Duplex Mitochondrial DNA PCR Amplification		
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Duplex Mitochondrial DNA PCR Amplification

1 Purpose

1.1 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.

2 Preparing the DNA aliquots for HVI-HVII amplification

2.1 Amplification can be performed with either Roche or Homebrew reagents.

2.2 When amplifying extracts which have nuclear DNA quantification data, the target amount of extract to be amplified is:

- 100 pg when performing Roche amplification
- 500 pg when performing Homebrew amplification

2.3 When amplifying samples that have not been quantified (e.g., hair shaft samples), use 20 µL of the extract.

2.4 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 µL)	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	

3 Amplification Test Batch Creation

3.1 Refer to the Forensic Biology LIMS Process Manual for general procedures within the LIMS.

3.2 Use batch configurations "MitoAmpHomebrew" and "MitoAmpHVIHVII" for Homebrew and Roche amplifications, respectively. When generating output samples, use 1:1 for Roche amplifications and Homebrew for Homebrew amplifications.

3.3 Fill out the "Conc, Nuclear DNA" column in the Data Entry section for the output samples as follows:

3.3.1 For control samples enter "5" (pg/uL) for the Amp Pos and and "-" for the Amp Neg samples. Enter the value of the ENeg if present in the "concentration" column (e.g. "0.0" pg/uL); if not, enter a "-".

3.3.2 For samples that were quantitated, enter the sample concentration present in the "concentration" column.

3.3.3 For samples that were not quantitated (e.g. hair shafts), enter a "-".

3.4 Click [Save]. Once the page refreshes, this should automatically populate the "Dilution", "Vol, DNA", and "Vol, TE" columns. Check the resulting values.

3.4.1 Amp Neg samples should have "0.0 uL" and "20.0 uL" entered in the "Vol, DNA" and "Vol, TE" columns, respectively.

3.4.2 ENeg samples should have "20.0 uL" and "0.0 uL" entered in the "Vol, DNA" and "Vol, TE" columns, respectively.

3.4.3 Make any necessary corrections to the volume amounts of the Amp Neg and ENeg samples (sometimes the values described above may be reversed).

3.4.4 If a change needs to be made to the dilution of the ENeg sample (e.g. when all associated samples are being run at a dilution less than 1), it can be made in the "Dilution" column at this time. In addition, the Eneg sample name in the output sample table will need to be edited to show the correct dilution (e.g. E Neg1_01192021.085905_d100_HB).

4 Amplification Setup

4.1 Fill in the amplification performed by tab in LIMS. This will add the date and time to the positive control and amplification negative. Update the test batch description and plate name using the amplification date and time (e.g. A052821-1215).

4.2 Retrieve sample(s) and reagents needed for amplification. Record the reagent lot numbers in LIMS.

4.3 Label 0.2 mL PCR reaction tubes with the amp tube label generated by LIMS.

4.4 **Amplification WITNESS:** Have a witness verify your input tube tops, sample labels and PCR amp tube labels. The analyst must read the ***entire*** amp tube label.

4.5 If samples require dilution, prepare the aliquots in UV-treated 1.5 mL microcentrifuge tubes according to the data entry screen and using the table below. Place the neat samples back into storage once dilutions are made.

Dilution	Amount of DNA template (µL)	Amount of TE^{-4} (µL)
0.5	X*	X*
0.1	2	18
0.01	2	198
0.001	2 µL of 0.01 DNA dilution	18
0.0001	2 µL of 0.01 DNA dilution	198

***NOTE:** For 0.5 dilution, add X µL DNA extract to an equivalent volume of TE^{-4}

5 Master mix preparation

5.1 For amplification using Roche reagents:

5.1.1 Calculate Master Mix with Reaction Mix and Primer Mix:

- *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.

5.1.2 Add Reaction Mix and Primer Mix together to prepare Master Mix. For small test batches, reaction mix and primer mix can be aliquoted directly into the reaction tube.

5.1.3 Vortex the Master Mix and centrifuge briefly.

5.1.4 Aliquot 30 µL of the Master Mix into the bottom of each labeled 0.2 mL reaction tube.

5.1.5 Proceed to step 5.3.

5.2 For amplification with Homebrew reagents:

5.2.1 Prepare master mix according to the Homebrew amplification reagent list in the LIMS reagents tab.

- *Homebrew (HB) Master Mix:*
- *Number of samples + 1 (N + 1) x HB Reagent Amount = __ µL*

5.2.1.1 The following amounts of reagents per sample are used:

Note: Use the "Calculate Amount" function in the LIMS Reagent section to calculate the necessary total amounts of reagents needed. AmpliTaq Gold DNA polymerase should be the last component added to the master mix.

Do NOT vortex the AmpliTaq Gold DNA polymerase.

Homebrew Reagents	Volume per Sample
Irradiated dH_2O	9.7 µL
GeneAMP 10X PCR Buffer	5 µL
2.5mM dNTPs	4 µL
25mM $MgCl_2$	4.8 µL
10 µM HVIF	1 µL
10 µM HVIR	1 µL
10 µM HVIIF	1 µL
10 µM HVIIR	1 µL
5U/uL AmpliTaq Gold DNA Polymerase	2.5 µL

5.2.2 Vortex the master mix and centrifuge briefly.

5.2.3 Aliquot 30 µL of master mix into the bottom of each labeled 0.2 mL reaction tube.

5.3 Add the samples into the 0.2 mL tubes.

Note: **Use a sterile filter pipette tip for each sample addition. Open only one tube at a time for sample addition.**

5.4 Transfer the appropriate volume of target DNA or TE^{-4} to each respective sample tube. The final aqueous volume in the PCR reaction mix tube will be 50 µL. 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.**

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

6 Thermal Cycling

6.1 Turn on the Perkin Elmer 9700 Thermal Cycler.

6.2 Use the following settings to amplify the samples:

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

6.3 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.

6.4 Start the run by performing the following steps:

6.4.1 The main menu options are RUN CREATE EDIT UTIL USER. To select an option, press the F key directly under that menu option.

6.4.2 Verify that the user is set to "mtDNA" if not, select the USER option (F5) to display the "Select User Name" screen.

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

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

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

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

6.4.6.1 The run will start when the heated cover reaches 103°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.

6.5 Record the instrument usage in LIMS and enter "lamtdna" as the Program.

6.6 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 lint free wipe and pull the lid closed to prevent dust from collecting on the heat block. Turn the instrument off.

6.7 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.

7 Important Information

7.1 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.

7.2 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.