the extraction through the amplification steps, while the latter tests for the presence of any background DNA that was introduced during the amplification step 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

Control	Result	Action required
Amplification negative	No signal	Controls pass this stage; proceed with linear array and sequencing analysis.
Extraction negative	No signal	

Control	Result	Action required
Amplification negative	No signal	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 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, that sample is invalid. The first extraction negative may be sequenced for quality control purposes.
Extraction negative	Band seen	
Amplification negative	Band seen	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 or e neg extract are 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 samples in the case
Extraction negative	No signal	

Control	Result	Action required
Amplification negative	Band seen	Amplification fails and samples must be re-amplified. The negative controls may be sequenced for quality control purposes.
Extraction negative	Band seen	

2. Sequencing results

If an extraction or amplification negative control contains a sequence that matches a case sample, 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 100 bp or more of readable sequence in order to be used in sequence comparisons with case samples.

Control	Result	Action required
Amplification negative	Negative	Controls pass this stage; proceed with interpretation and/or sequencing analysis.
Extraction negative	Negative	
Amplification negative	Negative	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 and re-sequencing of the extraction negative may be performed to ensure that the contamination is present.
Extraction negative	Readable sequence for some but not all primers	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 required
Amplification negative	Readable sequence for some but not all primers	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 and re-sequence this 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 case samples.
Extraction negative	Negative	
Amplification negative	Negative	If possible, repeat extraction and testing of samples. Reamplification and re-sequencing 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 case samples.
Extraction negative	Readable sequence for all primers	
Amplification negative	Readable sequence all primers	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 case samples.
Extraction negative	Negative	

Control	Result	Action required
Amplification negative	Readable sequence for some or all primers	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.
Extraction negative	Readable sequence for some or all primers	

NOTE: If it is necessary to re-sequence a casework sample from the cycle sequence step, a new amplification negative control 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 16024-16365 and 73-340. 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

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 3100 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 3 N's (~ 1% of total HVI or HVII sequence) 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 a repeat injection will result in the failing of the positive control and all of the associated sample runs.

GUIDELINES FOR REPORTING

A. Reporting of Base Calls

1. Sequence data should be determined from both complementary strands of DNA for mtDNA regions HVI and HVII.

   a. All good quality data that shows concordance for both complementary DNA strands can be reported. A list of reported differences from the rCRS must be accompanied by the read length of the region that was sequenced.

   b. For sequence where an ambiguous calling situation occurs for one of the complementary strands, it must be left unresolved and called an "N".

   c. If an ambiguous "N" base call is made on one of the DNA strands (eg., due to an electrophoretic artifact), this base can still be reported as long as (i) the data on both strands are not in conflict with each other, and (ii) the data generated from the complementary DNA strand is clean and there is no question regarding its base call. **No more than 3 un-editable N calls are acceptable within a contiguous 10 base stretch of data. If so, the reaction should be repeated.**

d. Unambiguous data from both complementary DNA strands are required in order to report a difference from rCRS or sequence heteroplasmy. Only under special circumstances (see below) can data be reported for single-stranded regions of DNA.

e. A minimum read length of 100 contiguous base pairs of double-stranded or confirmed data that forms a contig will be valid for interpretation and for generating weight assessment. Any strand with less than 100 contiguous bases will be deemed inconclusive.

2. Special circumstances will arise (eg., length heteroplasmy) when data from only one DNA strand can be obtained.

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 independent cycle sequencing reactions) must be clean and 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.

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 more than 3 un-editable "N" calls within a 10 base pair region in either HVI or HVII are inconclusive and if possible should be repeated, or the data should be.**

B. 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.

C. 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 controls to be valid. It can then be concluded that the sample DNA source(s) is not the contaminating DNA and is valid for sequence comparison.

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 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 in sequence due to the absence of a common length variant are exclusionary.

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).

For sequencing data, the laboratory will adopt the database and the procedure suggested by the FBI.

1. Database

The database that is being 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/april2002/mtDNA.htm.

A copy of the database including the search window will be maintained on all mtDNA analyst computers. The database contains HVI (16024-16365) and HVII (73-340) sequences from a variety of individuals of Asian, African or European ancestry. The database will be updated upon release of new versions.

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 individual differences
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: - load Internet Explorer, Excel, and Word

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 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 the different ethnic groups.

ATTENTION: Even though an HVII polycytosine length variant is entered, these C-stretch positions are ignored 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 are not considered for comparison purposes.

3. Frequency estimate

a. Frequency estimate when the mtDNA sequence is observed at least once in database.

Raw frequency estimates for the occurrence of a given mtDNA profile in the general population are 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 presented as the maximum frequency of individuals within each population who are estimated to be included as potential contributors of a questioned profile.

The upper bound estimate can be calculated automatically using the POPSTATS spreadsheet (Filename: popstats.xls) found on the Forensic Biology network drive, in the Mitochondrial DNA folder.

Example #1: The mtDNA sequence is observed 3 times in the mtDNA database containing 2000 sequences. 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%. In other words, at least 99.68% of the population can be excluded as the source of the evidence.

b. Frequency estimate when mtDNA sequence is not observed in the database.

The following equation is used when a mtDNA sequence profile is not found in the database:

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

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

The upper bound estimate can be calculated automatically using the POPSTATS spreadsheet (Filename: popstats.xls) found on the Forensic Biology network drive, in the Mitochondrial DNA folder.

Example #2: The mtDNA sequence is observed 0 times in the mtDNA database containing 2000 sequences. 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 sequence profiles, the frequency of a mtDNA sequence profile not observed in the database is 0.001 or 0.1%; or, with 95% confidence, 99.9% of the population can be excluded as being the source of the evidence.

c. The mtDNA population database search software will supply separate results of the frequency estimates for the four major population groups in the U.S. (African American, Hispanics and Caucasians, and Asians).

DNA Sequencing

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome Nature 1981; 290: 457-465. MEDLINE- 81173052 PUBMED-7219534

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.

Applied Biosystems. ABI Prism® 3100 Genetic Analyzer User Guide. 2002. Part Number 4334785 Rev. A. Applied Biosystems, Foster City, CA.

Applied Biosystems. DNA Sequencing Analysis Software User Guide: Version 5.1 for Windows® XP and 2000 Platforms. 2003. Part Number 4346866 Rev. B. Applied Biosystems, Foster City, CA.

Budowle B, Wilson MR, DiZinno JA, Stauffer C, Fasano MA, Holland MM, Monson KL. Mitochondrial regions HVI and HVII population data. Forensic Sci Int 1999; 103: 23-35.

Carracedo A, Baer W, Lincoln P, Mayr W, Morling N, Olaisen B, Schneider P, Budowle B, Brinkmann B, Gill P, Holland MM, Tully G, Wilson MR. DNA commission of the International Society for Forensic Genetics: Guidelines for mitochondrial DNA typing. Forensic Sci Int 2000; 110: 79-85.

Gene Codes. Sequencher™ 4.0 User Manual. 1999. Gene Codes Corporation. Ann Arbor, MI.

Holland MM, Parsons TJ. Mitochondrial DNA sequence analysis - Validation and use for forensic casework. Forensic Science Review 1999; 11: 21-50.

Monson KL, Miller KWP, Wilson MR, DiZinno JA, Budowle B. The mtDNA population database: An integrated software and database resource for forensic comparison, vol. 4, no. 2. Forensic Science Communications 2002.
http://www.fbi.gov/hq/lab/fsc/backissu/april2002/index.htm.

Sanger FS, Nilken S, Coulson AR. Sequencing with chain terminating inhibitors. Proc Nat Acad Sci U.S.A. 1977; 74: 5463-5467.

Scientific Working Group on DNA Analysis Methods (SWGDAM). Guidelines for mitochondrial DNA (mtDNA) nucleotide sequence interpretation, vol. 5, no.2. Forensic Science Communications 2003. http://www.fbi.gov/hq/lab/fsc/backissu/april2003/index.htm.

Stewart JEB, Aagaard PJ, Pokorak EG, Polanskey D, Budowle B. Evaluation of a multicapillary electrophoresis instrument for mitochondrial DNA typing. J Forensic Sci 2003; 48: 571-580.
Wilson MR, DiZinno JA, Polanskey D, Replogle J, Budowle B. Validation of mitochondrial DNA sequencing for forensic casework analysis. Int J Leg Med 1995; 108: 68-74.

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

Wilson MR, Allard MW, Monson KL, Miller KWP, Budowle B. Recommendations for consistent treatment of length variants in the human mitochondrial DNA control region. Forensic Sci Int 2002; 129: 35-42.

Wilson, MR., Allard, MW, Monson, KL, Miller KWP, Budowle B. Further discussion of the consistent treatment of length variants in the human mitochondrial DNA control region, vol. 4, no.4. Forensic Science Communications 2002.
http://www.fbi.gov/hq/lab/fsc/backissu/oct2002/index.htm.

ExoSAP-IT (PCR Product Clean-up)

Dugan, KA, Lawrence, HS, Hares, DR, Fisher, CL, Budowle, B. An improved method for post-PCR purification for mtDNA sequence analysis. J Forensic Sci 2002; 47:811-18.

Product Gel

Roche Diagnostics Corp. *DNA molecular weight marker XIV*. Package Insert, Version 3. November 2002. Cat. No. 1 721 933. Roche Diagnostics Corporation, Indianapolis, IN.

Roche Diagnostics Corp. *Linear Array Mitochondrial DNA HVI/HVII Region-Sequence Typing Kit.* Package Insert, Version 3. September 2003. Cat. No. 03 527 867 001.

Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 1989. Cold Spring Harbor, NY.

APPENDIX A – OLIGONUCLEOTIDE PRIMER SEQUENCES[1]

Region	Primer	Nucleotide (base) Sequence	Size (no. of bases)
HVI	A1	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	5'- CTC CAC CAT TAG CAC CCA A -3'	19
	HVIR	5'- ATT TCA CGG AGG ATG GTC -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.

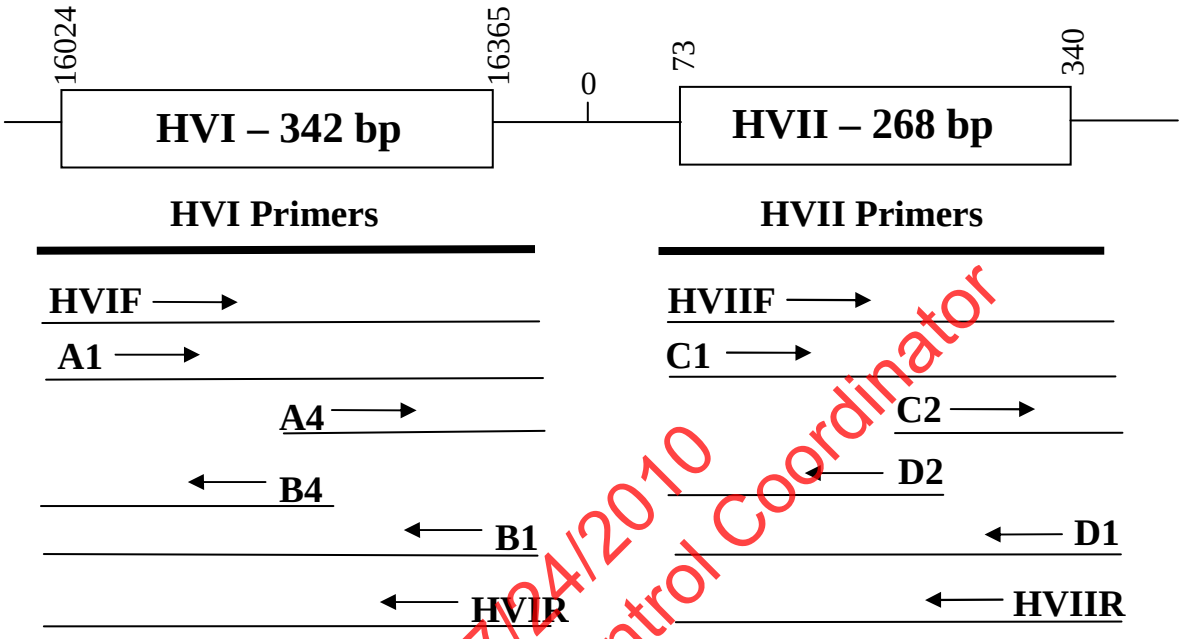
Nucleotide sequences for oligonucleotide primers HVIF, HVIR, HVIIF, HVIIR are from the product insert for the LINEAR ARRAY Mitochondrial DNA HVI/HVII Region-Sequence Typing Kit that is available from Roche Applied Sciences (Cat. No. 03-527-867-001; product information is available at www.roche-applied-science.com). 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.

Kline MC, Vallone PM, Redman JW, Duewer DL, Calloway CD, Butler JM. Mitochondrial DNA typing screens with control region and coding region SNPs. J Forensic Sci 2005; 50:377-385.

APPENDIX B – MITOCHONDRIAL DNA PRIMER LOCATIONS[4]

DATE EFFECTIVE	VERSION	PAGE
04-08-2005	2.0	1 OF 1



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.
[4] 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.

APPENDIX C – REVISED CAMBRIDGE REFERENCE SEQUENCE[1]

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)**

[1] Human Mitochondrial DNA Revised Cambridge Reference Sequence,
from http://www.gen.emory.edu/MITOMAP/mitoseq.html
LOCUS HUMMTCG 16568 bp ds-DNA Circular PRI 27-Sept-2001
DEFINITION- Human mitochondrion, complete genome.
ACCESSION- This sequence is a modified version of the 2001 Revised Cambridge Reference Sequence (GenBank #NC_001807, a derivation of #J01415)
SOURCE Human placenta mitochondrial DNA
ORGANISM Mitochondrion Homo sapiens- Eukaryota; Animalia; Chordata; Vertebrata; Mammalia; Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae.
REFRENCES

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome Nature 1981; 290: 457-465. MEDLINE- 81173052 PUBMED-7219534

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

APPENDIX D – DETAILED CYCSEQ SPREADSHEET CALCULATIONS

Example:

Sample	# of Gels	# of LA's*	LA* input vol. (ul)	Misc. vol. (ul)	Gel Value/4 ul	ExoSap-It (ul)	Template (ul)	Comment	Water (ul)
Amp. Neg	1	0	0	0	0	9.2	3.0	Control	7.8
Sample	1	1	5.4	1	56	7.9	4.3	1/10 dil.	6.5

* LA = linear array analysis, currently only used for quality control purposes

Calculations for Amplification Negative:

1. The amplification negative was run only once on a gel at the usual volume of 4 ul. The gel value was zero for that sample, and a linear array was not run. Therefore, the analyst enters 1 into the "# of Gels" field and 0 each into the "# of LA's", "LA input vol.", "Misc vol.", and "Gel value" fields.
2. The total remaining reaction volume is calculated by the spreadsheet to be 46 ul (50 ul starting volume minus 4 ul used for gel analysis).
3. The amount of ExoSAP-IT required is calculated by the spreadsheet according to the following guideline: 1 ul of ExoSAP-IT for every 5 ul 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 ul into the template field. The spreadsheet also enters "Control" into the comments field based on the 0 gel 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 ul. This DNA/water mixture is now ready to be added to the cycle sequencing reaction.

Calculations for Sample:

1. In this example, in addition to the first gel another gel was run using only 1 ul of amplified sample. This was done since the result of the first gel was off-scale relative to the upper molecular mass gel standard. Also, 5.4 ul 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 ul. The user also inputs 1 into the "# of LA's" field along with the volume of 5.4 (ul) used for the Linear Array analysis into the "LA input vol." field. To account for the additional 1 ul that was run on the second gel, this volume is inputted into the "Misc. Vol." field. Finally, the user inputs the gel value into the "Gel Value" field. This field is based on the amount of sample DNA in a volume of 4 ul. In this case, the gel rerun yielded a value of 14 ng/ul. This value is then corrected by the user and multiplied by 4 to yield the final value of 56.
2. The total reaction volume is calculated by the spreadsheet to be 39.6 ul, which is equal to the starting volume minus 4 ul for the first gel, 5.4 ul used for the Linear Array, and 1 ul that was used for the second gel (50 - 4 - 5.4 - 1 = 39.6).
3. The amount of ExoSAP-IT required is then determined as before. The spreadsheet calculates that the reaction requires 7.9 ul 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 ul)(39.6 ul)] by the new volume (47.5 ul). The new concentration (11.67 ng/ul) is then used to calculate the volume of sample needed to equal 5 ng of sample DNA [(5 ng)/(11.67 ng/ul) = 0.428 ul]. If the final volume is less than 1 ul, 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 ul of sample volume for the next step of cycle sequencing.