Post-Amplification Quantification of Mitochondrial DNA

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1 INTRODUCTION

These procedures describe the process for quantification of amplified mitochondrial deoxyribonucleic acid (mtDNA) following post-amplification purification using the Agilent 2100 Bioanalyzer and a DNA1000 or 7500 Series II LabChip kit.

2 SCOPE

These procedures apply to DNA personnel that perform quantification of purified mtDNA amplicons and DNA personnel that perform the associated quality control procedures. These procedures are not performed on known samples that are amplified using automation.

3 EQUIPMENT

3.1 Equipment/Materials

- General laboratory supplies (e.g., pipettes, centrifuge)
- 2100 Bioanalyzer, Agilent Technologies
    - 2100 Expert software (Rev. B.02.03 or above).
- Bioanalyzer Chip priming station, Agilent Technologies
- Vortex mixer
- DNA LabChip®, Agilent Technologies

3.2 Reagents

- DNA1000 or 7500 Kit, Agilent Technologies.
    - DNA1000 or 7500 Dye concentrate, if needed
    - DNA1000 or 7500 DNA gel matrix, if needed
    - DNA1000 or 7500 gel-dye mix
    - DNA1000 or 7500 markers
    - DNA1000 or 7500 ladder
- Water, Reagent Grade or equivalent

4 STANDARDS AND CONTROLS

All corresponding reagent blanks (RB) and amplification controls (Negative Control [NC] and Positive Control [i.e., HL60]) to a sample undergoing quantification must also be quantified.

For evaluation of the quantification data, refer to the section 5.4 of this procedure.

5 PROCEDURE

Refer to DNA Procedures Introduction (i.e., BIO-100) and follow applicable general precautions and cleaning instructions.

The Mito 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 workbook.

5.1 DNA 1000 Series II Kit

The DNA 1000 kit is typically used for samples amplified with HV1A, HV1B, HV2A, HV2B, HV1, HV2 or mini-primer sets.

5.1.1 DNA 1000 Gel-Dye Mix Preparation

1.	• Allow dye concentrate and DNA gel matrix to equilibrate to room temperature for approximately 30 minutes. • Add 25 µL of dye concentrate into DNA 1000 gel matrix vial. • Cap and vortex well.
2.	• Transfer gel-dye mix to spin filter. • Spin at approximately 2240 X g for 15 minutes. • Discard spin filter. • Label tube (include date of preparation).

Use gel-dye mix within 4 weeks of preparation. Protect from light and store refrigerated.

5.1.2 DNA 1000 Chip Loading

1.	• Add 9 µL gel-dye mix to the well marked "G" (Circled G). • Place chip into priming station (syringe clip adjusted to lowest notch) with syringe set at 1 mL. • Close priming station and press plunger until held by syringe clip. • Wait 60 seconds, then release clip and pull back syringe plunger to 1 mL. • Open priming station and remove chip.
2.	• Add 9 µL gel-dye mix to 2 wells marked "G". • Add 1 µL of DNA 1000 ladder (yellow tube) to ladder well. • Add 5 µL of DNA 1000 markers (green tube) to ladder well. • Add 5 µL of DNA 1000 markers (green tube) to each sample well to be used. • Add 6 µL of DNA 1000 markers (green tube) to any unused sample well. • Add 1 µL control/sample to appropriate wells.
3.	• Place chip into vortex mixer and vortex for 60 seconds at 2400 rpm (speed may be lowered).

The chip should be run within 5 minutes of preparation to avoid failure due to evaporation.

Proceed to section 5.3.

5.2 DNA 7500 Series II Lab Kit

The DNA 7500 kit is typically used for samples amplified with WCR primers.

5.2.1 DNA 7500 Gel-Dye Mix Preparation

1.	• Allow dye concentrate and DNA gel matrix to equilibrate to room temperature for approximately 30 minutes. • Add 25 µL of dye concentrate into DNA 7500 gel matrix vial. • Cap and vortex well.
2.	• Transfer gel-dye mix to spin filter. • Spin at approximately 1500 X g for 10 minutes. • Discard spin filter. • Label tube (include date of preparation).

Use gel-dye mix within 4 weeks of preparation. Protect from light and store refrigerated.

5.2.2 DNA 7500 Chip Loading

1.	• Add 9 µL gel-dye mix to well marked "G". (Circled G) • Place chip into priming station (syringe clip adjusted to highest notch) with syringe set at 1 mL. • Close priming station and press plunger until held by syringe clip. • Wait 30 seconds, then release clip and pull back syringe plunger to 1 mL setting. • Open priming station and remove chip.
2.	• Add 9 µL gel-dye mix to 2 wells marked "G". • Add 1 µL of DNA 7500 ladder (yellow tube) to ladder well. • Add 5 µL of DNA 7500 markers (green tube) to ladder well. • Add 5 µL of DNA 7500 markers (green tube) to each sample well to be used. • Add 6 µL of DNA 7500 markers (green tube) to any unused sample well. • Add 1 µL control/sample to appropriate wells.
3.	• Place chip into vortex mixer and vortex for 60 seconds at 2400 rpm (speed may be lowered).

The chip should be run within 5 minutes of preparation to avoid failure due to evaporation.

5.3 Quantification Using Agilent 2100 Bioanalyzer

1.	• Insert chip into Bioanalyzer and close lid. • Select DNA 1000 II or DNA 7500 II, accordingly, from Assay Selection button at upper right of Instrument screen. • Enter Laboratory number (and sample name, if possible) in file prefix box in Destination section. • Enter number of wells to be run. • Click Start button at upper right (under Assay Selection button). • Fill out Sample Information table by clicking on "Data File: name" or by clicking on Data and Assay icon in Contexts panel.

Once loaded, the software should display a chip in upper left. If a chip is not displayed, carefully open then close the instrument lid.

If the volume is incorrect on the chip, upon beginning the run, the chip will typically fail with a voltage error. Inspect the chip for appropriate volume. It may be necessary to discard and prepare a new chip. Case notes will reflect when a new chip is prepared.

2.	• When run is complete, discard chip and place electrode cleaner (containing water) in Bioanalyzer and close lid for ~10 seconds. • Remove electrode cleaner and let dry ~10 seconds before closing lid.

5.4 Evaluation of Results

- The software calculates sample concentration. Multiple peak concentrations should be added together when appropriate. If a single peak concentration exceeds 50 ng/µL, a dilution will be prepared and quantified. (See Limitations)
- If a peak of interest is not integrated, it can be manually integrated by decreasing analysis thresholds. In most instances decreasing Height Threshold is sufficient for integration. If this is not successful, decrease Height Threshold and adjust one or more thresholds as detailed below.
- Click on expansion tab on right side of sample electropherogram panel and select LOCAL tab.
- Change value of following threshold(s) as appropriate and press ENTER

Threshold	Default Value	Allowable Value Range
Height	20	19 – 0.02
Slope	0.5	0.45 – 0.05
Area	0.1	0.09 – 0.02
Width	0.5	0.45 – 0.1

- Do NOT manually integrate the peak of interest by changing the start and stop points for integration on the electropherogram of the peak of interest.
- Save file and export results.

6 Acceptance Criteria

- Samples may proceed to cycle sequencing of a region when:
  - The sample and positive control concentrations are ≥ 1 ng/µL AND
  - The NC and RB concentrations are < 10% of the corresponding sample concentration.
- Samples that do not meet these criteria will not be taken on to cycle-sequencing. Sample and positive control concentrations may be rounded up from 0.96 ng/µL.

7 Limitations

- The maximum quantitative range for the DNA 1000 and DNA 7500 is 50 ng/µL. If a single peak concentration exceeds 50 ng/µL, a dilution of that sample will be prepared and quantified. The diluent used for the sample will be used in the master mix for sequencing.
- Minimum sample and positive control concentration and maximum RB and NC concentrations in the Acceptance Criteria above are empirically determined to provide a level at which the cycle-sequence data is expected to be uninterpretable.

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.
- Procedural Specific Chemical Hazards:
  - Agilent kit components contain dimethyl sulfoxide (DMSO). This dye binds to nucleic acids and is treated as a potential mutagen.

9 References

Agilent 2100 Bioanalyzer 2100 Expert User's Guide. Available online at www.agilent.com.

Agilent 2100 Bioanalyzer Maintenance and Troubleshooting Guide. Available online at www.agilent.com

10 Revision History

Revision	Issued	Changes
00	7/1/2022	Reformatted DNA 406-0 into new template and assigned new Doc ID. Minor modifications to text. Added QC procedures to appendix.

11 APPENDIX A: QUALITY CONTROL PROCEDURES

11.1 Agilents and Chip Priming Station

11.1.1 General Maintence and Cleaning

Agilent bayonet cartridges (pin sets) and lenses will be cleaned quarterly. Agilent chip priming station syringes and gaskets will be replaced quarterly. A Test Chip Kit will be run on each instrument annually following cleaning. Records will be maintained.

Diagnostic and maintenance procedures are detailed in "Agilent 2100 Bioanalyzer: Maintenance and Troubleshooting Guide" chapters "Maintenance of the Electrode Cartridge", "Maintenance of the Chip Priming Station," "Maintenance of the Pressure Cartridge," "Maintenance of the Agilent 2100 Bioanalyzer," and "Hardware Diagnostics".

11.1.1.1 Cleaning the Pin Set of the Electrode Cartridge:

- Turn off power to the 2100 bioanalyzer. The switch is located at the rear of the instrument.
- Open the lid and pull the metal lever on the inside left of the lid to the vertical position. When the lever is in the vertical position, the cartridge will be released from the lid by about 10 mm.
- Gently pull the cartridge out of the lid.
- Open the bayonet socket of the pin set by turning the plastic lever to the left.
- Remove the cover of the bayonet socket by gently pulling the plastic lever. The pin set may stick to the electrode base. Remove it by carefully pulling it off. Be careful not to bend the pins. This would lead to poor quality results or pre-terminated assay runs.
- Sonicate the pin set for 15 minutes in deionized analysis-grade water in a tabletop sonicator. While sonicating, clean dust from the inside of the chip compartment, including the lens, with an isopropanol wipe.
- Rinse pin set thoroughly with deionized analysis-grade water.
- Let the pin set dry completely. Make certain that the pin set is completely dry before placing it back into the electrode base. Even small amounts of liquid on the pin set can damage the high voltage power supply.
- Put the pin set on the cartridge base and the bayonet cover on the pin set. Lock the pin set to the electrode base by pushing the plastic lever of the bayonet cover to the right.
- Slide the electrode cartridge with the pin set into the bioanalyzer lid and move the metal lever to the flat (closed) position.
- Push the metal front of the electrode cartridge to ensure a tight connection.
- To verify that the electrodes are completely dry, perform the Short Circuit diagnostic test. This test can be found in the Diagnostics tab under the Instrument context in the Agilent software. The Short Circuit test takes approximately three minutes to complete and the software will walk you through the steps.

- If the test fails, the assembly may still be wet. Take the assembly out of the instrument, dry it with oil-free compressed air, and repeat the test. If test continues to fail, install a new pin set in the Agilent.

11.1.1.2 Replacing the Syringe and Gasket of Chip Priming Stations:

- Unscrew the old syringe from the lid of the Chip Priming Station.
- Release the syringe from the clip. Dispose of it. Remove the plastic packaging of the new syringe and insert it into the clip.
- Slide it into the hole of the luer lock adapter and screw it tightly to the priming station.
- The silicone gasket ensures a tight connection between chip and syringe adapter. Remove the old gasket from the chip priming station with your fingers.
- Insert a new gasket and gently push into place, ensuring that it is seated evenly.
- Check the Chip Priming Station for proper performance. Pull the plunger of the syringe to the 1.0 mL position.
- Place an unused chip in the chip priming station.
- Close the chip priming station and make sure to lock it by pressing the cover. The lock of the latch will audibly click when it closes.
- Press the plunger down until it is locked by the clip.
- Wait five seconds and press the side of the clip to release the plunger.
- Appropriate sealing will be verified if the plunger moves back up to the 0.3 mL mark within less than one second.

11.1.2 <u>Performance Verification</u>

The performance verification requires a single-use Test Chip Kit for each Agilent to be tested.

- Access the hardware diagnostic tests by selecting the Diagnostics tab in the instrument context of the 2100 Expert Software.
- Select “All” to run all tests. If you later wish to run only a specific subset of tests, select any you want to apply from the list.
- Select “Start” and follow the instructions as given by the 2100 Expert Software.
- At the end of the procedure all tests must be passed. Records will be maintained.
- If the instrument does not pass, rerun the failed tests at least twice prior to contacting Agilent customer service as tests are extremely sensitive to local conditions and often pass on subsequent attempts.