Procedures for the Amplification and Amplicon Purification of Mitochondrial DNA

1 Scope

These procedures apply to DNA personnel who perform amplification of mitochondrial deoxyribonucleic acid (mtDNA) via the polymerase chain reaction (PCR) and the process for purification of amplicons and DNA personnel that perform the associated quality control procedures.

2 Equipment/Materials/Reagents

Equipment/Materials

- Thermal cycler, ABI 9700 or equivalent
- General laboratory supplies (e.g. tubes, pipettes, vortexer)
- PCR tube rack
- 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

Reagents

- Water, Reagent Grade or equivalent
- GeneAmp® 10X PCR Buffer I
- 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®
- 3% bleach (reagent grade or equivalent)
- 10% bleach (reagent grade or equivalent)
- Isopropyl alcohol, 70%
- Purified water or equivalent, available at laboratory sinks
- RoboScrub solution (Liquinox™ or equivalent)

3 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. 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 evaluation of the amplification controls, refer to the appropriate interpretation procedure of the *DNA Procedures Manual*.

4 Procedures

Refer to DNA QA 600 in the *DNA Procedures Manual* for applicable general precautions and cleaning instructions.

For water that will come into contact with the DNA samples (e.g., for dilutions), reagent grade, or equivalent, water 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 following information, along with the pre-amplification quantitation (qPCR) results and/or autosomal DNA results, should be used to determine the mtDNA region to amplify and the primers to use. The two primers listed represent the light (L) and heavy (H) strand primers that are added to the master mix for the amplification of the listed region.

1. For samples from which abundant amounts of mtDNA template are expected, the whole control region (WCR) can be amplified using primer sets listed below. This amplification may be done using the Tecan robot platform.

Region	Primers
WCR	A1, 617

2. To obtain the maximum possible sequence from a sample, flexibility in the choice of primer sets is allowed. This may include the two hypervariable (HV) regions, HV1 and HV2, listed below.

Region	Primers
HV1	A1, B1
HV2	C1, D1

3. For samples from which lower amounts of mtDNA template are expected, HV1A, HV1B, HV2A, and HV2B can be amplified using primer sets listed below.

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

4. For samples from which extremely degraded DNA template is expected, MPS1-MPS5 can be amplified using mini-primer sets (MPS) listed below.

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

5. For samples that have been extracted on the EZ1, only WCR or HV1/HV2 can be used. For samples that have been extracted on the QIASymphony, only WCR can be used.

4.1 Manual Amplification with WCR

4.1.1	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	30.0	150

4.1.2	Add 30 µL of master mix to each tube.	

4.1.3	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.

4.1.4	Place tubes in the thermal cycler.
	Start the "WCR32" or "WCR36" program:
	• 95ºC for 9 minutes
	• 32 or 36 cycles:
	  o 95ºC for 10 seconds
	  o 53ºC for 30 seconds
	  o 72ºC for 30 seconds
	• 72ºC for 10 minutes
	• 4ºC hold indefinitely	

Record of the thermal cycler program used will be in the case notes.

Amplicons can then be purified using ExoSAP-IT.

4.2 Automated Amplification with WCR

4.2.1	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 prompt	

The daily start up script prompt "Check syringes and tips," refers to checking that the tubing and syringes (plunger lock screws) are tight and not introducing air bubbles, and that the tips are tight, free of clogs, and not leaking.

4.2.2	Create the appropriate volume of master mix based on the number of samples to be amplified (with overage). Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin.	

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

4.2.3	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: • Place two uncapped tubes containing equal volumes of master mix in positions 3 and 4. Place empty tubes in positions 1-2 and 5-16. Sample Rack: • Ensure all DNA extracts, reagent blanks, and positive controls are in Tecan compatible tubes. • Place uncapped DNA sample tubes in the desired sequence starting in position 1 of sample rack 1 and continue on to additional racks as needed.	

4.2.4	• Run Scan Script and import scan file into workbook.	

4.2.5	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. • Each sample tube should contain at least 22 µl, including positive and negative controls.	

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.

4.2.6	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.

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

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

4.2.8	Proceed to amp room. 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.	

4.2.9	Start the "WCR32" or "WCR36" program:
	• 95ºC for 9 minutes
	• 32 or 36 cycles:
	  o 95ºC for 10 seconds
	  o 53ºC for 30 seconds
	  o 72ºC for 30 seconds
	• 72ºC for 10 minutes
	• 4ºC hold indefinitely	

Record of the thermal cycler program used will be in the case notes.

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

4.3.1	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

4.3.2	Add 15 µL of the appropriate master mix to the respective tubes.	

4.3.3	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 water to each NC tube.	

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

4.3.4	Place tubes in the thermal cycler.
	Start the "TAQ 32" or "TAQ 36" program:
	• 95ºC for 9 minutes
	• 32 or 36 cycles:
	  ○ 95ºC for 10 seconds
	  ○ 60ºC for 30 seconds
	  ○ 72ºC for 30 seconds
	• 4ºC hold indefinitely	

Record of the thermal cycler program used will be in the case notes.

Amplicons can then be purified using ExoSAP-IT.

4.4 Amplification with Mini-primer Sets

4.4.1	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

4.4.2	Add 15 µL of the appropriate master mix to respective tubes.	

4.4.3	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 water to each NC tube.	

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

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

Amplicons can then be purified using ExoSAP-IT.

4.5 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.

4.5.1	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

4.5.2	Add 9 µL of the appropriate master mix into respective tubes.	

4.5.3	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 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. (For example: 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.

4.5.4	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.

4.6 Manual Amplicon Purification by ExoSAP-IT

4.6.1	Add ExoSAP-IT to the amplicon tubes: For standard or mini-primer sets, add 10 µL For WCR sets, add 20 µL *Half the listed volumes of ExoSAP-IT have been validated for use and may be used as appropriate for lower yield samples.* Vortex or pipette mix. Place tubes in the thermal cycler and run "EXOSAPIT" program: • 37ºC for 15 minutes • 80ºC for 15 minutes • 4ºC hold indefinitely	

Samples are ready for post-amplification quantitation.

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

4.7 Automated Amplicon Purification by ExoSAP-IT

4.7.1	Ensure the Tecan is prepared to run. Prior to daily use: • Make ~100 mL of 3% bleach to replace bleach in *center* 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 prompt	

4.7.2	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: Vortex and spin down one tube of ExoSAP-IT. Place into position 1 of tube rack.	

4.7.3	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.	

4.7.4	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: • 37ºC for 15 minutes • 80ºC for 15 minutes • 4ºC hold indefinitely	

Samples are ready for cycle sequencing. *Post-amplification quantitation 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.

5 Sampling

Not applicable.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

8.1 The post-amplification yields of the positive and negative amplification controls indicate whether the amplification process is successful.

8.2 Samples that have been extracted on the EZ1 are only approved for amplification of WCR and HV1/HV2. Samples that have been extracted on the QIASymphony are only approved for amplification of WCR.

9 Safety

9.1 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures.

10 References

FBI Laboratory Quality Assurance Manual

FBI Laboratory Safety Manual

DNA Procedures Manual

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

Rev. #	Issue Date	History
1	02/28/18	Adjusted scope to apply to DNA personnel Adjusted applicability of primer set section. Added guidance about appropriate water to section 4. Added automated procedures as sections 4.2 and 4.7. Renumbered existing sections. Added allowance for repeating ExoSAP-IT process Added Appendix A from Mito QC instructions in STACS
2	10/28/20	2: Update to reagent grade bleach. 4.1: Added manual to header. 4.2.7: Added RB to note. 4.4.1 and 4.5.1: Added/reworded to region for consistency w/4.3.1. 4.5: Edited the intro. 4.5.3: Added to the note to clarify the addition of water. 4.6: Added manual to header. 4.6.1: Doubled volumes as standard procedure and added caveat to allow for use of half the volume. Both volumes were validated. 4.7.4: Changed final note from this section to purification to allow for manual purification if repeated. App A: Revised critical reagent instructions and added miniprimers

Approval

Redacted - Signatures on File

DNA Technical Leader Date: 10/27/2020

DCU Chief Date: 10/27/2020

DSU Chief Date: 10/27/2020

Appendix A: Quality Control Procedures

1. Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., DNA QA 608) for minimum frequency of performance verifications and additional requirements.

A. Performance Verification (PV) of the Tecan Robotic Workstation

Refer to the nuclear DNA quantification procedure (i.e., nDNA 226) for instructions for the PV of the Tecan Robotic Workstation.

B. Performance Verification of the 9700 Thermal Cycler

1. 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.

2. 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.

3. 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.

4. 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.

2. Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., DNA QA 609) for additional requirements.

A. Amplification Reagents

The following reagents will be tested by amplifying a positive (HL60) and negative control sample with the WCR for 32 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.

1. 10X PCR Buffer
2. AmpliTaq Gold
3. Deoxyribonucleotide triphosphate mix (dNTPs)
4. Bovine Serum Albumin (BSA)
5. ExoSAP-IT

B. Positive Control

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

C. 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	MPS1, 2, 3, 4, 5