Cycle-Sequencing		
Status: Published		Document ID: 1160
DATE EFFECTIVE 09/14/2017	APPROVED BY mtDNA Technical Leader	PAGE 1 OF 3

Cycle-Sequencing

1 Purpose

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

2 Procedure

2.1.1 The cycle sequencing reactions are done in a 96-well plate. Prepare the samples and reagents needed for cycle sequencing and be witnessed according to the sample names and order listed on the Cycle Sequencing documentation.

2.1.2 The amount of template DNA and water needed for each sample is calculated by the LIMS system. This calculation takes into account the total volume and concentration of amplified product present in the sample tube following the ExoSAP-IT procedure.

- The target amount for cycle sequencing is 5 ng of amplified product.
- The following formula is then used to create each sample for cycle sequencing:

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

- Samples with less than 5 ng of amplified product in 3 uL may be cycle-sequenced using 3 uL of the sample.

2.1.3 If a dilution of template DNA is necessary, it will be indicated on the cycle sequencing documentation as "*x* @ 1/10th" where *x* is the input volume. Any sample requiring dilution should be diluted in TE^{-4}. If no dilution is necessary, the notation will be "neat." The amount of water sufficient to make 20 uL reaction volume is then calculated.

2.1.4 If a sample is a negative control, the mtDNA concentration of zero will result in a default template input of 3 uL (per reaction), with the notation of "control" attached to the sample.

2.1.5 The documentation cannot indicate dilution factors greater than 1/10. For situations where the amount of DNA indicated is less than 1 uL @1/10 dilution, calculate the volume of extract required @1/100 dilution (multiplication by 10), and use this volume of a 1/100 dilution. Also calculate, by subtraction, the correct volume of water to add to the reaction. Note the correct aliquots on the documentation as a deviation of the procedure.

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

For (N+2) samples, add:

- 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)

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

For N samples, add:

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

3 Cycle-Sequencing

3.1 Print out mtDNA Cycle Sequencing Setup Sheet.

3.2 Obtain and label 1.5 mL microcentrifuge tubes for your primers and samples.

3.3 **Tube Setup WITNESS:** Have a witness verify the input PCR tube labels (corresponding to the tube label and amp tube label in LIMS) and the output tube labels and associated primers used according to the sample plate loading order.

3.4 Prepare sample dilutions as necessary with TE^{-4} based on the amount of DNA needed as listed on the mtDNA Cycle Sequencing Setup Sheet.

3.5 Each Primer Mix contains three components and has to be prepared separately. Prepare a Master Mix for each primer by adding Big Dye, Sequencing Buffer and the chosen Primer in the amounts listed on the mtDNA Cycle Sequencing Setup Sheet.

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

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

3.7.1 Add 10.6 µL – 10.8 µL of appropriate sample to its wells (according to plate set-up in LIMS); be sure to include all controls for each primer to be used.

3.7.2 Add 9.2 uL of appropriate primer to its wells (according to plate set-up in LIMS).

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

3.8 Vortex the plate and spin in the Eppendorf 5810 on program 1 (1000 rpm for 1 min).

3.9 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

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 control for each primer used in re-cycle sequencing to account for the cycle sequencing reagent.**

4.3 **A positive control, 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.