Amplification of Mitochondrial DNA

Table of Contents

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1 INTRODUCTION

These procedures describe the processes for amplification of mitochondrial deoxyribonucleic acid (mtDNA) via the polymerase chain reaction (PCR) and the PowerSeq™ CRM Nested System. The PowerSeq™ CRM Nested System produces sample libraries by converting a DNA sample into a library of fragments covering the control region of the mitochondrial genome (MCR) in one multiplex. The PCR amplifies the target amplicons and incorporates indexed sequencing adapters. The DNA Casework Unit (DCU) uses robotic workstations to automate the amplification.

2 SCOPE

These procedures apply to DNA personnel who perform amplification of mtDNA 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 (ProFlex PCR System 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
- Stratalinker
- STACS™ Casework Software (STACS), Sample Tracking and Control Solutions [STACS DNA Inc.] part of InVita Healthcare Technologies, version 5.1 or higher

3.2 Reagents

Refer to the appropriate DNA QA procedure [i.e., BIO-103] for reagent and control preparation information.

- Water (molecular grade or equivalent)
- PowerSeq™ CRM Nested System (Promega)
    - 5× Master Mix
    - 10× Primer Pair Mix
- Prepared index plate of PowerSeq® Nested System Index Primers (Promega)
- Positive control DNA 2800M (prepared to 2,000 cn/µL), (Promega or equivalent)
- 3% bleach (reagent grade or equivalent)
- Purified water or equivalent, available at laboratory sinks
- RoboScrub solution (Liquinox™ or equivalent)

4 STANDARDS AND CONTROLS

A positive amplification control (2800M) and negative amplification control (also referred to as the AMPBLANK or NC) must be processed in parallel with each set or batch of evidentiary samples subjected to polymerase chain reaction (PCR) amplification. The positive amplification control is 2800M at 2,000 copy number (cn)/µL. The negative amplification control is the maximum volume of molecular grade water that can be accommodated by the PCR volume (i.e., 12.5 µL). The positive and negative amplification controls must be amplified concurrently (i.e., in the same instrument) with the forensic samples to which they will be associated.

At least one reagent blank (RB) from an extraction batch must be amplified using the same primers, same instrument, and same concentration conditions as required by the sample(s) in the extraction batch 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. The RB is not required to be reamplified with samples in the associated extraction batch as long as an RB has been amplified using the same typing test kit, instrument model, and sensitivity conditions as the samples within the extraction batch. Amplification reworks must contain RB controls if the reworks are being processed with an increased level of sensitivity (e.g., lower number of total pool samples.)

Each sample and control in an amplification batch will have a unique combination of indexing primers added during amplification that will be used to identify samples during subsequent sequencing.

For evaluation of the amplification controls, refer to the appropriate mtDNA interpretation procedure [i.e., BIO-571].

5 PROCEDURE

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), molecular 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.

Ensure the appropriate fields (i.e., instruments, reagents) in STACS are completed from any network computer, as necessary.

5.1 Preparation of the Tecan Robotic Workstation

1.	Prior to daily use: • Clean the outside of the Tecan tips with 70% isopropyl alcohol • Decontaminate the Tecan work deck with 10% bleach. • Run the appropriate daily start up script.

	• The 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. Prior to each mtDNA Amplification run: • Make ~100mL of 3% bleach to replace bleach in front trough. • Bleach must be made and replaced at the beginning of each run. • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. If replacing the carboy, rerun the daily start up script. When a carboy is refilled, it should be allowed to de-gas overnight before use. • Check volume of waste container and empty if needed. • Ensure the tubing is properly submerged in the intake water and not coiled or kinked. As needed: • Clean barcode scanners with a lint free cloth.

5.2 Prepare Sample Racks/Create a Scan File Import

Ensure all DNA extracts and reagent blanks (aka sample tubes) are in Tecan compatible tubes and are appropriately barcoded.

If needed, molecular grade water may be added to a sample and/or RB in order to obtain a necessary volume. 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 AMPBLANK and, if applicable, the RB.

1.	Place tubes into Tecan sample rack(s): • Sample tubes start in position 1 of sample rack 1 and continue on to a second rack as needed (for up to 30 samples). • A tube of positive control DNA (with an appropriate [i.e., UW] barcode) is in the position immediately after the last sample tube. • A new tube with an "AMPBLANK" barcode is in the position immediately after the positive control DNA. • Any rack position(s) unfilled by a tube as described above must contain empty tubes with unique “BL” barcodes.

A batch must have a minimum of 8 samples and maximum of 32 samples [including controls].

“BL” barcode tubes may be reused; however, each “BL” barcode on the Tecan must be unique.

2.	• Load the sample racks onto the Tecan. • Use the appropriate script to scan the sample racks and generate a .csv scan file. • Import the scan file into STACS.

5.3 Master Mix Preparation

Master Mix may be prepared at most convenient point prior to loading on the Tecan robot.

1.	• Prepare master mix (MM) based on volumes below. • Equally distribute the master mix between two labeled 2 mL v-bottom tubes. • Vortex and quick spin tubes.

PowerSeq™ CRM Master Mix Component	µL per Sample
5x Master Mix	5
10x Primer Pair Mix	2.5
TOTALS	7.5

Master mix volume calculations should account for the number of samples, RB(s), positive control (2800M), AMPBLANK, and an overage of ~6 samples per amplification plate.

5.4 Preparing the Tecan Deck

The below steps may be performed in any order prior to running the Tecan robot. Positions of racks may vary between instruments. The robotic script will direct the placement.

1.	Bleach Rack: • Ensure the 3% bleach solution in the front trough was replaced prior to each mtDNA amplification run.

2.	Water Rack: • Prior to each use, replace the molecular grade water in the center trough. Ensure the water is filled above the line (i.e., contains at least ~200 mL). • UV the water trough containing molecular grade water for 15 minutes (900 seconds) prior to use.

3.	Plate Rack: • Place a 96-well plate into a base. Place into the front position of the plate rack. Ensure an amplification batch barcode (i.e., AMC) is on the right side of the base. • Place a 96-well 2.0 mL deep well plate with a working plate barcode (i.e., WKP) on the right side of the plate into the center position of the plate rack. • Place a sealed index plate in the rear position of the plate rack. *Do not remove the seal until prompted by the script.* ◦ Ensure the index plate barcode is moved to the right side of the plate base.

4.	Master Mix Rack (see Figure 1): • Place two uncapped tubes (with "C1" barcodes) containing equal volumes of master mix in positions 3 and 4. • Place empty tubes (with unique "BL" barcodes) in positions 1-2 and 5-16.

"BL" barcode tubes may be reused; however, each "BL" barcode on the Tecan must be unique.

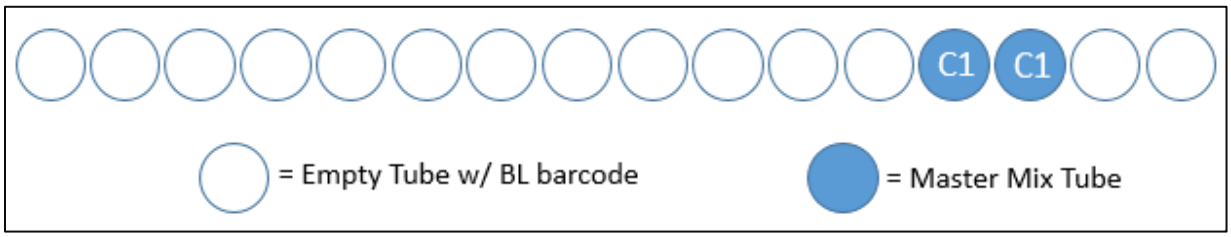

Figure 1 - Master Mix Rack

5.5 Amplification Plate Setup

1.	Run the current version of AMPSTR for the amplification setup file and then the index plate file.

The amplification setup file must be processed before the index plate file to obtain all needed worklists.

2.	Run the current NGS Amp script and follow the prompts to ensure the appropriate racks are on the deck as required. • Enter number of sample tubes [i.e., samples and RB(s)] when prompted. **(DO NOT include amplification controls.)**

3.	Follow the script prompts for the dilution plate. The script will include prompts to perform the associated tasks: • Seal the dilution plate with a foil cover. • Ensure that the edges of each well are well-sealed. • Invert the dilution plate several times, attempting to shake the liquid off the bottom of the plate. Firmly seat the dilution plate on the Te-shake and follow computer prompts to turn on the plate shaker.

If no dilutions were prepared, the dilution plate does not need to be manipulated while following the script prompts.

The seal may be applied with the Thermal Microplate Sealer or, if needed, manually.

The plate will shake for ~1.5 minutes. If necessary, the dilution plate may be manually vortexed with a corresponding batch comment in the notes.

4.	• Remove the dilution plate from the Te-shake and invert several additional times, attempting to shake the liquid off the bottom of the plate. • Centrifuge the dilution plate for ~1.5 minutes at ~2000 rpm. • Remove the dilution plate from the centrifuge and carefully remove the foil seal. • Return the dilution plate to its original deck position **in the correct orientation**. • Ensure that the dilution plate is properly positioned **BEFORE** selecting "OK" to continue with the script.

5.	When prompted by the script: • Remove the index plate from the Tecan, vortex, and spin down. • Carefully remove the index plate seal and return the index plate to its original deck position in the correct orientation. • Ensure that the index plate is properly positioned **BEFORE** selecting "OK" to continue with the script.

The Tecan will prepare the amp plate so that each well contains 7.5 µL of master mix, 5.0 µL of index primer combination, and 12.5 µL of DNA template (i.e., neat sample, sample plus water, or diluted sample) or the appropriate control (See the Calculations and Limitations sections for more information.)

6.	• Upon completion of the Tecan run, seal the amplification plate with a clear seal. • Ensure that the edges of each well are well-sealed. • Quick spin (generally ~2,000 rpm for 5 seconds).

The seal may be applied with the Thermal Microplate Sealer or, if necessary, manually.

Unused wells on the index plate can be used for future amplifications. Seal the index plate and store frozen. Discard the index plate if there are fewer than 8 unused wells left on the plate.

Sample tubes should be removed from the Tecan deck and capped prior to proceeding to the Amplification (Amp) room. Ensure the amp batch barcode is on a side of the amplification plate and proceed to the Amp room.

5.6 PCR Amplification

1.	• Place the amp plate in an appropriate thermal cycler and close the lid by pressing the lever down completely. • Start the **PowerSeq mtDNA CR-30 Cycles** program and verify block.

The thermal cycler will run the following:

- 96°C for 10 minutes
- 30 cycles:
  - 96°C for 5 seconds
  - 60°C for 35 seconds
  - 72°C for 5 seconds
- 60°C for 2 minutes
- 4°C hold indefinitely

Upon completion of the PCR amplification, the amp plate should be stored until needed for library purification. Plates should be stored refrigerated overnight or frozen for longer periods of time.

6 CALCULATIONS

The volume of DNA extract used in the amplification is calculated using the quant results of each sample [from BIO-521], the maximum volume of sample allowed in the amplification reaction, and the target DNA amounts. An examiner may adjust the volume of sample and/or reagent blank amplified, as needed, provided the reagent blank is not diluted more than the associated sample. An examiner can override or adjust the dilutions that the Tecan will make within STACS (See Limitations).

The following is the basis for determining the volume of sample to use for amplification:

$$C_1V_1 = C_2V_2 \rightarrow V_1 = (C_2V_2)/C_1$$

Where C_1 = Quant result (cn/µL)

V_1 = Volume of sample to add to amplification reaction (µL)

C_2 = Target concentration (i.e., 2000 cn/µL = 25,000 cn/12.5 µL)

V_2 = Maximum input volume (i.e., 12.5 µL)

Example inputs for PowerSeq CRM:

Average sample quant result (cn/µL)	Volume to add to amplification for target concentration of 25,000 total copies per 12.5 µL
<2,000	12.5 µL sample
2,000	12.5 µL sample
10,000	2.5 µL sample +10 µL water

7 LIMITATIONS

- Successful amplification is dependent upon the quantity or quality of DNA in the sample.
- Target DNA amounts are determined during validation and set in STACS. STACS uses the quantitation results and the target copy number (cn) of DNA to determine the volume of input DNA or the default dilution calculation; however, the input volumes may be adjusted by an examiner such that more or less DNA sample is used.
    - Generally, 25,000 copies of DNA is targeted for PowerSeq CRM.
    - For samples that quant at less than 2000 cn/µL, the default is the maximum input of 12.5 µL.
- The Tecan is not maintained to pipette less than 2 µL. As a result, with a target of 25,000 copies per 12.5 µL, the default is to make a dilution for any sample with a quant value >12,500 cn/µL. An examiner should adjust the amplification setup sheet information, as appropriate, to prevent the Tecan from using more sample than necessary to make a dilution while ensuring an appropriate dilution volume is queued.
    - When adjusting the amplification setup information, generally the minimum volume accepted for a dilution is 40 µL total (i.e., diluent plus sample) and the accepted maximum volume of diluent is 1200 µL.
    - The Tecan has a maximum dilution that can be made. Generally, samples with quant values that would require exceeding the maximum dilution need to be manually diluted and requanted prior to amplification.
        - Samples may be diluted and quanted concurrently with the neat extract to reduce the need to requant.

8 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.
- Avoid reaching into the Tecan robot while it is running as personal injury could result from moving robot accessories

9 REFERENCES

Applied Biosystems. ProFlex PCR System User's Guide (Pub no. MAN0007697, rev B.0, 2016).

ARTEL. *MVS Multichannel Verification System User Guide.* 2006

Promega Corporation. *Massively Parallel Sequencing of Mitochondrial Control Region using the PowerSeq® CRM Nested System. Promega Application Note AN322.*

10 REVISION HISTORY

Revision	Issued	Changes
00	12/16/2024	New procedure.

11 APPENDIX A: QUALITY CONTROL PROCEDURES

11.1 Instruments

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

11.1.1 Tecan Robotic Workstation

A. General Maintenance of the Tecan Robotic Workstation
    1. RoboScrub cleaning should be performed weekly, generally at the end of a workday:
        i. Make ~3.5 L of diluted Liquinox (see instructions on the label of the bottle for preparation)
        ii. ~3.5 L purified water in a separate container is needed
        iii. Run the RoboScrub Clean script, and follow the prompts
B. Refer to the nuclear DNA quantification procedure (i.e., BIO-520) for instructions for the Performance Verification (PV) of the Tecan Robotic Workstation

11.1.2 ProFlex™ Cleaning and Performance Verification

Refer to the ProFlex™ PCR System User Guide for instructions on how to perform the following procedures.

A. Cleaning – Refer to the chapter for maintaining the instrument for instructions on how to clean the sample wells and heated cover.
B. Verify Block Temperature –These tests are found in the Block Verification Test screen and requires the use of a Temperature Verification System for the following test types:
    1. Heated Cover Test - This test verifies the proper functioning of the heated cover.
    2. Verification Test - This procedure verifies that the thermal cycler remains within the temperature accuracy specification.
    3. Temperature Non-Uniformity Test - This procedure verifies the temperature uniformity of the sample wells in the thermal cycler.

11.2 Critical Reagents

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

11.2.1 Amplification Reagents

The following reagents will be tested using the PowerSeq CRM Nested System and processed through sequencing. New lots of reagent may be concurrently tested or tested with in-use lots of the counterpart reagents. The test batch will include at least one positive control (2800M) at 2,000cn/ul (or another sample with known types) and a negative control (AMPBLANK) sample. Testing may be conducted using replicates of 2800M (or other samples with known types) or

may be run in parallel with casework reference samples (but NOT forensic samples) to complete the batch.

A. 5 × Master Mix
B. 10× Primer Pair Mix
C. 2800M (prepared to 2000 cn/µL)
    1. When practicable, an in-use lot of 2800M will be run in a batch with a new lot of 2800M when run in parallel with casework reference samples.
D. PowerSeq Nested System Index Primers [Set 1: D701-D712; Set 2:D501-D508]:
    1. Each index primer must be tested in at least one combination with a positive control or known sample.
    2. Every combination of set 1 with set 2 will not be tested.
    3. A negative control (AMPBLANK) will be run with the QC batch, but each primer does not need to be run with a negative control.

Passing Criteria:

- All 2800M (or known) samples must yield data of the expected sequence and ≥200 reads.
- The AMPBLANK should result in no reads ≥200.
    - If the AMPBLANK results in ≥200 reads it will be evaluated on an individual basis and may be used according to the following guidelines:
        - The AMPBLANK results in ≥200 reads and is <10% of the read depth for the corresponding portion of the associated samples.
        - The AMPBLANK results in ≥200 reads and ≥10% of the read depth for the corresponding region of the associated samples but the read depth is < the associated sample read depth at all positions ≥200 reads and not in concordance with the sample in one or more amplicons.

Samples run in parallel with a new lot of critical reagents may be interpreted after the successful evaluation of the QC samples.

If the data does not meet the above listed criteria, the Technical Leader will be consulted and the assessment will be repeated, as necessary. The use of data for samples run in parallel with a new lot of critical reagent(s) that do not meet the passing criteria will be determined in consultation with the TL.