Amplification and Amplicon Purification of Mitochondrial DNA

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Amplification and Amplicon Purification of Mitochondrial DNA

1 Introduction

These procedures describe the processes for amplification of mitochondrial deoxyribonucleic acid (mtDNA) via the polymerase chain reaction (PCR) and the purification of amplified PCR product through enzymatic cleanup.

2 Scope

These procedures apply to DNA personnel who perform amplification of mtDNA and/or purification of amplicons and DNA personnel that perform the associated quality control procedures.

3 Equipment

3.1 Equipment/Materials

- General laboratory supplies (e.g. tubes, pipettes, vortexer, PCR tube rack)
- Thermal cycler, ABI 9700 or equivalent
- Tecan robot model 'Freedom EVO'
  - Tecan EvoWare software, version 2.3 or higher
- 96-well plates, Applied Biosystems MicroAmp® optical or equivalent
- Clear and/or foil plate seals
- Thermal microplate sealer

3.2 Reagents

- Water, Reagent Grade or equivalent
- GeneAmp® 10X PCR Buffer
- Bovine Serum Albumin (BSA), 1.6 µg/µL
- GeneAmp® Deoxyribonucleotide Triphosphates (dNTP) blend, 2.5 mM each
- mtDNA Amplification Primers, 30 µM or 10 µM
- AmpliTaq Gold®, 5U/µl
- Positive control DNA, HL60 or equivalent
- ExoSAP-IT®
- 10% bleach (reagent grade or equivalent)
- Isopropyl alcohol, 70%
- Purified water or equivalent, available at laboratory sinks
- RoboScrub solution (Liquinox™ or equivalent)

4 Standards and Controls

At least one negative control (NC) and one positive control (i.e., HL60) must be processed concurrently in the same instrument with the samples for each amplified region. Typically a 100 pg/µL concentration of HL60 is used for whole control region (WCR) amplifications and a 20 pg/µL concentration is used for the hypervariable (HV) regions and mini-primer set (MPS) regions.

An associated reagent blank (RB) must be amplified with the same primers and at the most sensitive concentration conditions as required by the sample(s) containing the least amount of DNA. For extraction batches with multiple RBs, at least the RB that demonstrates the greatest signal, if any, must be amplified.

For evaluation of the amplification controls, refer to the appropriate DNA interpretation procedure (i.e., BIO-571).

5 Procedure

The contextual information in the case, along with the amplification strategy guidance in Table 1 using the pre-amplification quantitation (qPCR) results and/or the quantity and quality of autosomal DNA results, should be used to determine the mtDNA region(s) to amplify. For samples that have been extracted on the EZ1, only WCR or HV1/HV2 can be used.

	Sample Type		**Degradation Index (DI)**		
	Bone	*Hair*	**<2.5**	**2.5 – 5**	**>5**
mtDNA copy #	--	>4K	WCR	WCR	WCR
	>30K	--	WCR	HV1/HV2	HV1/HV2
	>400	>100	HV1/HV2	HV1/HV2	HV1A/1B/2A/2B or MPS
	<400	<100	HV1A/1B/2A/2B or MPS	HV1A/1B/2A/2B or MPS	HV1A/1B/2A/2B or MPS

Table 1 - Amplification Strategy Guidance

The two primers listed in Table 2 represent the light (L) and heavy (H) strand primers that are added to the master mix for the listed region in accordance with the applicable amplification section below.

Region	Primers
WCR	A1, 617

Region	Primers
HV1	A1, B1
HV2	C1, D1

Region	Primers
HV1A	A1, B2
HV1B	A2, B1
HV2A	C1, D2
HV2B	C2, D1

Region	Primers
MPS1	1A, 1B
MPS2	2A, 2B
MPS3	3A, 3B
MPS4	4A, 4B
MPS5	5A, 5B

Table 2 - Primer Selection

Refer to the DNA procedures introductions (i.e., BIO-100) for applicable general precautions and cleaning instructions.

For water that will come into contact with the DNA samples (e.g., for dilutions), reagent grade water, or equivalent, will be used. The purified water available via faucets (typically labeled DE) at the laboratory sinks is used for Tecan operation and is also called Tecan system liquid.

The Mito workbook is used to record the applicable case notes, to facilitate the transfer of data between instruments and equipment, and to generate examination records. Ensure the relevant run information (i.e., instruments and reagents) are recorded in the appropriate fields in the Mito workbook.

5.1 Manual Amplification with WCR

1.	Prepare WCR amplification master mix.

Typically, a 5-reaction master mix is sufficient to account for a sample, RB, positive control, NC, and appropriate overage. When manually amplifying a batch of samples, the volume of master mix should be calculated to account for the number of samples, RB(s), positive control, NC, and overage. Components should be added in order listed below.

Master Mix Component	µL per Sample	µL for 5 reactions
Water	12.0	60.0
10X PCR Buffer	5.0	25.0
BSA (1.6 µg/µL)	5.0	25.0
dNTP mix	4.0	20.0
A1 primer 30 µM	1.0	5.0
617 primer 30 µM	1.0	5.0
AmpliTaq Gold	2.0	10.0
TOTALS	30	150

2.	Add 30 µL of master mix to each tube.
3.	• Add 20 µL of sample extract (or extract dilution) to the sample tube. • Add 20 µL of RB (or RB dilution) to the RB tube. • Add 20 µL of positive control DNA to the positive control tube. • Add 20 µL of water to the NC tube.

When amplifying a batch of samples, add all samples and applicable blanks prior to adding the positive and negative controls.

4.	• Place tubes in the thermal cycler.
	• Start the "WCR36" program:
	    • 95°C for 9 minutes
	    • 36 cycles:
	        ○ 95°C for 10 seconds
	        ○ 53°C for 30 seconds
	        ○ 72°C for 30 seconds
	    • 72°C for 10 minutes
	    • 4°C hold indefinitely.

WCR was also validated with a 32 cycle amplification. If needed, a 32 cycle amplification may be performed using the program "WCR32". The cycle number used will be recorded in the case notes.

Amplicons can then be purified using ExoSAP-IT (Section 5.6).

5.2 Automated Amplification with WCR

<table>
<tr><td>1.</td><td>Ensure the Tecan is prepared to run.

Prior to daily use:
• Make ~100 mL of 3% bleach to replace bleach in front trough
• Clean the outside of the Tecan tips with 70% isopropyl alcohol
• Decontaminate the Tecan work deck with 10% bleach
• Run the daily start up script

Prior to each run:
• Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. When a carboy is refilled, it should be allowed to de-gas overnight before use.
• Check volume of waste container and empty if needed

Weekly, generally at the end of the workday:
• Make ~3.5 L of diluted Liquinox solution (see instructions on bottle)
• ~3.5 L purified water in a separate container is needed
• Run the RoboScrub Clean script, and follow the prompts</td></tr>
<tr><td>2.</td><td>Create the appropriate volume of master mix based on the number of samples to be amplified. Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin.</td></tr>
</table>

This step may be performed any time prior to loading the master mix on the Tecan robot. When amplifying a batch of samples, the volume of master mix should be calculated to account for the number of samples, RB(s), NC, positive control, and an overage of two samples per amplification plate. Components should be added in order listed below.

Master Mix Component	µL per Sample
Water	12
10X PCR Buffer	5
BSA (1.6 µg/µL)	5
dNTP mix	4
A1 primer (30 µM)	1
617 primer (30 µM)	1
AmpliTaq Gold	2
TOTALS	30

3.	Prepare the Tecan Deck. *The steps below may be performed in any order prior to running the Tecan robot.* Bleach Rack: • Ensure the 3% bleach solution in the front trough was replaced prior to daily use. Plate Rack: • Place a 96-well plate into a base. Place into the rear position of the plate rack. Master Mix Rack (Grid 12): • Place two uncapped tubes containing equal volumes of master mix in positions 3 and 4. • Place empty tubes (with “BL” barcodes) in positions 1-2 and 5-16. Sample Rack (Grid 4): • Ensure all DNA extracts, reagent blanks, and positive controls are in Tecan compatible tubes. • Place uncapped DNA sample tubes istarting in position 1 of sample rack 1 and up to position 6. Place positive and negative controls in rack 1 positions after all sample tubes (e.g., positions 7-8 for a full run). *Each tube should contain at least 22 µl.*
4.	Run Scan Script and import scan file into workbook.
5.	Run Amplification Script. • Follow the prompts to ensure the appropriate racks are on the deck as required. • Enter number of samples to be amplified when prompted.

The Tecan will prepare the amp plate so that each well contains 30 µL of master mix and 20 µL of DNA template or control.

6.	Upon completion of the Tecan run, seal the amplification plate with a clear or foil seal.

The seal may be applied with the Thermal Microplate Sealer or, if necessary, manually. Ensure that the edges of each well are properly sealed.

7.	Quick spin (generally ~2,000 rpm for 5 seconds).

Sample tubes should be removed from the Tecan deck and capped prior to proceeding to the Amplification (Amp) room.

8.	• Place the amp plate in an appropriate thermal cycler. • Place an optical compression pad, gold side up, onto the top of the sealed plate and close the lid by pressing the lever down completely.

9.	Start the “WCR36” program:
	• 95°C for 9 minutes
	• 36 cycles:
	  ○ 95°C for 10 seconds
	  ○ 53°C for 30 seconds
	  ○ 72°C for 30 seconds
	• 72°C for 10 minutes
	• 4°C hold indefinitely

WCR was also validated with a 32 cycle amplification. If needed, a 32 cycle amplification may be performed using the program “WCR32”. The cycle number used will be recorded in the case notes.

Amplicons can then be purified using ExoSAP-IT (Section 5.7 or 5.6).

5.3 Amplification of HV1, HV2, HV1A, HV1B, HV2A, HV2B

1.	Prepare an amplification master mix for each region.

Typically, a 5-reaction master mix is sufficient to account for sample, RB, positive control, NC, and appropriate overage. Components should be added in order listed below.

Master Mix Component	µL per Sample	µL for 5 reactions
Water	6.0	30.0
10X PCR Buffer	2.5	12.5
BSA (1.6 µg/µL)	2.5	12.5
dNTP mix	2.0	10.0
(L) primer 30 µM	0.5	2.5
(H) primer 30 µM	0.5	2.5
AmpliTaq Gold	1.0	5.0
TOTALS	15.0	75.0

2.	Add 15 µL of the appropriate master mix to the respective tubes.
3.	• Add 10 µL of sample extract (or sample dilution) to each sample tube. • Add 10 µL of RB (or RB dilution) to each RB tube. • Add 10 µL of positive control DNA to each positive control tube. • Add 10 µL of reagent grade or higher water to each NC tube.

To use additional extract volume, see section 5.5 for using 16 µL of extract.

<table>
<tr><td>4.</td><td>

Place tubes in the thermal cycler.
Start the “TAQ 36” program:

95°C for 9 minutes
36 cycles:

95°C for 10 seconds
60°C for 30 seconds
72°C for 30 seconds
4°C hold indefinitely

</td></tr>
</table>

The HV regions were also validated with a 32 cycle amplification. If needed, a 32 cycle amplification may be performed using the program “TAQ 32”. The cycle number used will be recorded in the case notes.

Amplicons can then be purified using ExoSAP-IT (Section 5.6).

5.4 Amplification with Mini-primer Sets

1.	Prepare an amplification master mix for each mini-primer set region.

Typically, a 5-reaction master mix is sufficient to account for the sample, RB, positive control, NC, and appropriate overage. Components should be added in order listed below.

Master Mix Component	µL per Sample	µL for 5 reactions
Water	6.0	30.0
10X PCR Buffer	2.5	12.5
BSA (1.6 µg/µL)	2.5	12.5
dNTP mix	2.0	10.0
(L) primer 10 µM	0.5	2.5
(H) primer 10 µM	0.5	2.5
AmpliTaq Gold	1.0	5.0
TOTALS	15.0	75.0

<table>
<tr><td>2.</td><td>Add 15 µL of the appropriate master mix to respective tubes.</td></tr>
<tr><td>3.</td><td>

Add 10 µL of sample extract, or extract dilution, to each sample tube.
Add 10 µL of RB, or RB dilution, to each RB tube.
Add 10 µL of positive control DNA to each positive control tube.
Add 10 µL of reagent grade or higher water to each NC tube.

</td></tr>
</table>

To use additional extract volume, see section 5.5 for using 16 µL of extract.

4.	• Place tubes in the thermal cycler.
	• Start "MINI" program:
	  • 95°C for 12 minutes
	  • 36 cycles:
	    ○ 95°C for 15 seconds
	    ○ 56°C for 30 seconds
	    ○ 72°C for 45 seconds
	    ○ 4°C hold indefinitely

Amplicons can then be purified using ExoSAP-IT (Section 5.6).

5.5 Amplification using 16 µL of Extract

The following section may be used for increasing the sample volume for amplifications of HV1A, HV1B, HV2A, HV2B, HV1, HV2 or the mini-primer set regions.

1.	Prepare an amplification master mix for each region.

Typically, a 5-reaction master mix is sufficient to account for the sample, RB, positive control, NC, and appropriate overage. Components should be added in order listed below.

Master Mix Component	µL per Sample	µL for 5 reactions
10X PCR Buffer	2.5	12.5
BSA (1.6 µg/µL)	2.5	12.5
dNTP mix	2.0	10.0
(L) primer 10 µM	0.5	2.5
(H) primer 10 µM	0.5	2.5
AmpliTaq Gold	1.0	5.0
TOTALS	9.0	45.0

2.	Add 9 µL of the appropriate master mix into respective tubes.
3.	• Add 16 µL of sample extract to each sample tube. • Add 16 µL of RB to each RB tube. • Add 16 µL of positive control DNA to each positive control tube. • Add 16 µL of reagent grade or higher water to each NC tube.

If needed, water may be added to a sample and/or RB in order to obtain a total volume of 16 µL. (e.g., If only 15 µL of sample extract is available, 1 µL water may be added to the sample tube.) However, the RB must not be diluted more than the sample. The same water used for the sample must also be used for the associated NC and, if applicable, the RB.

4.	• Place tubes in the thermal cycler.
	• Start appropriate program:
	  • For HV1A, HV1B, HV2A, HV2B, HV1, HV2, use “TAQ36”
	  • For Mini-primers, use “MINI”

Amplicons can then be purified using ExoSAP-IT (Section 5.6).

5.6 Manual Amplicon Purification by ExoSAP-IT

1.	• Add ExoSAP-IT to the amplicon tubes:
	  • For standard or mini-primer sets, add 10 µL
	  • For WCR sets, add 20 µL
	• Vortex or pipette mix.
	• Place tubes in the thermal cycler and run “EXOSAPIT” program:
	  • 37°C for 15 minutes
	  • 80°C for 15 minutes
	  • 4°C hold indefinitely

Note: Half the listed volumes of ExoSAP-IT have been validated for use and may be used as appropriate for lower yield samples.

If needed based on the sequencing results, this step may be repeated and the sample(s) re-processed as appropriate.

5.7 Automated Amplicon Purification by ExoSAP-IT

1.	Ensure the Tecan is prepared to run. See section 5.2 step 1.
2.	Set up Tecan deck: Bleach Rack: • Ensure the 3% bleach solution in the *center* trough was replaced prior to daily use. Plate Rack: • Spin down 96-well amplification plate and carefully remove seal. Place into the rear position of the plate rack. ExoSAP-IT Rack (Grid 5): • Vortex and spin down one tube of ExoSAP-IT. Place into position 1 of tube rack.
3.	Start the ExoSAP-IT script. • The script will prompt the user to enter the number of occupied wells in the amplification plate. The Tecan will add 10 µL of ExoSAP-IT to each well indicated and mix thoroughly.

4.	• Seal plate.
	• Place an optical compression pad, gold side up, onto the top of the sealed plate and close the lid by pressing the lever down completely.
	• Place in the thermal cycler, and run "EXOSAPIT" program:
	  ◦ 37°C for 15 minutes
	  ◦ 80°C for 15 minutes
	  ◦ 4°C hold indefinitely

Following purification by ExoSAP-IT, samples are ready for post-amplification quantification (see BIO-541) and/or cycle sequencing (see BIO-551). Post-amplification quantification is not required for samples processed with the automated procedures.

If needed based on the sequencing results, purification may be repeated and the sample(s) re-processed as appropriate.

6 LIMITATIONS

- Samples that have been extracted on the EZ1 are only approved for amplification of WCR and HV1/HV2.
- Automation can only be used for known reference samples and other samples expected to yield high quantity DNA (e.g., blood stains). Automation is not used for calcified or kertinized tissue samples (e.g., bone or hair) even with a high quantity yield.

7 SAFETY

- All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material.
- Refer to the FBI Laboratory Safety Manual for information on personal protection, the proper disposal of the chemicals used in these procedures, as well as the biohazardous wastes generated.

8 REFERENCES

Applied Biosystems. GeneAmp® PCR System 9700 User's Manual Set. 1997.

9 REVISION HISTORY

Revision	Issued	Changes
00	07/01/2022	Reformatted DNA 405-2 into new template and assigned new Doc ID.

10 APPENDIX A: QUALITY CONTROL PROCEDURES

10.1 Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., BIO-104) for minimum frequency of performance verifications and additional requirements.

10.1.1 Performance Verification (PV) of the Tecan Robotic Workstation

Refer to the nuclear DNA quantification procedure (i.e., BIO-520) for instructions for the PV of the Tecan Robotic Workstation.

10.1.2 Performance Verification of the 9700 Thermal Cycler

A. Cleaning - Refer to Cleaning the Sample Wells and Cleaning the Sample Block Cover procedures described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set.
B. Temperature Verification Test - This procedure verifies that the thermal cycler remains within the temperature accuracy specification. Refer to Running the Temperature Verification Test procedure described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set. This test procedure requires the use of a Temperature Verification System.
C. Temperature Non-uniformity Test - This procedure verifies the temperature uniformity of the sample wells in the thermal cycler. Refer to Running the Temperature Non-uniformity Test procedure described in the Maintenance section of the GeneAmp® PCR System 9700 User's Manual Set. This test procedure requires the use of a Temperature Verification System.
D. Rate Test and Cycle Test - These procedures verify the integrity of the cooling and heating system of a thermal cycler. Refer to Running System Performance Diagnostics procedure described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set.

10.2 Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., BIO-103) for additional requirements.

10.2.1 Amplification Reagents

The following reagents will be tested by amplifying a positive (HL60) and negative control sample with the WCR for the appropriate number of cycles and processed through sequencing (A1 and 617 primers only). Reagents may be concurrently tested or tested with in use lots of the counterpart reagents. Multiple positive control samples may be amplified; at least one must yield data of acceptable quality and result in the expected positive control sequence. The negative control should result in no sequence data.

A. 10X PCR Buffer
B. AmpliTaq Gold
C. Deoxyribonucleotide triphosphate mix (dNTPs)

D. Bovine Serum Albumin (BSA)
E. ExoSAP-IT

10.2.2 Positive Control

New lots and dilutions of HL60 DNA will be tested by amplifying a less sensitive region or the WCR , including a NC, and processed through sequencing. Typically HV2B at 36 cycles is used for the 20 pg/µL dilution and the WCR at 32 cycles for the 100 pg/uL dilution.

Multiple positive control samples may be amplified; at least one must yield data of acceptable quality and result in the expected positive control sequence. The negative control should result in no sequence data.

10.2.3 Amplification Primers

New lots and dilutions (30 µM and 10 µM) of amplification primers will be tested by amplifying a positive (HL60) and negative control sample for all regions in which the primer being tested is used for. The resulting amplicon(s) will be processed through sequencing with all primers typically used in those amplicon(s). Multiple positive control samples may be amplified; at least one must yield data of acceptable quality and result in the expected positive control sequence. The negative control should result in no sequence data.

Primer for QC	Amplification Regions
A1	HV1, HV1A, WCR
A2	HV1B
B1	HV1, HV1B
B2	HV1A
C1	HV2, HV2A
C2	HV2B
D1	HV2, HV2B
D2	HV2A
617	WCR
Miniprimers	See Table 2