Cycle-Sequencing		
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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 base 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 in the LIMS.

2.1.2 The amount of template DNA and water needed for each sample is calculated by the LIMS. 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 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 of reaction volume is then calculated.

2.1.4 If a sample is an amplification or extraction negative control, the mtDNA concentration of zero will result in a default template input of 3 uL per.

2.2 Create a ***master mix for each primer*** using 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)
- 9.2 uL of primer master mix will be added to each sample.

2.3 Create a ***master mix for each sample DNA*** using the following formula:

For (N+1) samples, add:

- *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 Cycle-Sequencing

3.1 Print out the test batch-specific mtDNA Cycle Sequencing Setup Sheet. **Note: Use the Cycle Sequencing Setup Sheet only for the amounts of reagents needed for each primer master mix.**

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 the 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 in the input sample data entry section in LIMS after entering the following mandatory fields: number of samples, concentration of mtDNA, and dilution. Then in a separate tube, prepare the sample master mix as described above guiding on the amounts indicated in the input sample data entry section in LIMS.

3.5 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 in the amounts listed on the mtDNA Cycle Sequencing Setup Sheet as described above.

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

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

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

3.8 Vortex the plate and shake in a plate shaker at 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 (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.