Duplex Mitochondrial DNA PCR Amplification

PURPOSE: To increase the amount of available mtDNA for the purposes of analysis, by performing an *in vitro* replication of template DNA using oligonucleotide primers, thermostable DNA polymerase and deoxynucleoside triphosphate bases (dNTPs) within a thermal cycler.

PROCEDURE:

A positive control, an amplification negative, and an extraction negative control (if applicable) should be included with each batch of samples being amplified to demonstrate procedural integrity. The positive control is a laboratory grade cell line, for which the mtDNA type is known.

Follow the mtDNA pre-amplification guidelines for handling the tubes and cleaning of the work surfaces. The following steps have to be performed in the appropriate dedicated areas. Evidence samples and exemplar samples should not be handled at the same time.

A. Preparing the DNA aliquots for HVI-HVII amplification

- Amplification can be performed with either Roche or Homebrew reagents. **Homebrew reagents should be used for Missing Person's testing, body identifications, and kinship testing where the sample type is other than a hair shaft sample.**
- When amplifying extracts which have nuclear DNA quantification data, the target amount of extract to be amplified is:
  - 100 pg when using Roche reagents
  - 500 pg when using Homebrew reagents
- When amplifying samples that have not been quantified (e.g., hair shaft samples), use 20ul of the extract and Roche reagents, only.
- Table I refers to the preparation of the control samples for the amplification.

Table I – Control samples for amplification.

Sample	DNA (extract)	TE^{-4}
HL60 Positive Control DNA (100 pg/20 □l)	20 µL	---
Amplification Negative Control	---	20 µL
Extraction Negative Control,(s) when sample amplified neat	20 µL	---
Extraction Negative Control (s) when a dilution/concentration of sample extract is amplified	Submit 20 µL of the extraction negative at the same dilution/concentration factor or more concentrated than the sample	

B. Amplification Setup

1. For each amplification set, record the lot numbers and samples in the documentation. Label 0.2 ml PCR reaction tubes with sample label name and with date and time for the positive and negative controls.

2. If samples require dilution, prepare the aliquots in UV'ed 1.5mL tubes, and place the neat samples back into storage.

3. Master mix preparation:

a) For amplification using Roche reagents:

- Prepare a Master Mix with Reaction Mix and Primer Mix, The following calculations are used:
  - Reaction Mix: number of samples N x 20 µL Reaction Mix = __ µL
  - Primer Mix: number of samples N x 10 µL Primer Mix = __ µL

Note: For ≤6 samples, use N, for ≥ 6 samples, use N+1. To save on reagents, individual aliquots of Reaction and Primer Mix can be made.

- Add Reaction Mix and Primer Mix together to prepare Master Mix.
- Vortex the Master Mix and centrifuge briefly.
- Aliquot 30 µL of the Master Mix into the bottom of each labeled 0.2 ml reaction tube.

b) For amplification with Homebrew reagents:

- Prepare Reaction Mix and Primer Mix master mixes according to Homebrew amplification documentation. The following amounts of reagents per sample are used:

Reaction Mix Reagents	**Volume per sample**	**Primer Mix Reagents**	**Volume per sample**
UltraPure dH2O	3.7 µL	UltraPure dH2O	6 µL
GeneAMP 10X PCR Buffer	5 µL	10□M HVIF	1 µL
2.5mM dNTPs	4 µL	10□M HVIR	1 µL
25mM $MgCl_2$	4.8 µL	10□M HVIIF	1 µL
5U/□L AmpliTAQ Gold DNA Polymerase	2.5 µL	10□M HVIIR	1 µL
Total Reaction Mix volume per sample	20 µL	**Total Primer Mix volume per sample**	10 µL

The following calculations are used:

- Reaction Mix: number of samples + 1 (N + 1) x Reaction Mix Reagent Amount = __ µL
- Primer Mix: number of samples + 1 (N + 1) x Primer Mix Reagent Amount = __ µL

- Vortex the Reaction and Primer Mix and centrifuge briefly
- Aliquot 20 µL of the Reaction Mix and 10 µL of the primer mix into the bottom of each labeled 0.2 ml reaction tube.

4. Add samples into the 0.2ml tubes. **Use a sterile filter pipette tip for each sample addition. Open only one tube at a time for sample addition.** The final aqueous volume in the PCR reaction mix tube will be 50 µL. Transfer the appropriate volume of target DNA or TE^{-4} to each respective sample tube. After the addition of the DNA, cap each sample before proceeding to the next tube. **If necessary, spin down the tubes at 1000 rcf for a few seconds.**

5. When finished, place the rack with the 0.2ml tubes in the pre-amp room dumbwaiter. Send the samples up to the post-amp room.

C. Thermal Cycling

1. Turn on the Perkin Elmer 9700 Thermal Cycler.

2. Use the following settings to amplify the samples

9700 Thermal Cycler User: mtDNA File: lamtdna	The amplification file is as follows- Soak at 94^{O}C for 14 minutes 34 cycles: - Denature 92 ^{O}C for 15 seconds - Anneal at 59 ^{O}C for 30 seconds - Extend at 72 ^{O}C for 30 seconds Incubation at 72 ^{O}C for 10 minutes Storage soak at 4 ^{O}C indefinitely

3. Place the tubes in the tray in the heat block, slide the heated lid over the tubes, and fasten the lid by pulling the handle forward. Place the microtube rack used to set up the samples for PCR in the post-amp room bleach bath

4. Start the run by performing the following steps:

   a. The main menu options are RUN CREATE EDIT UTIL USER. To select an option, press the F key directly under that menu option.
   b. Verify that the user is set to "mtDNA" if not, select the USER option (F5) to display the "Select User Name" screen.

   c. Use the circular arrow pad to highlight "mtDNA." Select the ACCEPT option (F1).

d. Press the RUN button (F1) and select the "lamtdna" file.

e. Verify that the reaction volume is set to 50 µL and the ramp speed is set to **9600 (very important)**.

f. If all is correct, select the START option (F1).

The run will start when the heated cover reaches 103 ^{O}C. The screen will then display a flow chart of the run conditions. A flashing line indicates the step being performed, hold time is counted down. Cycle number is indicated at the top of the screen, counting up.

g. Be sure to record the use of the thermal cycler in the documentation under the appropriate instrument name.

h. Upon completion of the amplification, press the STOP button repeatedly until the "End of Run" screen is displayed, and remove your samples. Select the EXIT option (F5). Wipe any condensation from the heat block with a Kimwipe and pull the lid closed to prevent dust from collecting on the heat block. Turn the instrument off.

i. After removing your samples, place them in the appropriate 2-8 °C refrigerator for storage. Samples should be separated according to sample type (exemplar, evidence, or quality control). Record the date and time of when samples were amplified on the cover of the 0.2 mL PCR storage box.

IMPORTANT:

Turn instruments off **ONLY** when the Main Menu is displayed, otherwise there will be a Power Failure message the next time the instrument is turned on. It will prompt you to review the run history. Unless you have reason to believe that there was indeed a power failure, this is not necessary. Instead, press the STOP button repeatedly until the Main Menu appears.

In case of a real power failure the 9700 thermal cycler will automatically resume the run if the power outage did not last more than 18 hours. The Uninterruptible Power Supply (UPS) present in the amplification room will power the thermal cyclers for about 2-3 hours in the case of a total

power outage. The history file contains the information at which stage of the cycling process the instrument stopped. Consult with the QA team and/or the Technical Leader on how to proceed.