DNA Casework Unit
Procedures for Pre-Amplification Quantification of Mitochondrial DNA

1 Scope

These procedures apply to DNA personnel performing pre-amplification quantification of mitochondrial deoxyribonucleic acid (mtDNA) extracts. Extracts may be quantified by Real Time Quantitative PCR (qPCR) and the results may be used as a guide for subsequent amplification. The DNA Casework Unit (DCU) uses robotic workstations to automate the set-up of the quantification (aka quant) plates.

2 Equipment/Materials/Reagents

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 Plates, Applied Biosystems MicroAmp® optical or equivalent
- Clear plate seals
- Thermal Microplate Sealer

Reagents
- mtDNA Quantitative PCR Standard Dilution Series - Double Stranded Synthetic Standard (dsT8sig), 10,000,000 copies → 10 copies, 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
- 20 pg/µL HL60 DNA (calibrator), ATCC or equivalent
- TE^{-4} Buffer (DNA Suspension Buffer), Fisher Scientific or equivalent
- 10% bleach, Daigger or equivalent
- 3% bleach, Molecular grade
- 70% Isopropyl alcohol, Sigma or equivalent
- Roboscrub solution, Liquinox™ or equivalent
- Purified water or equivalent, available at laboratory sinks
- Water, reagent grade or equivalent

3 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 processed 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 HL60 calibrator will be run in duplicate and is used in determining the degradation index for the samples but there is no evaluation criteria for this sample.

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

4 Procedures

Refer to the DNA Procedures Introduction (i.e., DNA QA 600) and follow applicable general precautions and cleaning instructions.
The purified water, available via laboratory sink faucets (typically labeled DE), is used for Tecan operation and is also called Tecan system liquid.
The Mito Quant 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 Quant workbook.

4.1 Preparing the Tecan Robotic Workstation
If necessary, turn on the Tecan, which will undergo an initialization routine. Log on to the Tecan computer, launch and logon to the current Tecan software.

| 4.1.1 | 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 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
As needed:
• Clean barcode scanners with a lint-free cloth | |

4.2 Preparing the Sample Rack and Creating a Scan File Import

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

4.2.1	Place DNA sample tubes in positions 1 through 16 in the sample racks. Use up to 2 sample racks (32 sample tubes), as needed. Any rack position(s) unfilled by a DNA sample tube must contain an empty tube with a unique "BL" barcode. Place sample racks in grids 4 and 5.	

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

4.2.2	Use the appropriate Tecan script to scan the sample racks and generate a .csv scan file. Import the file into the Mito Quant workbook.	

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

4.3.1	Prepare amplification master mix (MM) based on volumes below. Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin tubes.	

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

*Number of wells = [Number of sample tubes x 2] + [TE^{-4} control x 2, if needed] + [24 for standards, controls, and overage]

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

4.4 Preparing the Tecan Deck

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

4.4.1	Bleach/TE Rack: • Ensure the 3% bleach solution in the front trough was replaced prior to first daily use.	

| 4.4.2 | Place tubes in the Standards Rack (grid 3):
• Positions 1 through 7: the mtDNA Quantitative PCR Standard Dilution Series
• Position 8: an uncapped tube of 20 pg/µL HL60 containing at least 20 µL
• Position 16: if needed, the uncapped tube of TE^{-4} control | |

Before loading, vortex, quick spin, and uncap the prepared standard dilution series and HL60, as appropriate.

The TE^{-4} control is provided with the mtDNA Quantitative PCR Standard Dilution Series and must be processed with the first run of the dilution series. If a TE^{-4} control is not needed, position 16 will be empty.

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.

| 4.4.3 | Plate Rack:
• Place a 96-well plate into a base in the front position of the plate rack. | |

| 4.4.4 | Place tubes in the Master Mix Rack (grid 12):
• 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). | |

4.5 Tecan Plate Preparation

| 4.5.1 | Run the mtDNA qPCR Degradation Assay script.
The Tecan will add 18 µL of master mix and 2 µL of each standard, sample extract, and control, in duplicate, to the 96 well plate. | |

| 4.5.2 | 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.
The DNA sample tubes and standard dilution series tubes should be removed from the Tecan deck and capped prior to taking the sealed quant plate to the Amp room.

4.6 Real-Time PCR

4.6.1	Ensure the 7500 and the supporting computer are powered on. Place the sealed plate into the 7500 so that well A1 is in the back-left and the notched corner of the plate is in the back-right.	
4.6.2	In the 7500 software, open the Mito Degradation Template. (This can be done by selecting a custom assay and creating a New Experiment as From Template.) Import the file generated by the Mito Quant workbook.	
4.6.3	Save the run file, ensure the 7500 door is closed, and start the run.	

4.7 Data Evaluation

4.7.1	Using the 7500 software, review the results.	
4.7.2	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. Record the results for the slope, R^2, and Y-intercept in the Mito Quant workbook.	
4.7.2.1	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	
4.7.2.2	If the R^2 value is < 0.985, if the slope 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 if necessary*).	

To omit a replicate:
>   Right click on the well and choose omit, then reanalyze the data. (This may be done by clicking the green "Analyze" tab in the top right corner.)

4.7.2.3	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 requanted.	

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.

| 4.7.3 | Export the results from the 7500. (*This may be done by selecting Export from File menu, then choosing Results.*)
 Import the results file (.txt) into the Mito Quant workbook. | |

| 4.7.4 | Check the quantification results and internal PCR control (IPC) C_T 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. | |

| 4.7.5 | 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 control, a contaminant may be present. | |

If a DNA value of greater than 10 copies/µL appears in any of the negative controls, the samples may be re-quanted at examiner discretion.

If the HL60 calibrator does not yield results, the samples may be re-quanted at examiner discretion.

| 4.7.6 | The sample data should be evaluated for:
 • an indication of possible inhibition based on the IPC.
 • an indication of degradation based on the degradation index ($\Delta\Delta CT$). An examiner will review the quantification results and determine the mtDNA region(s) to amplify and the primers to use for amplification. | |

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-quanted. Reagent grade water is used to dilute samples as appropriate. Any dilution(s) made will be recorded in the case notes.

5 Sampling

Not applicable.

6 Calculations

$\Delta C_T = C_{T\ 300} - C_{T\ 100}$

$\Delta\Delta C_T = \Delta C_{T\ sample} - \Delta C_{T\ HL60\ calibrator}$

7 Measurement Uncertainty

Not applicable.

8 Limitations

The accuracy of the results obtained from qPCR are dependent upon the precision of the standard curve and the results should be reviewed in accordance with the parameters previously listed in the Data Evaluation section of this document. The resulting values are an estimate of the quantity of DNA in the sample.

9 Safety

Refer to the *FBI Laboratory Safety Manual*.

10 References

FBI Laboratory Safety Manual

DNA Procedures Manual

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*

Rev. #	Issue Date	History
0	02/05/16	Reformatted from Mitochondrial DNA Analysis Laboratory Procedures. qPCR Master Mix is now prepared in advance and instructions are in the QA reagent SOP and up to 4 freeze/thaws are approved. Extended allowable usage of previously prepared standards to up to 9 freeze/thaws.
1	02/15/19	Overhauled entire procedure for the new degredation assay including added sections to incorporate automated procedures. Added quality control procedures to Appendix A.

Approval   Redacted - Signatures on File

DNA Technical Leader   Date: 02/14/2019

DCU Chief   Date: 02/14/2019

QA Approval

 Quality Manager   Date: 02/14/2019

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 verification and additional requirements.

A. General Maintenance of the AB 7500 Sequence Detection System

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 standard dyes VIC, FAM, and NED

B. Performance Verification of the AB 7500 Sequence Detection System

The performance verification of the AB 7500 Sequence Detection System can be accomplished by running either the Quantifiler® DUO DNA Quantification Kit or the mtDNA qPCR Degradation Assay as both assays use the same dyes (FAM, VIC, NED). Instructions for performance verification using Quantifiler® DUO are in the procedure for quantitation of nuclear DNA (i.e., DNA 226). Follow the below instructions for running the performance verification with the mtDNA qPCR Degradation Assay:

1. Using in-use lots of reagents for the mtDNA qPCR Degradation Assay, run a plate containing the mtDNA Quantitative PCR Standard Dilution Series, the HL60 calibrator, and appropriate controls, all in duplicate.

2. The 7500 will be deemed suitable for casework analysis if:
    a. The slope is within the acceptable range of -3.200 to -3.600.
    b. The R^2 is ≥ 0.985.
    c. The Y-intercept is within the acceptable range of 36.100 to 39.600

C. General Maintenance of the Tecan Robotic Workstation

RoboScrub cleaning should be performed weekly, generally at the end of a workday:
1. Make ~3.5 L of diluted Liquinox (see instructions on the label of the bottle for preparation)
2. ~3.5 L purified water in a separate container is needed
3. Run the RoboScrub Clean script, and follow the prompts

D. Performance Verification of the Tecan Robotic Workstation

1. 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.

2. 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.

3. The results must be within the tolerance limits set by DCU for each volume. At times, it may be necessary to modify/optimize the Tecan liquid class parameters (e.g., offset and factor).

4. 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.

2. Critical Reagents

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

A. 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 HL60 calibrator, and appropriate controls, all in duplicate. 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 R^2 is ≥ 0.985.
    c. The Y-intercept is within the acceptable range of 36.100 to 39.600

More than one of the above reagents may be simultaneously tested for reliability.