Quantification of Human and Male Nuclear DNA

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1 INTRODUCTION

These procedures describe the process for quantification using real-time polymerase chain reaction (PCR) with the Quantifiler® Trio DNA Quantification Kit to determine the quantity of amplifiable human and male nuclear DNA (nDNA) detected in a sample.

2 SCOPE

These procedures apply to DNA personnel who perform quantification of human and male nuclear DNA, evaluate data, and/or perform the associated quality control procedures.

3 EQUIPMENT

3.1 Equipment/Materials

- Tecan robot model 'Freedom EVO'
    - Tecan EvoWare software, version 2.8 or higher
- 7500 Real-Time PCR System, Applied Biosystems
    - HID Real-Time PCR Analysis Software vs 1.2 or higher
- STACS™ Casework Software (STACS), Sample Tracking and Control Solutions [STACS DNA Inc.] part of InVita Healthcare Technologies, version 5.0 or higher
- General laboratory supplies (e.g., pipettes, tubes, vortex, centrifuge)
- Microcentrifuge tubes (robot compatible) (e.g., 2mL screw cap for master mix and 1.5mL screw cap for standards/samples)
- Speed-Vac, Vacufuge Concentrators, or equivalent
- 96-well Plates, Applied Biosystems MicroAmp® optical or equivalent
- Clear plate seals
- Thermal Microplate Sealer

3.2 Reagents

- Quantifiler® Trio DNA Quantification Kit
    - Prepared Quantifiler® Trio DNA standard calibrators, 1:10 (~10 ng/µl) and 1:50 (~2 ng/µl)
- Quantifiler® Automation Enhancer
- 3% bleach (reagent grade or equivalent)
- Purified water or equivalent, available at laboratory sinks
- Water (molecular grade or equivalent)
- Roboscrub solution (Liquinox™ or equivalent)
- Standard Reference Material (SRM) 2372 Human DNA Quantitation Standard (or equivalent)

4 STANDARDS AND CONTROLS

A master mix control and the prepared Quantifiler® Trio DNA standard calibrator samples will be run on each plate. Evaluation of these control samples can be found in the Data Evaluation section of this procedure.

The reagent blank(s) (RB) associated with each extraction batch are quanted to determine the RB with the greatest (if any) signal for amplification.

5 PROCEDURE

Refer to the DNA Procedures Introduction (i.e., BIO-100) for general precautions and cleaning instruction.

For water that will come into contact with the DNA samples (e.g., for reconstituted samples), molecular 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.

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

5.1 Preparing the Tecan Robotic Workstation

The below steps 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.

1.	Ensure the Tecan is prepared to run: ● Prior to daily use: ○ Make ~100mL of 3% bleach and 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 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: ○ 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 ● As needed: ○ Clean barcode scanners with a lint-free cloth
2.	If using the Tecan to reconstitute samples: ● Prior to each quant batch, replace the molecular grade water in the center trough. Ensure the water is filled above the line (i.e., contains at least ~200mL).

5.2 Concentrating Extracted Samples Using the Speed-Vac or Vacufuge

The Speed-Vac/Vacufuge should be turned on ~45 minutes prior to use. The Speed-Vac/ Vacufuge flask should be emptied as needed and the flask seal should be tight. Ensure the gasket on the centrifuge is in its proper position and that the rotor is properly tightened prior to sample processing.

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

- Samples from questioned items and corresponding RBs are generally concentrated to 15 µL or 25uL.
    - The volume of water to reconstitute will be recorded in STACS. This volume is determined by the type of sample or as requested by the examiner.
    - The volume used for the RB must be the same or less than the volume used for the associated samples.
- Known samples, female fractions from vaginal swabs, and similar sample types are generally not concentrated.

1.	Concentrate appropriate samples. • On the Speed-Vac with the heat set to "High", a 50 µL extract may take ~30-40 minutes to dry and a 100 µL extract may take ~60 minutes to dry. • On the Vacufuge with a setting of 60°C, a 50 µL extract takes ~45 minutes. • Samples should not be dried on "High" for more than four hours (maximum starting volume of ~400 µL).

2.	• Once dry, samples are reconstituted with molecular grade water.
	• If using the Tecan to add water:
	  • Load the dried tubes for a specific reconstituted volume group as recorded in STACS (i.e., all 15 µL samples or all 25 µL samples) onto Tecan sample rack(s) starting in position 1.
	  • Run the appropriate Tecan script to add water to the sample tubes.
	  • Repeat as needed for each different reconstituted volume, as appropriate.

3.	• Cap, vortex, and quick spin the tubes prior to preparing the sample rack(s) for the Quant Trio Plate Preparation in 5.3.

5.3 Preparing the Sample Racks and Creating a Scan File Import

1.	• Place sample tubes in positions 1 through 16 in the Tecan sample racks. Use additional sample racks as needed (up to 93 sample tubes on 6 racks). • 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 script to scan the sample racks and generate a .csv scan file. Import the file into STACS.

5.4 Master Mix Preparation

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

1.	• Create the master mix based on the volumes below. • Equally distribute the master mix between two labeled microcentrifuge tubes. • Vortex and quick spin.

Quantifiler Trio Master Mix Components

	µL per well*
PCR Reaction Mix	10.0
Primer Mix	8.0
Automation Enhancer	0.018**

*Number of wells = number of samples + 3 controls and appropriate overage (~6)

NOTE: *Master mix must be created for a minimum of 56 wells to prevent pipetting less than 1 uL of automation enhancer.*

**Round the total volume of automation enhancer to 2 decimals as appropriate for the pipette capability.

Vortex and quick spin the prepared Quantifiler® Trio DNA standard calibrators before loading. The 1:10 calibrator has an LU STACS barcode and the 1:50 calibrator has an LO STACS barcode. The calibrator samples will be labeled with Tecan compatible barcodes before loading. Calibrators may be used for 2 months after thawing for first use.

2.	Place the calibrators and master mix tubes in the master mix rack (see Figure 1): • Positions 1 and 2: 1:10 calibrator (with a “1:10” barcode) and 1:50 calibrator (with a “1:50” Barcode), respectively. • Positions 3 and 4: the two tubes containing equal volumes of master mix (with “C1” barcodes). • Positions 5 through 16: empty tubes (with unique “BL” barcodes).

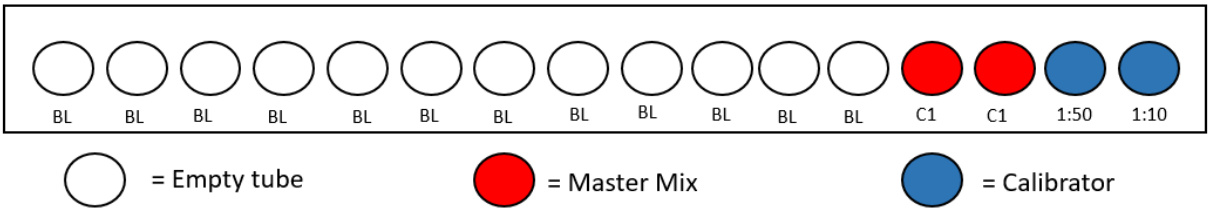

Figure 1 – Positioning for Master Mix Rack

5.5 Quant Trio Plate Preparation

Ensure all tubes are uncapped prior to run.

1.	• Ensure a 96-well plate, in a base if applicable, is in the front position of the plate rack so that well A1 is in the back-left and the quant batch barcode label is on the right side of the base or the plate, as appropriate.
2.	• Run the current version of the FBI Quantifiler Trio script and answer the prompts. • The Tecan will add 18 µL of Quantifiler® Trio master mix and 2 µL of each sample extract or control to the 96 well plate.
3.	• Seal the plate with a clear seal. • Quick spin (generally ~2,000 rpm for 5 seconds). • Ensure the quant plate barcode is on a side of the plate.

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

The sample tubes and calibrator 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 a new Trio run file. • Import the plate map (.txt file) generated by STACS for the quant batch as the plate setup file.
3.	• Save the run file (.eds) with the quant batch barcode in the file name, ensure the 7500 door is closed, and start the run.

5.7 Data Evaluation and Acceptance Criteria

1.	Review the results in the 7500 software. The data will be analyzed using the Virtual Standard Curve (VSC) settings.

If STACS prompts for the Standard Curve parameters, the targets below will be entered and "1" will be entered as the R^2 value.

Figure 2 – VSC Settings

2.	At least one calibrator must meet the C_T parameters for each of the targets below.

Calibrator Passing C_T Ranges

	1:10 (LU Barcode)	1:50 (LO Barcode)
T. Large Autosomal	19.6 - 22.3	21.8 - 24.0
T. Small Autosomal	21.6 - 24.5	23.7 - 26.7
T. Y	21.0 - 25.1	23.1 - 26.9
IPC	26.2 - 30.6	26.1 - 29.6

If either calibrator does not meet the above criteria, both calibrators should be discarded and a new set thawed for next use.

3.	• Export the results to the appropriate folder on the network. (Select Export from File menu, then choose Results.) • Import the results file (.txt) and run file (.eds) into STACS.
4.	Check the T. Large Autosomal, T. Small Autosomal and T.Y quantification results and IPC C_T of the master mix control. • If the master mix control displays a signal, the quantity value detected will be added as a comment in STACS.
5.	The plate will be successful if: • at least one calibrator meets the C_T parameters for each of the targets, and • the Master Mix control displays no quantifiable DNA (or if any detected value is noted in the comments, it was concluded to be spurious.)

The master mix control should display no quantifiable DNA. If a DNA concentration value appears in the master mix control, the concentration values obtained for the RB(s) run on the plate should be examined.

- If one or more of the RBs display no quantifiable DNA, the master mix value can be concluded to be spurious (i.e., not indicative of the presence of adventitious DNA) and the sample data should be used.
- The T. Large Autosomal target is not used for quantification. Values appearing in this target alone should not be considered.

The sample data can be evaluated to determine if any sample should be diluted and/or re-quanted. IPC C_T values are typically between 27 and 30. Undetermined IPC C_T values or values greater than 31 may indicate inhibition. At the discretion of the examiner:

- Samples that have an indication of possible inhibition may be diluted and re-quanted.
- Samples with excessive DNA (generally >300 ng/µL) should be diluted and re-quanted.

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

An examiner will review the quant results for each sample. STACS uses the quant results and the default amplification settings to determine the volume of sample to queue for amplification. Typically the T. Small quant value is used for STRs and the T.Y is used for YSTRs. An examiner should make adjustments to the amplification setup as necessary. Additional guidance is located in the nDNA amplification procedure (i.e., BIO-530).

6 CALCULATIONS

The 7500 software uses the standard curve equation:

$$C_T = m\ [\log (Qty)] + b$$

where m is the slope and b is the y-intercept as set for the virtual standard curve. The C_T is the cycle threshold measured during the Quantifiler Trio real-time PCR run and then used by the software to calculate the estimated starting DNA quantity (Qty). While this value is calculated by the software, by rearranging the standard curve equation the Qty can also be calculated with the formula:

$$Qty = 10\text{^}[(C_T\text{-}b)/m]$$

In general, a difference of 1 C_T equates to a two-fold difference in initial template amount. Therefore, if comparing quant data from 2 samples, a sample with 1 C_T higher will have a Qty ~1/2 that of the other sample and conversely a sample with 1 C_T lower will have a Qty ~2x that of the other sample.

7 LIMITATIONS

Trio may be affected by inhibition when amp kits are not. In such cases, it is possible that samples yielding no result at quant may yield DNA typing results.

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

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10 REVISION HISTORY

Revision	Issued	Changes
00	09/01/2022	Reformatted DNA 226-7 into new template and assigned new Doc ID. Consolidated reconstitution steps. Minor edits throughout.

11 APPENDIX A: QUALITY CONTROL PROCEDURES

11.1 Instruments

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

11.1.1 AB 7500 Real-Time PCR System

A. General Maintenance of the AB 7500 Real-Time PCR System
   1. Once a year, general maintenance is performed as part of the annual PM. 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:
      i. Regions of Interest (ROI) Calibration (Chapter 2)
      ii. Background Calibration and Optical Calibration (Chapter 3)
      iii. Dye Calibrations (Chapter 4) for standard dyes VIC and FAM and custom dyes ABY and JUN and, when applicable, for standard dye NED used for the mtDNA qPCR Degradation Assay.

B. Performance Verification (PV) of the AB 7500 Real-Time PCR System
   1. The performance verification of the AB 7500 Real-Time PCR System will be accomplished by running both the Quantifiler® Trio DNA Quantification Kit and, when applicable, the mtDNA qPCR Degradation Assay, as each assay uses different dyes. Refer to the above Quantifiler® Trio procedures and the procedures for the mtDNA qPCR Degradation Assay (i.e., BIO-521):
      i. Using an in-use lot of Quantifiler® Trio kit, run a plate containing the two calibrator samples, in triplicate, and appropriate controls.
      ii. 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:
      i. All replicates of the calibrator samples meet the C_T parameters in section 5.7 and
      ii. the slope, Y-intercept, and R^2 values for the mtDNA qPCR Degradation Assay meet the criteria of a passing run:
         a. $R^2 \geq 0.985$
         b. Slope in the range of -3.200 and -3.600
         c. Y-intercept in the range of 36.100 and 39.600
   3. If the performance verification of the 7500 does not meet the passing criteria for either assay, the unsuccessful plate(s) will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

11.1.2 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. 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 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.

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 Qualification of Quantifiler® Trio DNA Quantification Kit and Calibrators

A. Each new lot of Quantifiler® Trio kits will be evaluated by running a standard dilution series (50, 5, 0.5, 0.05, 0.005 ng/µl) of the NIST SRM 2372 Component A (in duplicate), the prepared calibrators, and appropriate controls (include a TE^{-4} control to account for prepared calibrator diluent) and analyzed with the VSC.
B. The new Quantifiler® Trio kit lot will be deemed suitable for casework analysis if:
    1. both replicates of three of the five SRM dilutions fall within 30 percent of the expected values for each of the three targets (small autosomal, large autosomal, and Y) and
    2. both calibrators meet the C_T parameters in section 5.7.

If the SRM dilutions do not meet the passing criteria, new dilutions will be prepared and the plate will be re-run. If the calibrators do not meet the passing criteria, the dilutions will be adjusted and the plate will be re-run. If the results are still deemed unsuitable, the Technical Leader will be consulted. The VSC will be reevaluated if there is an indication that it is no longer working appropriately.