

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

Mitochondrial DNA Sequencing Analysis		
Status: Published		Document ID: 1176
DATE EFFECTIVE 09/14/2017	APPROVED BY mtDNA Technical Leader	PAGE 1 OF 8

Mitochondrial DNA Sequencing Analysis

1 Purpose

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

2 Procedure

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

3 Transfer of the 3130xl run data into the master file

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

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

- 3.2 Once there, identify the injection folders of the runs you wish to analyze.
- 3.3 Copy these injection folders into the master file (e.g. Batman08-015 files) that was created earlier making sure that all of the run statistic files and log files are included.
- 3.4 Also copy the newly created run files into the respective archive files in the MITO DATA directory.

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

Mitochondrial DNA Sequencing Analysis		
Status: Published		Document ID: 1176
DATE EFFECTIVE 09/14/2017	APPROVED BY mtDNA Technical Leader	PAGE 2 OF 8

4 Sequence Analysis

- 4.1 Open the Sequence Analysis program by double clicking on the icon. Login using your username and password.
- 4.2 Click on the import samples icon in the upper left of the screen, or go to **Add Sample(s)** under the File menu.
- 4.3 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.
- 4.4 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.
- 4.5 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.
- 4.6 The **Analysis Status** window will indicate the progress of analyzing the samples. As samples are analyzed, the **BC** column will display a green, blue or yellow box around the check box indicating the quality of the base calling:

Green:	Indicates a successful base calling for that sample
Blue:	Indicates a problem in base calling the data for that sample
Yellow:	Indicates that there is no data to be analyzed for that sample
- 4.7 If the sequencing analysis is successful, click the yellow floppy-disk icon in the top left of the screen to **Save All Samples**, or click on **Save All Samples** under the File menu.

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

Mitochondrial DNA Sequencing Analysis		
Status: Published		Document ID: 1176
DATE EFFECTIVE 09/14/2017	APPROVED BY mtDNA Technical Leader	PAGE 3 OF 8

- 4.8 Print the analysis report page. Click on the **Analysis Report** button at the top of the page (white button with ^AR; A is in red and R in blue), or select **Analysis Report** under the Analysis menu at the top of the screen. When the report page opens, right click on the column headings for the analysis report. Deselect the following options- *PP Status, Peak 1, Base Spacing, # Low QV, # Med QV, # High QV, Sample Score, CR Start, and CR Stop*. The visible column headings will show the following- **BC Status, Well, Cap #, LOR, 'A' S/N, 'C' S/N, 'G' S/N, 'T' S/N, Avg S/N**. To ensure that all sample identification fits onto one row, deselect "Fit Columns to Window" (lower left of screen), select size "8" font, and increase the width of "Sample File Name" column.
- 4.9 Create a PDF file of the Analysis Report by clicking **Print** and send the file to **Adobe PDF**. Be sure the page set-up is set to portrait before creating the PDF file. Check the PDF file to make sure that the complete Sample Names and Sample Descriptions are present (e.g., not cut off). If necessary, make formatting adjustments, resend Adobe PDF file, and recheck. Following the successful creation of the PDF file, save it in the run folder (e.g., save in *B08-040* with run name *B08-040 Analysis Report*) that is contained within the Analyzed Archive.
- 4.10 Attach the PDF Analysis Report to the analysis test batch in the LIMS.
- 4.11 Click **Exit** under the File menu to close out the Sequencing Analysis program.

5 Sequencher

- 5.1 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 not allow you to save the resulting file.
- 5.2 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 create contigs are set to the proper settings. Click on the box in the upper left-hand corner that is marked "Assembly Parameters". The following settings should be selected:

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

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

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

<h3>Mitochondrial DNA Sequencing Analysis</h3>		
Status: Published		Document ID: 1176
DATE EFFECTIVE 09/14/2017	APPROVED BY mtDNA Technical Leader	PAGE 4 OF 8

- 5.4 Find the sequence files that were copied to the Analyzed Archive. To simplify, under File of Type select “With Chromatogram Sequences.” To select all of the files press and hold the Shift key, and click on the last file. Once the files are selected, click **Open**. Maximize the analysis window to view all of the samples and sample data.
- 5.5 Import the appropriate reference sequence into the project for every contig that needs to be built. The reference files (HVI.spf or HVII.spf) are located on the Forensic Biology network (Mito Data/Reference Seqs).
 - 5.5.1 These files can be saved to the desktop or local hard drive for easier access.
- 5.6 Holding down the Shift or the Control key, click on imported rCRS file, and the forward and reverse sequence files that will make up the contig.
- 5.7 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)
- 5.8 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**.
- 5.9 Double click on the Contig icon. When the contig diagram window opens, click on the **Bases** button at the top left of the screen.
 - 5.9.1 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.
 - 5.9.2 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.
- 5.10 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.**

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

Mitochondrial DNA Sequencing Analysis		
Status: Published		Document ID: 1176
DATE EFFECTIVE 09/14/2017	APPROVED BY mtDNA Technical Leader	PAGE 5 OF 8

- 5.11 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**.
- 5.12 **IMPORTANT**- In the instances where length and/or sequence heteroplasmy is present and yields single-stranded DNA regions and/or ambiguous bases, or un-editable N calls are present, the consensus setting on the Sequencher program must be set to Plurality mode to analyze that particular sample.
- 5.13 **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:

<u>Standard Codes</u>	<u>IUPAC Codes *</u>
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- <i>Ambiguous</i>	S- C or G
	W- A or T

*See Nomenclature section of this manual for further discussion.

- 5.14 **To delete a base**, click on the base in question and do one of the following:
- 5.14.1 To have the bases fill in from the left side of the strand, press the delete key.
- 5.14.2 To have the bases fill in from the right side of the strand, press the **backspace** key, and follow the on-screen instructions.
- 5.15 **To insert a base**, press the **Tab** key and follow the on-screen instructions.
- 5.16 **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.
- 5.17 **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.
- 5.18 **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.

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

Mitochondrial DNA Sequencing Analysis		
Status: Published		Document ID: 1176
DATE EFFECTIVE 09/14/2017	APPROVED BY mtDNA Technical Leader	PAGE 6 OF 8

- 5.19 All edits should be documented on the mtDNA Sequence Editing Worksheet. **To review edits press and hold Ctrl E.**
- 5.20 When the sequencing analysis and editing are completed, the contigs that were built need to be archived in the appropriate folders. To archive contigs, highlight contigs that are to be archived together, go to file → export → selection as subproject. Click on Browse button and select folder for contig to be archived. Make sure that the format is set to ‘Sequencher Project’. Click the Export button. Enter project name, e.g. Amp_Pos_ddmmyyyy.hhmmss for positive control contigs and FBYY-12345 for sample contigs.
- 5.20.1 Positive Control contigs should be archived as follows: M:/MITO_DATA/PC Archive/year/PCmmdyy-hhmm.
- 5.20.2 Samples contigs should be archived as follows: M:/MITO_DATA/Project Archives/sample type/FBYY-12345. Sample type should be ‘Casework’ for evidence and associated exemplars and should be ‘Missing Person cases’ for all Missing person samples.
- **From this point forward, the saved contigs can be further modified with additional primer runs, edited, and resaved as outlined above.**
 - **The following procedures should only be done once the analysis of the contig is complete and the contig is ready for review.**
- 5.21 Once contigs have been exported and archived, open appropriate project, go to the **Contig** menu and click on **Compare Consensus to Reference**. If necessary, widen the contig name column to view the entire contig name. To print, select **Reports, Entire Table, Open Report, and Print**. Select **portrait** as the orientation, and print.
- 5.22 At the top of the sequence comparison page, click on the **Summary** button. When the **Summary View** window opens, click on **File, Print Setup**, and select “landscape.” Then click on the **Ruler** button at the top of the page and adjust the margins (triangles on ruler) and adjust column spacing as needed to print entire sample ID.
- 5.23 Print the **Summary** page. To do this, select the **File** menu and select **Print**.
- 5.24 Click on the **Overview** button.
- 5.25 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.

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

Mitochondrial DNA Sequencing Analysis		
Status: Published		Document ID: 1176
DATE EFFECTIVE 09/14/2017	APPROVED BY mtDNA Technical Leader	PAGE 7 OF 8

- 5.26 Print out the **Contig Diagram**. Under the **File** menu, select **Print Setup**. Select **landscape** as the orientation, and print (select printer from the drop-down list). Click **OK**. Under the **File** menu, select **Print**. Confirm that the appropriate printer is selected and click **Print**.
- 5.27 The final step of Sequencher analysis is to prepare the export data file from Sequencher for the LIMS. This will allow the contig data to import into the contig sample within the LIMS. To do this, open the contig and select “Compare Consensus to Reference” from the Contig menu. Click on the “Reports” button at the top of the screen. Select the radio button for “Entire Table” and from the drop down menu, select the report format as “Variance Table Report.” Save the variance table as a text file to the appropriate network location and import into the LIMS for the appropriate contig.
- 5.27.1 *Note:* this is only necessary if samples are being sent to CODIS through the LIMS.
- 5.28 After the import is completed in the LIMS, and the contig data is saved, the analyst can run the reports for the mtDNA Run Review and Positive Control Reviews, and print these reports as necessary for review.

6 Run Review Construction

- 6.1 Arrange the paperwork in following order, from bottom to top:
- Sequencher Chromatogram printouts (if necessary)- *landscape*
 - Contig Diagram- *landscape*
 - Summary View- *landscape*
 - Editing Sheet
 - Compare Consensus to Reference- *portrait*
 - Run/re-run review sheets (to be included for each run of samples)
- 6.2 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.

7 Data Review

- 7.1 Once all of the Sequencher data has been completed and the file have been archived, pass the entire set of sample printouts for one 3130 run to another IA for data review.
- 7.2 For the IA performing the review, the following steps must be performed.
- 7.2.1 Open the respective file from the appropriate archive located in the MITO_DATA directory. Review all sequencher files.

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

Mitochondrial DNA Sequencing Analysis		
Status: Published		Document ID: 1176
DATE EFFECTIVE 09/14/2017	APPROVED BY mtDNA Technical Leader	PAGE 8 OF 8

- 7.2.2 For each sample, including Positive Control(s), ensure that all edits reflected in the editing documentation are valid and accounted for within the data set.
 - 7.2.3 For positive controls, ensure that the proper type is displayed on the Compare Consensus to Reference page.
 - 7.2.4 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.
 - 7.2.5 Ensure for every sample that the documentation is assembled in order and reflects the entire project and sample names.
- 7.3 If a problem is found, mark the occurrence within the documentation and return the documentation to the original analyst.

8 Archiving the Sequencer Data

- 8.1 Data will be archived by scanning the Sequencer print outs into LIMS as PDF files and saving the Sequencer electronic data in the MITO_DATA directory on the M drive.