PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

	MITOCHONDRIAL DNA SEQUENCING ANALYSIS	
DATE EFFECTIVE 06-20-2016	APPROVED BY MITOCHONDRIAL DNA TECHNICAL LEADER	PAGE 1 OF 12

Mitochondrial DNA Sequencing Analysis

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

PROCEDURE:

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

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

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

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

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

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

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

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

	MITOCHONDRIAL DNA SEQUENCING ANALYSIS	
DATE EFFECTIVE 06-20-2016	APPROVED BY MITOCHONDRIAL DNA TECHNICAL LEADER	PAGE 2 OF 12

B.   Sequence Analysis

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

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

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

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

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

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

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

8. Print the analysis report page. Click on the **Analysis Report** button at the top of the page (white button with ^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, Sample Score, CR Start, and CR Stop*. The visible column headings will show the following- **BC Status, Well, Cap #, LOR, 'A' S/N, 'C' S/N, 'G' S/N, 'T' S/N, Avg S/N**. To ensure that all sample identification fits onto one row, deselect "Fit Columns to Window" (lower left of screen), select size "8" font, and increase the width of "Sample File Name" column.

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

10. Attach the PDF Analysis Report to the LIMS for the cycle-sequencing batch. Close the PDF Analysis Report and the Analysis Report screen.

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

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

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

	MITOCHONDRIAL DNA SEQUENCING ANALYSIS	
DATE EFFECTIVE 06-20-2016	APPROVED BY MITOCHONDRIAL DNA TECHNICAL LEADER	PAGE 4 OF 12

C.   Sequencher

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

1.   Open Sequencher. Under the **Contig** menu, select **Consensus to Forensic Standards**. This only needs to be done after opening Sequencher for the first time. Once set, it will remain until the program is closed. Also, check to make sure that the Assembly parameters used to 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)

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

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

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

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

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

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

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

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

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

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

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

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

    <u>IMPORTANT</u>- In the instances where length and/or sequence heteroplasmy is present and yields single-stranded DNA regions and/or ambiguous bases, or un-

editable N calls are present, the consensus setting on the Sequencher program must be set to Plurality mode to analyze that particular sample.

11. Within the LIMS, create the appropriate contig samples, and indicate which primer samples are associated with each appropriate contig. All edits, trims, and modifications to the contig sequence must be recorded within the LIMS documentation.

12. **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- *Ambiguous*   S- C or G

                              W- A or T

*See Nomenclature section of this manual for further discussion.

13. **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.

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

15. **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.

16. **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.

17. **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.

18.   **To review edits press and hold Ctrl E.**

19.   When the sequencing analysis and editing are completed, the contigs that were built need to be archived in the appropriate folders. To archive contigs, hightlight contigs that are to be archived together, go to file → export → selection as subproject. Click on Browse button and select folder for contig to be archived. Make sure that the format is set to 'Sequencher Project'. Click the Export button. Enter project name, e.g. PCddmmyy-hhmm for positive control contigs and FBXX-12345 for sample contigs.

- Positive Control contigs should be archived as follows: M:/MITO_DATA/PC Archive/*year/PCmmddyy-hhmm.*
- Samples contigs should be archived as follows: M:/MITO_DATA/Project Archives/*sample type/FBXX-12345*. Sample type should be 'Casework' for evidence and associated exemplars and should be 'Missing Person cases' for all Missing person samples.

      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.

20.   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.

21.   Print the **Compare Consensus to Reference** page again, but this time send the file to **Adobe PDF**. Click **Print.** Save the PDF file in the master run file**,** named as the sample name eg. "FB05-0005m hair 1 HVI" Close the Adobe window. Close the Difference Review window. Attach this PDF to the LIMS system within the contig sample.

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.

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

	MITOCHONDRIAL DNA SEQUENCING ANALYSIS	
DATE EFFECTIVE 06-20-2016	APPROVED BY MITOCHONDRIAL DNA TECHNICAL LEADER	PAGE 8 OF 12

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

24. 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. Attach this PDF to the LIMS within the contig sample.

25. Click on the **Overview** button. In the Contig Diagram, double click on the individual sequence files. The sequence window of the file will open. Click on the **Show Chromatogram** button at the top of the screen. A new window will open. In the upper left corner is a slider bar, the four-color bases and two buttons: a dot and a vertical bar. **DO NOT CLICK THE VERTICAL BAR, AS THIS WILL ERASE THE HEADER FROM THE PRINTED PAGE.**
    Print this chromatogram as edited data, trimmed to the hypervariable region of interest. To do this, select the **File** menu and select **Print Setup**. Select **landscape** as the orientation, and print.

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

    Repeat step 25 for every separate chromatogram file.

26. 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

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

	MITOCHONDRIAL DNA SEQUENCING ANALYSIS	
DATE EFFECTIVE 06-20-2016	APPROVED BY MITOCHONDRIAL DNA TECHNICAL LEADER	PAGE 9 OF 12

master run file**,** named as the sample name plus the primer, e.g. "FB05-0005m hair 1 HVI A4." Close the Adobe window. Attach this PDF to the LIMS within the contig sample.

Repeat step 26 for every separate chromatogram file.

27. Click on the **Overview** button.

28. 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.

29. 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.**

30. 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. Attach this PDF to the LIMS within the contig sample.

31. 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.

32. 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.

D. File Output and Construction

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

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

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

E. Data Review

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

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

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

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

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

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

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

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

	MITOCHONDRIAL DNA SEQUENCING ANALYSIS	
DATE EFFECTIVE 06-20-2016	APPROVED BY MITOCHONDRIAL DNA TECHNICAL LEADER	PAGE 11 OF 12

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

F.   Archiving the Sequencer Data

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

ARCHIVED IN **BINDERS**:

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

PC Binder
        For each positive control:
        Positive control review report
        Contig diagrams, original
        Sequence summaries, original
        Editing reports, original
        Difference review sheets, original

FB **CASEFILES**:

        3130xl report, copy
        Run review report(s), copy
        Positive control review report, copy
        Positive control editing report, copy
        FB sample contigs, originals
        Contig diagrams
        Sequence summaries
        Editing reports

Difference review sheets

ARCHIVED IN **ELECTRONIC FILES**:

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

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

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

> **NOTE: If a positive CTR is sequenced twice (e.g., during QC tests), the name will stay the same as in PCmmddyy-hhmm but a lettered suffix will be added after each new sequencing as in PC-mmddyy-hhmm-A, PC-mmddyy-hhmm-B….**

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