Pre-Amplification Quantification of Mitochondrial DNA

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1 INTRODUCTION

These procedures describe the pre-amplification quantification of mitochondrial deoxyribonucleic acid (mtDNA) extracts. Extracts are quantified by quantitative real-time polymerase chain reaction (qPCR) and the results used as a guide for subsequent amplification. The DNA Casework Unit (DCU) uses robotic workstations to automate the set-up of the quantification plates.

2 SCOPE

These procedures apply to DNA personnel that perform pre-amplification qPCR of mtDNA extracts and DNA personnel that perform the associated quality control procedures.

3 EQUIPMENT

3.1 Equipment/Materials

- General laboratory supplies (e.g., tubes, pipettes, vortex, centrifuge)
- Tecan robot model 'Freedom EVO'
    - Tecan EvoWare software, version 2.3 or higher
- 7500 Sequence Detection System, Applied Biosystems
    - HID Software, version 1.2 or higher
- Microcentrifuge tubes (robot compatible)
- 96-well Plate, Applied Biosystems MicroAmp® optical or equivalent
- Clear plate seals
- Thermal Microplate Sealer
- 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.

- mtDNA Quantitative PCR Standard Dilution Series - Double Stranded Synthetic Standard (dsT8sig), 10,000,000 to 10 copies/uL (Integrated DNA Technologies)
- TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific)
- mtDNA Quantitative PCR Primer/Probe/IPC Mix (PPI Mix)
    - Primers: Qfor8, Qrev8, L, M, G, B (Integrated DNA Technologies)
    - Probes: QRL8, C, U (Thermo Fisher Scientific)
    - Internal Positive Control (IPC), (Integrated DNA Technologies)
- 2800M (2,000 cn/uL) calibrator (Promega)
- TE^{-4} Buffer (DNA Suspension Buffer), (Fisher Scientific or equivalent)
- 3% bleach, Molecular grade
- Roboscrub solution (Liquinox™ or equivalent)
- Purified water or equivalent, available at laboratory sinks
- Water, molecular grade or equivalent

4 STANDARDS AND CONTROLS

The standard dilution series will be run in duplicate on each plate to generate the standard curve that is used to extrapolate the quantity of DNA in each sample. Two Master Mix (MM) controls will be run on each plate as a negative control. A TE^{-4} control must be evaluated with the first run of a prepared standard dilution series. Evaluation of these standards and controls can be found in the Data Evaluation section of this procedure.

The 2800M calibrator will be run in duplicate and is used in determining the degradation index.

The reagent blank(s) (RB) associated with each sample or batch will be quantified to determine the RB with the greatest (if any) signal.

5 PROCEDURE

Refer to DNA Procedures Introduction [i.e., BIO-100] for applicable laboratory quality assurance 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 laboratory sink faucets (typically labeled DE), is used for Tecan operation and is also called Tecan system liquid.

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

5.1 Preparing the Tecan Robotic Workstation

1.	Prior to daily use: • Make ~100mL of 3% bleach to replace in front trough. • 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 run: • Ensure the 3% bleach solution in the front trough was replaced prior to first daily use. • 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 • 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 Preparing the Sample Rack and Creating a Scan File Import

DNA extracts can be diluted with molecular grade water (generally 1:50) and run concurrently with the neat DNA extract (See Limitations).

Ensure all sample tubes (i.e., DNA extracts, diluted extracts, and reagent blanks) are Tecan compatible and appropriately barcoded. Ensure all tubes have been vortexed, spun down, and are uncapped prior to run.

1.	• Place sample tubes in positions 1 through 16 in the sample rack(s). Use up to 2 sample racks (32 sample tubes total), as needed. • Any rack position(s) unfilled by a sample tube must contain an empty tube with a unique "BL" barcode.

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

2.	• Use the appropriate Tecan script to load and scan the sample rack(s) and generate a .csv scan file. • Import the file into STACS. • A plate map file will be generated by STACS.

5.3 Master Mix Preparation

This step may be performed any time prior to loading the master mix on the Tecan.

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.

MM Component	**µL per well***
TaqMan® Fast Advanced Master Mix	10
mtDNA Quantitative PPI Mix	8

*Number of wells = [Number of samples x 2] + 14 for standards + 3 for controls + overage (~8)

The PPI Mix is stored frozen until first use. Once thawed, the PPI Mix is stored refrigerated and may be used for up to one month.

5.4 Preparing the Tecan Deck

The steps below 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.

Before loading, vortex and quick spin the prepared standard dilution series and 2800M and ensure all tubes are uncapped.

1.	Prepare the Standards Rack (See Figure 1): • Positions 1 through 7: the mtDNA Quantitative PCR Standard Dilution Series • Position 8: a tube of 2800M containing at least ~10 µL • Position 16: the tube of TE^{-4} control

The TE^{-4} control is provided with the mtDNA Quantitative PCR Standard Dilution Series and must be evaluated with the first run of the dilution series.

The mtDNA Quantitative PCR Standard Dilution Series may be used up to one month from the date of preparation. Prepared standards will be stored refrigerated and labeled with the preparation and expiration dates.

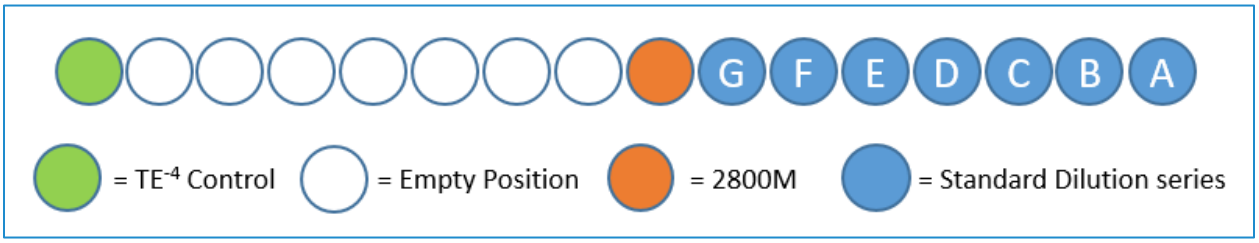

Figure 1 - Standards Rack

2.	Plate Rack: • Place a 96-well plate into a base in the front position of the plate rack. Ensure a quant batch barcode (i.e, QMQ) is on the right side of the plate base.
3.	Place tubes in the Master Mix Rack (See Figure 2): • Positions 3 and 4: the two tubes containing equal volumes of master mix. Ensure tubes are uncapped. • Positions 1, 2, and 5 through 16: empty tubes (with unique "BL" barcodes).

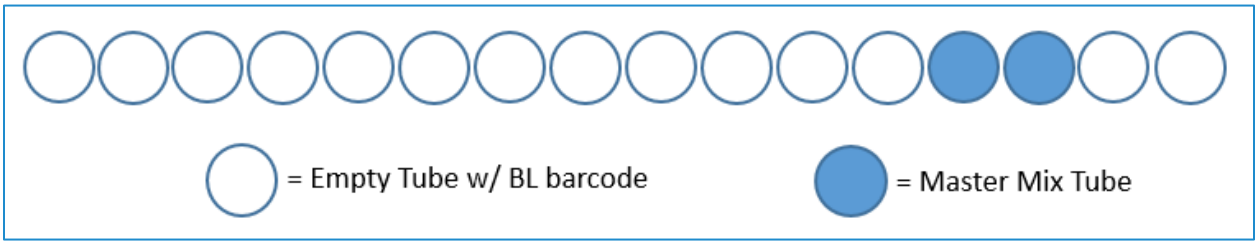

Figure 2 - Master Mix Rack

5.5 Tecan Plate Preparation

1.	• Run the current **NGS mtDNA qPCR Degradation Assay** script.

The Tecan will add 18 µL of master mix and 2 µL of each standard, sample, RB, 2800M, and master mix control (in duplicate) and of one TE^{-4} control to the 96-well plate.

2.	• Seal the plate with a clear seal. • Quick spin (generally ~2,000 rpm for 5 seconds).

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

Reagents and sample tubes should be removed from the Tecan deck and capped prior to taking the sealed quant plate to the Amp room.

5.6 Real-Time PCR

1.	• Place the sealed plate into the 7500 so that well A1 is in the back-left
2.	• In the 7500 software, open the Mito Degradation Template. • Import the plate map file generated by STACS.
3.	• Save the run file (.eds), ensure the 7500 door is closed, and start the run.

5.7 Data Evaluation/Acceptance Criteria

Use the 7500 software to review the results.

1.	• Review the Standard Curve plots of C_T (cycle threshold) versus Quantity (DNA concentration). • Use the Target dropdown menu to view the "QRL" curve results. • Review the parameters of the Standard Curve (i.e., slope, R^2, and Y-intercept).
1A.	A passing run will have: • $R^2 \geq 0.985$ • Slope in the range of -3.200 and -3.600 • Y-intercept in the range of 36.100 and 39.600
1B.	If the R^2 value is < 0.985, if the slope or Y-intercept is out of range, or if there is a visible outlier, omitting a poor replicate of a standard(s) and reanalyzing may result in passing values for the standard curve. (*For each standard pair in the dilution series, only one of the replicates may be omitted*).

To omit a replicate, right click on the well and choose omit, then reanalyze the data.

1C.	If the R^2, slope, or Y-intercept do not meet the required values, the plate fails, the data is not suitable for evaluation, and the samples must be re-quantified.

If two or more wells are omitted from the standard curve or if the R^2, slope, or Y-intercept do not meet the required values, the standard dilution series should be discarded.

2.	• Export the results from the 7500. In STACS: • Import the results files (.txt and .eds). • Record the results for the slope, R2, and Y-intercept of the QRL detector only.
3.	Check the quantification results of the Master Mix control. • The Master Mix control should display no quantifiable DNA. • If a DNA value of greater than 10 copies/µL appears in the master mix control, a contaminant may be present. For plates using a new DNA standard dilution series, check the quantification result and IPC C_T of the TE^{-4} Control. • If a DNA value of greater than 10 copies/µL appears in the TE^{-4} control, a contaminant may be present.

Samples can proceed to amp even if a DNA value of greater than 10 copies/µL appears in any of the negative controls.

4.	The sample data should be evaluated for an indication of possible inhibition based on the IPC.

The IPC is generally expected to be between 29 and 31. An IPC >31 may indicate inhibition.

Samples that have an indication of possible inhibition may be diluted and re-quantified. Molecular grade water is used to dilute samples as appropriate. Any dilution(s) made will be recorded in the case notes.

If both of the 2800M calibrators do not yield results, the samples should be re-quantified to allow for calculation of the degradation index.

The degradation index (ΔΔCT) can be used to inform amplification strategy. (See Calculations)

6 CALCULATIONS

- Degradation index (ΔΔCT):

$$\Delta C_T = C_{T\,300} - C_{T\,100}$$
$$\Delta\Delta C_T = \Delta C_{T\,\text{sample}} - \Delta C_{T\,\text{2800M calibrator}}$$

- Guidance for use of the pre-amplification quant data to determine the sample volume for amplification is in the mitochondrial DNA amplification procedure [i.e., BIO-532].

7 LIMITATIONS

- The accuracy of the results obtained from the qPCR are dependent upon the precision of the standard curve and the results should be reviewed in accordance with the parameters listed in the Data Evaluation section of this document.
- The resulting values are an estimate of the quantity (copies/µL) of mitochondrial DNA in the sample.
- Samples with DNA quantities outside of the range of the standard curve may be less accurately estimated. Since this can have an impact in downstream dilutions of high quantity samples, samples with quant values that exceed 10,000,000 cn/µL (the highest quantity sample in the standard curves) should be diluted and requanted.
    - The DNA extracts may be diluted prior to quantitation and quanted concurrently with the neat extracts to prevent 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.

9 REFERENCES

Applied Biosystems. *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide*. 2006.

Applied Biosystems. *Applied Biosystems 7500/7500 Fast Real-Time PCR System Maintenance Guide*. 2010.

Kavlick, M.F. *Development of a triplex mtDNA qPCR assay to assess quantification, degradation, inhibition, and amplification target copy numbers.* Mitochondrion, *in press.* Available online at: *https://doi.org/10.1016/j.mito.2018.09.007*

10 REVISION HISTORY

Revision	Issued	Changes
00	07/01/2022	Reformatted DNA 404-1 into new template and assigned new Doc ID. Minor edits throughout.
01	12/16/2024	Revisions for the use of STACS and subsequent NGS typing.

11 APPENDIX A: QUALITY CONTROL PROCEDURES

11.1 Instruments

Refer to Instrument Calibration and Maintenance procedure [i.e., BIO-104] for the minimum frequency and additional requirements.

11.1.1 General Maintenance of the AB 7500 Real-Time PCR System

A. Once a year, general maintenance is performed as part of the annual PM.
B. For semi-annual general maintenance, refer to the instructions in the Applied Biosystems *7500/7500 Fast Real-Time PCR System Maintenance Guide* to perform the following:
    1. Regions of Interest (ROI) Calibration (Chapter 2)
    2. Background Calibration and Optical Calibration (Chapter 3)
    3. Dye Calibrations (Chapter 4) for the dyes used in the assays run on the instrument.

11.1.2 Performance Verification of the AB 7500 Real-Time PCR System

A. Refer to the Quantifiler TRIO QC procedures [i.e., BIO-520] and the PowerSeq Quant QC procedures [i.e., BIO-542].

11.1.3 General Maintenance of the Tecan EVO Robotic Workstation

RoboScrub cleaning should be performed weekly, generally at the end of a workday:

A. Make ~3.5 L of diluted Liquinox (see instructions on the label of the bottle for preparation)
B. ~3.5 L purified water in a separate container is needed
C. Run the RoboScrub Clean script, and follow the prompts

11.1.4 Performance Verification of the Tecan EVO Robotic Workstation

A. An Artel MVS Multichannel Verification System and NIST traceable standards will be used to test the accuracy and precision of the liquid handling by the Tecan. Refer to the Artel MVS Multichannel Verification System User Guide for operation of the Artel MVS.
B. The Tecan Robotic workstations are typically configured with eight (8) fixed tips and there are multiple volumes aliquoted during each procedure. A minimum of 6 repetitions will be performed with each tip for each volume.
C. The results must be within the tolerance limits set by DCU within the Artel software for each volume. At times, it may be necessary to modify/optimize the Tecan liquid class parameters (e.g., offset and factor).
D. If the performance verification of the Tecan does not meet the above listed criteria, the performance verification will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

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 Performance Verification of the mtDNA qPCR Degradation Assay Reagents

Each new lot of dsT8sig standard secondary stock, TaqMan® Fast Advanced Master Mix, and mtDNA Quantitative PCR Primer/Probe/IPC Mix (PPI Mix) will be evaluated by running the standard dilution series, the 2800M calibrator, and appropriate controls, all in duplicate. Reagents may be simultaneously tested for reliability.

The new lot of reagents will be deemed suitable for use in casework if:

A. The slope is within the acceptable range of -3.200 to -3.600.
B. The R2 is ≥ 0.985.
C. The Y-intercept is within the acceptable range of 36.100 to 39.600

If the performance verification does not meet the above listed criteria, the performance verification will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.