Mito Cycle Sequencing, Cleanup, and 3130xl Sequencing		
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1 Overview

1.1 Cycle Sequencing: 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 base chain terminators. The Applied Biosystems Big Dye Terminator Cycle Sequencing Kit is used.

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

1.3 Centri-Sep Sample Filtration: Prior to sample electrophoresis, sequenced products must be purified to remove unincorporated dye terminators.

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

2 Test Batch Creation

2.1 Refer to the Forensic Biology LIMS Process Manual for general guidelines within the LIMS.

2.2 During the test batch setup, the Cycle Sequencing Amplification Negative (CAN) control will automatically be added to the test batch. If it is not needed, delete it from the test batch page prior to filling out the Data Entry section for the input samples.

2.3 Fill out the Data Entry section for the input samples as follows:

2.3.1 Enter the number of primer specific reactions needed for each sample in the "# of Samples" column. This number is usually 8 for routine sequencing, however, it may be (i) more if confirmatory primers are being used or (ii) less when samples are being re-cycle sequenced.

2.3.2 Enter the sample concentration in the "Conc. MtDNA Calc" column. Copy by retyping this information from the "Concentration" column located in the same table. Enter a value of "0" into the "Conc. MtDNA Calc" column for the CAN control sample, if present.

2.3.3 Enter the sample dilution from the drop-down menu of 1, 0.1, or 0.01 in the "Dilution" column.

2.3.3.1 A dilution value of "1" will be appropriate for most samples that are being sequenced with at least several primers. If the resulting calculated DNA sample volume is less than 1 uL, recalculate using a dilution of 0.1.

2.3.4 Alternatively, use the table below for selecting an appropriate dilution value.

Sample Concentration	Dilution
6.7-67 ng/4 uL	**1 (Neat)**
67-670 ng/4 uL	**0.1**
670-6,700 ng/4 uL	**0.01**

Note: The target amount of amplified product for cycle sequencing is 5 ng in 3 uL which is equivalent to 6.7 ng/4 uL. Samples with less than 5 ng of amplified product in 3 uL (< 6.7 ng/4 uL) may be cycle-sequenced using 3 uL of the sample. In these situations, the sample concentration will need to be adjusted to 6.7 ng/4 uL for the calculated sample volume to be equal to 3 uL. Obtain permission from a supervisor to proceed.

2.4 Click [Save] and the table will populate the total sample and water volumes needed to create a n+1 master mix where n is the total number of primer-specific reactions being run for a given sample.

2.4.1 When the data entry section for the input samples is completed, select "All" in the dropdown and click the Release icon and then click [Save].

2.4.2 When generating output samples, select the appropriate output sample template from the Output Sample List ID as follows:

2.4.2.1 Choose "MitoCycSeqPrimers" for primers with no prefix (initial cycle sequencing runs).

2.4.2.2 Choose "MitoCycSeqConfPrimer" for primers with the "conf" prefix (confirmatory primer runs).

2.4.2.3 Choose "MitoCycSeqRecycPrimer" for primers with "recyc" prefix (re-cycle sequencing primer runs).

2.4.3 Remove any primers from the Reagents tab in LIMS that are not needed for cycle sequencing. This is done by selecting all unused primers and clicking on the [Remove Primers] box shown below the reagents table. Click [Save] when done.

3 Procedure

3.1 Fill in the Cycle Sequencing Performed By tab in LIMS. In the LIMS plate load screen, download the plate to the instrument by clicking [Download Instrument].

3.2 Print out the mtDNA Cycle Sequencing Setup Sheet for the test batch from the "Choose Report" tab in the Test Batch List tram stop. Use the Cycle Sequencing Setup Sheet for the amounts of reagents needed for each primer master mix and the amounts of reagents needed for each sample DNA master mix.

3.3 Obtain and label 1.5 mL microcentrifuge tubes for your primers and samples. Obtain the necessary primers and reagents and record the lot numbers in LIMS. If the TE^{-4} reagent is not needed for the run (e.g. none of the samples requires a dilution), leave this reagent field blank.

3.4 **Tube Setup WITNESS:** Have a witness verify the input amp tube labels, and the output tube labels and associated primers used according to the load plate screen in LIMS. For the input amp tube labels, the analyst must read the ***entire*** amp tube label.

3.5 If necessary, prepare sample dilutions with TE^{-4} based on the amount of DNA needed as indicated on the Cycle Sequencing Setup Sheet (this information is also listed in the input sample data entry section in LIMS). Then in a separate tube, prepare the sample master mix using the amounts indicated on the Cycle Sequencing Setup Sheet for the volume of sample and the volume of water needed.

3.6 The formula for each ***sample DNA master mix*** is:

For (N+1) samples:

- *X* uL x (N+1) mtDNA sample DNA, where *X* is the amount of mtDNA needed per sample as calculated by the LIMS
- *Y* uL x (N+1) Water, where *Y* is the amount of water needed per sample as calculated by the LIMS

10.8 uL of sample master mix will be added to each sample

3.7 Each Primer Mix contains three components and needs to be prepared separately. Prepare a Master Mix for each primer by adding Big Dye, sequencing buffer and the chosen primer, using the amounts listed on the mtDNA Cycle Sequencing Setup Sheet.

3.8 The formula for each ***primer master mix*** is:

For (N+2) samples:

- 4 uL x (N+2) Big Dye Terminator Ready Reaction Mix
- 2 uL x (N+2) Sequencing Buffer
- 3.2 uL x (N+2) primer (1uM concentration)

9.2 uL of primer master mix will be added to each sample.

3.9 Vortex and briefly spin all primers prior to plate loading.

3.10 Obtain a 96-well plate, label with the run name, and load as follows:

3.10.1 Add 10.8 µL of the appropriate sample master mix to its wells (according to the plate set-up in the LIMS).

3.10.2 Add 9.2 uL of the appropriate primer master mix to its wells (according to the plate set-up in the LIMS).

3.10.3 Use 8-strip caps to seal each row of wells as they are filled.

3.11 Vortex the plate and shake in a plate shaker at 1000 rpm for 1-2 min. and spin down plate in a centrifuge at 1000 rpm for 1 min.

3.12 Place sample plate on Thermal Cycler using 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

3.13 Record the instrument usage in LIMS. Enter the plate name as the Purpose and “bdt cyc seq” as the Program.

4 Instructions for re-cycle sequencing:

4.1 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.

4.2 A new cycle sequencing amplification negative (CAN) control will be set up for each primer used in re-cycle sequencing; this will serve as a negative control for each respective primer that is used. Each CAN control consists of 10.8 uL of water in addition to 9.2 uL of primer reaction mix.

4.3 A positive control will be set up for each primer used in re-cycle sequencing to report on the integrity of the reaction.

4.4 Samples can be re-cycle sequenced with more (-recych) or less (-recycl) input DNA if necessary. Based on validation, up to 90ng of DNA can be used for recych. If recych sample volume would be more than 3 µL, similar conditions must be used for the associated negative controls. For each of these scenarios, speak with a supervisor before proceeding.

5 SDS Cleanup

5.1 Notes:

5.1.1 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.

5.1.2 If necessary, transfer 1 mL of 2% SDS to a 1.5 mL microcentrifuge tube and place the tube into a thermomixer set at 37ºC to ensure the solution is at least at room temperature.

5.1.3 Allow the cycle-sequenced DNA to equilibrate to room temperature before adding SDS.

5.1.4 If necessary, place the plate containing your samples on a thermocycler set to 37ºC for 5-10 minutes to ensure that the samples are at least at room temperature.

5.2 Fill in the Clean Up Performed By tab in LIMS.

5.3 Retrieve the SDS and record the lot number in LIMS.

5.4 Using a multi-channel pipette, add 2 µL of 2% SDS to each well of cycle-sequenced DNA in a 96-well plate.

5.5 Use new 8-strip caps to re-seal each row after the SDS has been added.

5.6 Vortex the plate in a plate shaker at 1000 rpm for 1 minute and spin down the plate(s) in a centrifuge.

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

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

5.8 Record the instrument usage in LIMS. Enter the plate name for the Purpose and "SDS" for the Program.

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

6 Centri-Sep Sample Filtration

6.1 ****It is important that the Centri-Sep Strips are at room temperature before being used for filtration**.

6.2 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. Record the lot number in LIMS.

6.3 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.

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

6.5 Arrange the newly drained strips on a new 96-well plate, labeled with the plate name, date, and analyst's initials.

6.6 Add the amplified sample to each column, taking care not to touch the gel with the pipet tip.

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

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

6.9 Evaporate the samples in the 96-well plate at 75 °C in a thermal cycler with the lid open. Evaporation may take 1-2 hours.

6.9.1 The plate should not evaporate for more than 3 hours.

6.10 Record the instrument usage in LIMS. Enter the plate name for the Purpose and "evaporate" for the Program.

6.11 If the samples are not going to be loaded immediately, they should be stored as dried pellets at 4 °C for no longer than 7 days. When ready, proceed to 3130*xl* setup.

7 ABI 3130xl Sequencing

7.1 Setting up a 3130*xl* Run

7.1.1 Turn on the computer. Make sure the 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).

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

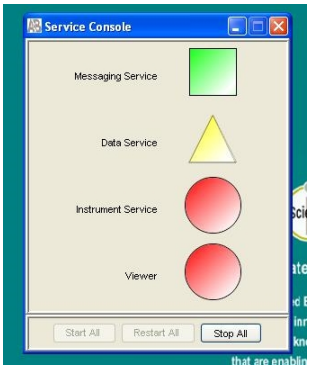
E:\Applied Biosystems\UDC\data collection\data\ga3130xl*Instrumentname*

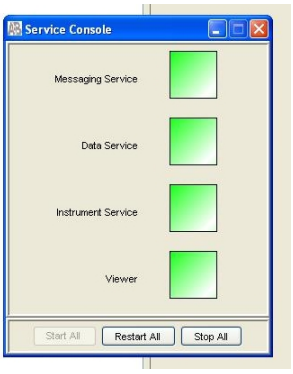
7.1.3 Once there, create a master file using the following format:

7.1.4 "*InstrumentnameYear-Run Number* Files" (e.g. Batman14-015 Files) within the appropriate archive folder (e.g. Batman 2014). Move the 3130xl mtDNA files from the LIMS fileshare (L:\FB\LIMS to Instrument\3130\[Instrument Name] into the master folder just created.

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

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

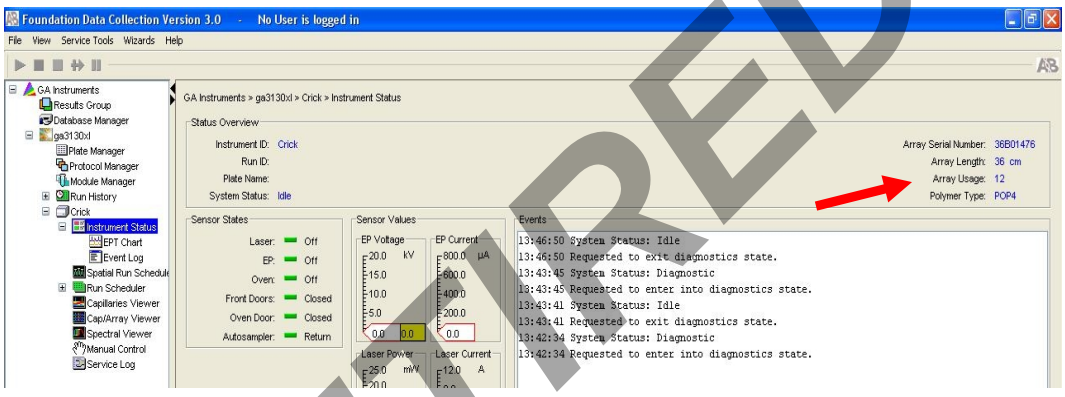




7.1.5.2 This process could take several minutes. The Service Console must not be closed or it will shut down the application.

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

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



7.1.7 Check the LIMS to see when the POP6 was last changed. If it is >7 days, proceed with POP6 change (See QC135 Water Wash and POP Change Procedure).

7.1.8 Check the level of POP6 in the bottle to ensure there is enough for your run (approximately 600 uL is needed per injection). If there is not, proceed with POP6 change (See QC135 Water Wash and POP Change Procedure).

7.1.9 If you are the first run the instrument that day, proceed with step 7.1.10 . If a run has already been performed on the instrument that day, skip to **Section** 8 Creating a Plate ID**.**

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

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

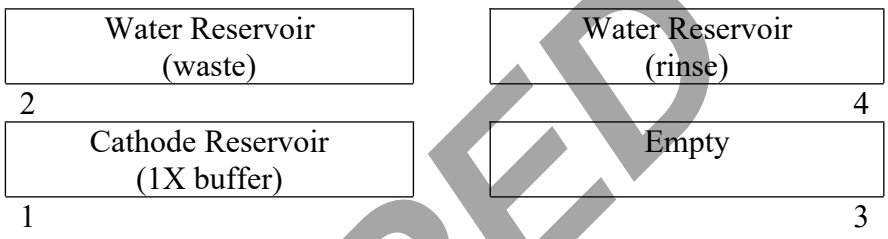
7.1.12 Remove the three plastic reservoirs from the sample tray and anode jar from the base of the lower pump block and dispose of the fluids.

7.1.13 Rinse and fill the "water" and "waste" reservoirs to the line with sterile deionized water.

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

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

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



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

7.1.18 Close the instrument doors.

8 Creating a Plate ID

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

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

8.3 If the text file is not immediately apparent, find it by going to My Computer → E drive → Applied Biosystems → UDC → data collection → data → ga3130xl → [Instrument Name].

8.4 Click on **OK.**

8.5 Preparing the DNA Samples for Sequencing

8.6 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.

8.7 Remove the Hi-Di formamide from the freezer and allow it to thaw. Record the lot number in LIMS.

8.8 Using the multi-channel pipette if desired, add 10 uL of formamide to each dried sample and mix to bring the sample into solution. Be sure to fill any unused wells that are part of an injection set (eg. containing <16 samples) with at least 10 uL of Hi-Di formamide.

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

8.9 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.

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

8.11 Prepare thermal cyclers for snap de-chill step. Set one thermal cycler to 95°C (denature program) and one thermal cycler to 4°C (chill program). Denature samples at 95°C for 2 minutes followed by a quick chill at 4°C for 5 minutes. Centrifuge the tray for one minute after the snap de-chill.

8.12 While plates are denature/chilling, set up the 3130 for run.

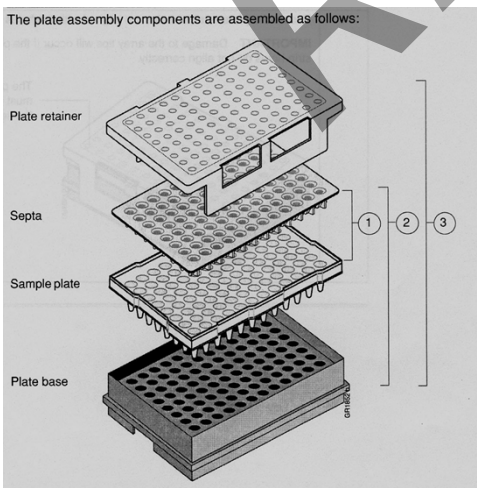
8.12.1 Turn on oven and set for 50◦

8.12.1.1 Manual Control → Send Defined Command For: click on Oven.

8.12.1.2 Command Name click on Turn On/Off oven → Send Command

8.12.1.3 Command Name click on Set Oven Temperature →50→Send Command

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



IMPORTANT:

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

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

9 Placing the Plate onto the Autosampler (Linking and Unlinking Plate)

9.1 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.

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

9.3 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.

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

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

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

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

10 Linking/Unlinking the Plate record to Plate

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

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

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

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

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

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

11 Viewing Run Schedule and Starting Run

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

11.2 The RunID column indicates the folder number(s) associated with each injection in your run (e.g. *Batman-2008-0114-1600-0197)*. The folder number(s) and the run ID should be recorded in the LIMS.

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

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

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

11.5.1 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.

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

11.6 The visible setting should be:

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

11.7 Expected values are: EP current constant around 40-60 µA starting current

EP current constant around 70-80 µA running current
Laser current: 5.0 A ± 1.0 A

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

11.9 Record the run folder start and run folder end in the QC Batch Params in LIMS.