Quantitation using Agilent 2100 Bioanalyzer		
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1 Purpose

1.1 To quantify the amplified product of the mitochondrial hypervariable regions I and II, in order to establish the input of DNA for cycle sequencing. The DNA 1000 assay is capable of analyzing amplified DNA fragments in the range of 25-1000 bp, and in the concentration range of 0.5-20 ng/uL.

2 Preparing the documentation

2.1 Prepare a new Agilent Test Batch, and select samples for analysis

2.2 Exemplar samples and positive controls should be run at 2-fold (d2) and 5-fold (d5) dilutions. Hair and evidence samples should be run d1 (neat) and d2. Negative controls should be run d1 (neat). Prepare output samples accordingly.

2.3 Exemplar and evidence samples may be quantitated on the same Agilent run; however, they must be aliquoted for quantitation separately.

2.4 Prepare the plate record, and manually link the samples into the associated plate wells.

2.5 Download the plate record, and confirm the .CSV file is saved in the appropriate network location.

3 Preparing the samples

3.1 Follow the documentation to prepare dilutions. Add H_2O first in all tubes where needed.

3.2 Vortex and centrifuge tubes between serial dilutions

3.3 Aliquot all volumes less than 2 µl using a 2 µl pipette.

3.4 When pipetting sample for dilution or quantitation, pick up from the top of the solution (directly on the meniscus) to avoid carrying sample on the outside of the tip.

3.5 Use 1µl for quantification. Vortex and centrifuge every tube before use.

4 Procedure

4.1 If the Gel-Dye mix is not prepared, proceed to Section 5.

4.2 If the Gel-Dye mix is already prepared, proceed to Section 6.

4.3 For analysis of data only, proceed to Section 7.

POWDER-FREE GLOVES ARE REQUIRED FOR THE HANDLING OF AGILENT CHIPS. POWDER FROM LATEX GLOVES COULD CLOG THE MICRO-CHANNELS ON A CHIP.

5 Preparing the gel-dye mix

5.1 Allow the DNA dye concentrate (blue tube) and the DNA gel matrix (red tube) to come to 37°C in the heat block.

5.2 ALWAYS PROTECT THE DYE CONCENTRATE FROM THE LIGHT. Vortex the DNA dye concentrate (blue tube) and spin down. Add 25 µL of the dye concentrate (blue tube) to the DNA gel matrix vial (red tube).

5.3 Vortex the mixture for 10 seconds to ensure complete mixing, and transfer the entire mixture to the top receptacle of a spin filter.

5.4 Centrifuge for 15 minutes at 6000 rpm. Discard the filter and label the gel-dye mix tube with the lot numbers of the DNA dye concentrate, the DNA gel matrix, and your initials and the date.

5.5 One tube of gel-dye mix is enough for 10 runs, and will last for 4 weeks. Discard the gel-dye mix 4 weeks after the date of preparation. Protect the gel-dye mix from light, and store at 4 ^{O}C. Record the creation of the gel-dye mixture in the LIMS.

6 Loading and running of the Agilent Bioanalyzer 2100

6.1 2100 Expert System Setting (left inside of the window, click on system) are saved by default as:

6.1.1 Data Files Name: serial number, data, time are checked.

6.1.2 Data Files directory: “Create Daily subdirectories” is checked.

6.1.3 Data File format: “Binary format” is checked.

6.1.4 Nothing is checked in “Run and Results”, “Auto Export,” and, “Default Export Directories”.

ALL PIPETTING INTO THE CHIP MUST BE DONE DIRECTLY ON THE GLASS AT THE BOTTOM OF THE WELL, NEVER ON THE SIDES OF THE WELL.

6.2 Allow the gel-dye mix to equilibrate to room temperature.

6.3 Have a witness check samples and worksheet. Record the run name, the lot#, analyst, date, time, and the Agilent machine, in the documentation. Open a new DNA chip

6.4 Pipette 9 µL of gel-dye mix into the bottom of the well marked **G**. Make sure there are no bubbles, if any use a 1µl pipette tip to remove them. Place it in the priming station, and fill out usage log. Make sure the base plate of the station is set to position C, and the clip on the syringe trigger is set to the lowest position. Make sure the syringe piston is pulled back to the 1 ml mark, and close the lid of the priming station. (Listen for the "click.")

6.5 Grab the syringe with your index fingers under the fins on the syringe body and thumbs on the plunger. Swiftly and steadily, press down on the plunger until it locks under the silver trigger. Make sure your thumbs are not in the way of the trigger lock or it will not work. Let the chip pressurize for 60 seconds.

6.6 Release the syringe with the trigger, and make sure the syringe comes back to 0.3-0.4 ml. Wait for 5 seconds, pull slowly the syringe back to 1 ml, and open the chip priming station. Turn the chip over and inspect the capillaries for proper filling.

6.7 Pipette 9 µL of gel-dye mix into the two wells marked **G**. Make sure there are no bubbles, if any use a 1µ tip to remove them.

6.8 Vortex and spin down the DNA marker (green tube), and pipette 5 µL of marker into each of the 12 sample wells and ladder well. Each well must be filled, even if it will not be used.

6.9 Vortex and spin down the DNA ladder (yellow tube), and pipette 1 µL of ladder into the lower right well, marked with the ladder symbol.

6.10 Add 1 µL of amplified DNA to each well. If a well is not used, add 1 µL of dH_2O into the well.

6.11 Place the chip in the IKA vortexer and vortex at ~2200 rpm for 60 seconds.

6.12 Run chips within 5 minutes.

6.13 Start the collection software by clicking on the symbol on the desktop.

6.14 Click on "instrument" on the left panel and place the cursor in one cell of the Sample Name column, click on the right button of the mouse, select import, choose the .CSV text file that was created for the run (see A7). The software might give a warning "failed to import the text file." Ignore, and press OK.

6.15 Once the machine is highlighted in the upper left-hand corner of the screen, open the lid. The icon should now show the lid open as well. Insert the DNA 1000 chip and carefully close the lid. The machine icon will now change to a blue chip on the screen. Make sure the "Assay Class" in the

"Assay Details" panel (middle right panel) is "DNA 1000". If the assay class is different than DNA 1000 see a supervisor before starting the run.

6.16 Adjust the sample # in the Data Acquisition Parameters field, if necessary.

6.17 Click on the START button.

6.18 The run will begin with a pre-heat and should take approximately 35-40 minutes for a full chip.

6.19 The ladder sample will process first. It is a good idea to monitor this sample to make sure the upper and lower markers come out correctly (15 bp and 1500 bp).

6.20 **Immediately** after the run (less than 5 minutes), remove the sample chip. The electrodes need to be cleaned with the clear electrode cleaner chip within 5 minutes after the run. To do this, begin by filling one of the large wells with 350 µL of deionized water. Place the electrode cleaner in the Agilent 2100 Bioanalyzer and close the lid for 10 seconds (and **not more than 10s**). Open the lid, remove the electrode cleaner chip, and let the pin set dry for another 10 seconds (and **not more than 10s**), then close the lid. Drain and dry the electrode cleaner chip.

7 Data collection, analysis, electronic filing

7.1 When the Bioanalyzer 2100 run is complete, go to the Data & Assay field. The main window will show the gel image, sample list, and chip summary. Any problems detected by the software will be indicated with yellow triangles above the lanes in the gel image (see Section 12 Troubleshooting).

7.2 **Ladder**

7.2.1 Select the Ladder sample on the sample list. The main window should show the following peaks (11 ladder peaks plus lower-LM- and upper-UM- markers):

15bp	Lower Marker
25bp	Ladder
50bp	Ladder
100bp	Ladder
150bp	Ladder
200bp	Ladder
300bp	Ladder
400bp	Ladder
500bp	Ladder
700bp	Ladder
850bp	Ladder
1000bp	Ladder
1500bp	Upper Marker

7.3 **Samples**

7.3.1 Click on the individual sample on the sample list. The positive control and sample lanes should show two peaks, indicating the HVII and HVI amplified products (around 420-490 bp) for samples amplified with HVI and HVII multiplex primers. All samples should have the lower marker (~15bp) and upper marker (~1500bp).

7.4 **Manual editing**

7.4.1 If the upper marker (UM) or lower marker (LM) is present but not labeled properly, right click on the peak cell "size bp" in the table and select "manually set upper marker" or "manually set lower marker," respectively.

7.4.2 If a ladder peak, HVI and/or HVII are present but not labeled or if an extra peak is present: right click on the peak, select "manual integration", add or remove peak at that position (bp).

7.4.3 Smaller amplified product peaks in samples with severely unbalanced HVI and HVII peak heights due to potential length heteroplasmy may be manually edited. Be sure to record any and all edits within the run documentation.

7.5 **Export data to the network**

7.5.1 The data are automatically saved in a "yyyy-mm-dd" folder as "DES547045xx\yyyy-mm-dd_hh-mm-ss.xad" (a shortcut on the desktop). If from AG1: DES547045**xx** is DES54704**15**, if from AG2:DES547045**xx** is DES54704**24**.

7.5.1.1 Open the yyyy-mm-dd_hh-mm-ss.xad file, make edits if necessary, save the .xad file as DES547045xx\yyyy-mm-dd_hh-mm-ss-**analystinitials.**xad. Transfer the .xad file(s) in M:\MITO_DATA\Agilent Archive\yyyy\ yyyy-mm-dd-hh-mm-ss folder.

7.5.1.2 Create a PDF file by going to the "file" menu and selecting "print." When the print window opens, select "Run Summary", "electropherograms" and "Results Table", choose "all wells" if it is a full chip, or fill in the well numbers of used wells for a partial chip. Select "Include Ladder", one per page, PDF. Click on "… " to select the drive. Select the appropriate folder on the network. Add analyst initials before ".pdf" in the path name. Click save.

7.5.1.3 Check that PDF and.xad file (s) are present in M:\MITO_DATA\Agilent Archive\yyyy\ a new yyyy-mm-dd-hh-mm-ss folder.

7.6 2100 expert software

7.6.1 If analysis or review is done at a different time than the run or from another computer, open the 2100 Expert software.

- Select "data" in the "contexts" column on the left side of the window.
- Go to file
- Open
- Select the folder with the run you want to review/analyze in M:\MITO_DATA\Agilent Archive\YEAR\xxxxxxxxxxxxx
- Select the appropriate.xad file of the run
- Click open

7.6.2 After analysis/review: IF ANY EDITS/CHANGES ARE MADE, do not forget to re-save the .xad file with **-reviewerinitials**" at the end of its name. Create a new PDF file. Print and initial.

8 Data Entry, Review, Filing, Rerun

8.1 DATA TAB ENTRY

8.1.1 Open the appropriate Agilent batch to review and edit, as necessary.

bp * : peak position manually called
bp ** : peak position edited out manually
bp ^ : spikes position manually removed
LM*: sample lower maker manually called
UM*: sample upper maker manually called

8.2 Samples

8.2.1 HVII and HVI peaks should be discrete and approximately 400 to 500bp respectively or the peak will be inconclusive. Samples with multiple peaks or peak imbalance due to potential sequence length heteroplasmy may be manually edited.

8.2.2 The "Vol, Used" refers to the volume aliquoted of the original amplified sample.

8.3 Sample edits:

8.3.1 If a sample is edited, enter the location in bp followed by one of the symbols from the table above. If special edits are needed, document them in the sample edits column.

Indicate if editing was necessary for any sample, including the ladder, and if so, record the edits in the documentation.

8.3.2 Once the editing is complete in the Agilent Software, save the file, and export the final results to the appropriate network location.

8.4 Import this Agilent data file into the LIMS system against the Agilent batch data. This will automatically update the dilution factors, and input the HVI and HVII initial values. This, in turn, will calculate the value of HVI and HVII, and output the final "Conc, Mean HVI-HVII" value for each sample in the batch.

9 Review Tab Entry

9.1 Fill the "Interpretation" column following the guidelines in the table below:

one dilution			
HVII	**HVI**		**Comments**
[0.5-20]	[0.5-20]	HVII and HVI mean concentration will be used for further testing	**USE**
INC	[0.5-20]	non-INC peak concentration value will be used for further testing	**USE**
[0.5-20]	INC	non-INC peak concentration value will be used for further testing	**USE**
INC	INC	Rerun	**RQ**

2 dilutions				
				Comments
dilution A	pass	dilution A within ± 2.5 X dilution B	use lower value	**USE**
dilution B	pass			**-**
dilution A	pass	dilution A outside ± 2.5 Xdilution B	Rerun	**RQ**
dilution B	pass		Rerun	**RQ**
dilution A	pass	n/a	use for further testing	**USE**
dilution B	INC		n/a	**-**
dilution A	INC	n/a	n/a	**-**
dilution B	pass		use for further testing	**USE**
If both dilutions are INC				
dilution A	INC	n/a	Rerun at appropriate dilution	**RQ, dx**
dilution B	INC		Rerun at appropriate dilution	**RQ, dx**
appropriate dilution example 1				
dilution 2	<0.5	n/a	Rerun at appropriate dilution	**RQ, d1**
dilution 5	<0.5		Rerun at appropriate dilution	**RQ, d1**
appropriate dilution example 2				
dilution 2	>20	n/a	Rerun at appropriate dilution	**RQ, d10**
dilution 5	>20		Rerun at appropriate dilution	**RQ, d100**
If both dilutions are INC for any other reason than concentration value out of range, see supervisor				

INC = sample inconclusive
USE = concentration will be sued for further testing
RQ = sample will be requantified
dx = sampel will be requantified at dilution x

- If the ladder fails (e.g., discrete bands) or upper or lower markers are not present (e.g., can't be edited) the run is inconclusive, all samples have to be requantified, indicate as such in each sample Interpretation cell.

- If one or both markers are not called, the run is inconclusive, all samples have to be requantified, indicate as such in each sample Interpretation cell.

- If a sample peak concentration value is out of range it will appear as INC

- If a sample was called INC for any other reason than the value range it will appear as INC. A comment can be added to explain why the peak was called INC in the "notes" of the review sheet. In both cases the concentration of only one peak could be used for further testing instead of the mean concentration of both peaks and **USE** added in the comments column (see table above).

- If the ladder fails manually select "fail" as the "Ladder Result", if not select "Pass".

- If the ladder marker (s) fail(s) circle "fail" as the "Ladder Result", if not select "Pass".

10 Review

10.1 The reviewer will review the documentation, as well as any comments based on the parameters in section 8.

10.2 After review, the reviewer will indicate the appropriate samples for the next process step, as indicated by the Interpretation results of each sample.

11 Reruns

11.1 After review, the analyst will set up a new Agilent batch for the necessary samples that were indicated as reruns.

12 Troubleshooting

12.1 For troubleshooting, refer to the Agilent 2100 Bioanalyzer Maintenance and Troubleshooting Guide Edition 11/November 2003.pdf archived in MITO_DATA folder.