CYCLE-SEQUENCING		
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Cycle-Sequencing

PURPOSE: 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.

PROCEDURE:

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

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

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. If no dilution is necessary, the the notation will be "neat." The amount of water sufficient to make 20µL l reaction volume is then calculated. If a sample is a negative control sample, the mtDNA concentration of zero will result in a default template input of 3µL, with the notation of "control"attached to the sample The documentation cannot indicate dilution factors greater than 1/10. For situations where the amount of DNA indicated is less than 1µL @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.

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

   a. For (N+2) samples, add:
   - 4 µLx (N+2) Big Dye Terminator Ready Reaction Mix
   - 2 µLx (N+2) Sequencing Buffer
   - 3.2 µLx (N+2) primer (1uM concentration)

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

   b. For N samples, add:
   - X µLx (N) mtDNA sample DNA, where X is the amount of mtDNA needed as calculated by the system.
   - Y µLx (N) Water, where Y is the amount of water needed as calculated by the system

   Note: The calculations for the two master mixes mentioned above are done by the LIMS system. They are located by clicking on the "Reagents" tab.

   c. Include all controls for each primer that is used for a sample. 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.

   d. **The re-cycle sequencing step requires the following:**

   - **A new cycle sequencing amplification negative control for each primer used in re-cycle sequencing to account for the cycle sequencing reagent.**
   - **A positive control, for each primer used in re-cycle sequencing to report on the integrity of the reaction.**
   - 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 3µL, see supervisor.

5. Use 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 ^{O}C for 1 minute 25 cycles: - Denature 96 ^{O}C for 15 seconds - Anneal at 50 ^{O}C for 1 seconds - Extend at 60 ^{O}C for 1 minutes Storage soak at 4 ^{O}C indefinitely