Quantitation using Agilent 2100 Bioanalyzer		
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Quantitation using Agilent 2100 Bioanalyzer

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

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

2.3 Prepare output samples accordingly:

2.3.1 Exemplar samples, positive controls and all blood samples including evidence should be run at 2-fold (d2) and 5-fold (d5) dilutions. Choose “Agilent Dil Exemplar” in the Output Sample list ID.

2.3.2 Hair and evidence samples (of unknown concentrations) should be run d1 (neat) and d2. Choose “Agilent Dil Evidence” in the Output sample List ID.

2.3.3 Negative controls should be run d1 (neat).

2.4 Load the samples on the load plate screen three samples at a time, horizontally. Alternatively, the samples can be added one at a time and saved after loading every sample. Save changes after loading is complete.

2.5 Download the plate record and upload the .csv file from LIMS onto the Agilent instrument’s computer.

3 Preparing the samples

3.1 Fill in the quantitation Performed By tab in LIMS.

3.2 Retrieve all reagents and record all lot numbers in LIMS.

3.3 Follow the table below to prepare dilutions. Add water first in all tubes where needed.

	Sample	Water
Neat	n/a	n/a
d2	1.5 neat	1.5
d5	1 d2	1.5
d10	1.5 d5	1.5
d100	1 d10	9

NOTE:

- Vortex or pipette up and down and centrifuge tubes before use and between serial dilutions.
- Aliquot all volumes less than 2 µL using a 2 µL pipette.
- 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.

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

4 Quantitation

4.1 If the Gel-Dye mix is not prepared, prepare fresh Gel-Dye mix according to the Agilent Gel Dye Mix Reagent sheet.

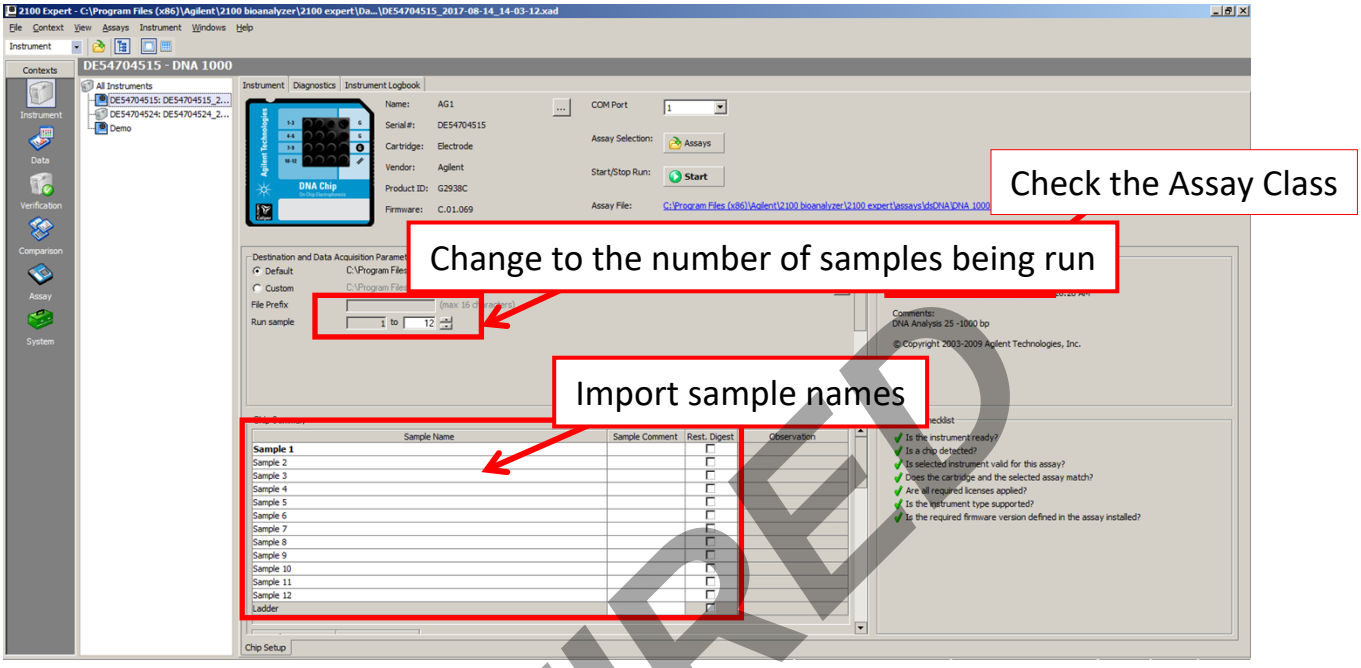
4.2 If the Gel-Dye mix is already prepared, proceed to SECTION 5.

4.3 For analysis of data only, proceed to SECTION 6.

5 Loading and running of the Agilent Bioanalyzer 2100

5.1 Start the collection software by clicking on the Agilent icon on the desktop.

5.2 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.

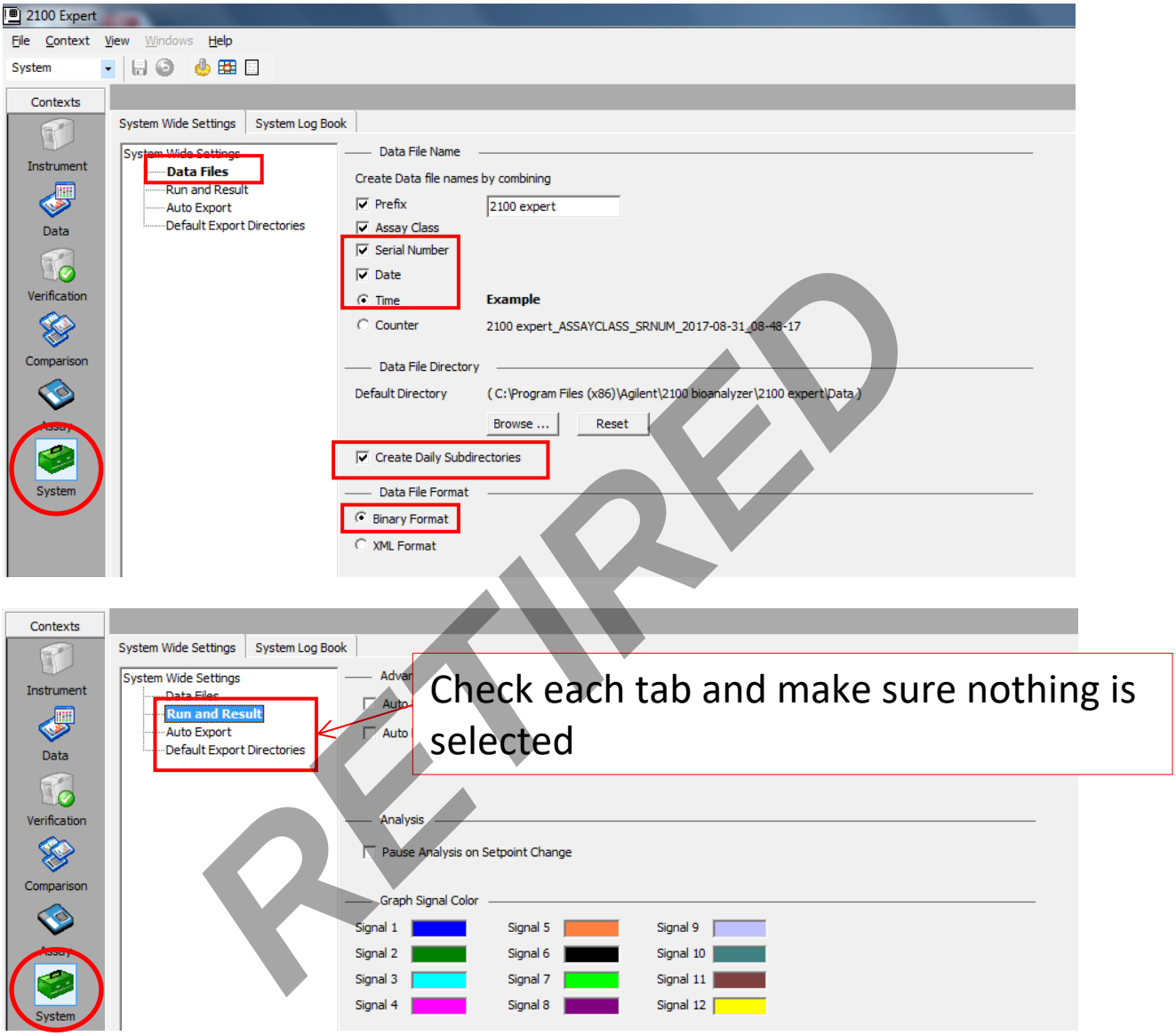


5.3 Once the machine is highlighted in the upper left-hand corner of the screen, open the lid. The icon should now show the lid open as well. Insert the DNA 1000 chip and carefully close the lid. The machine icon will now change to a blue chip on the screen. Make sure the "Assay Class" in the "Assay Details" panel (middle right panel) is "DNA 1000". If the assay class is different than DNA 1000 see a supervisor before starting the run.

5.4 Adjust the sample # in the run sample field of the Destination and Data Acquisition Parameters section, if necessary.

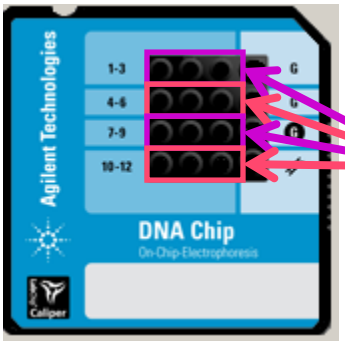
5.5 **2100 Expert System Settings** (left inside of the window, click on "**System"**) are saved by default as:

- Data ***Files Name***: serial number, data, time are checked.
- Data ***Files directory***: "Create Daily subdirectories" is checked.
- Data ***File format***: "Binary format" is checked.
- Nothing is checked in "***Run and Results***", "**Auto Export**," and, "**Default Export Directories**".
- System → Data File → Data File Name/Data File Directory/Data File Format

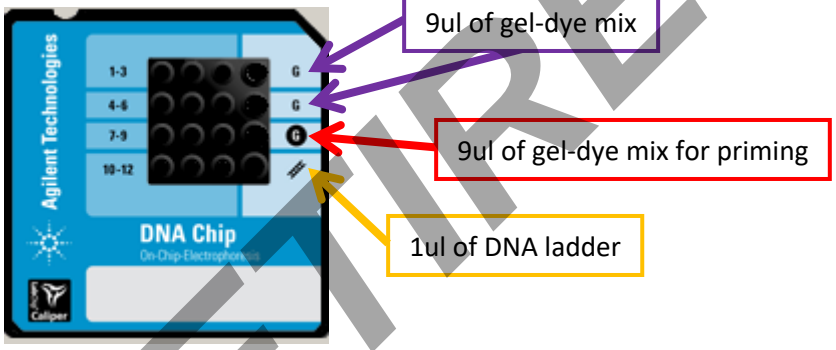


ALL PIPETTING INTO THE CHIP MUST BE DONE DIRECTLY ON THE GLASS AT THE BOTTOM OF THE WELL, NEVER ON THE SIDES OF THE WELL.

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

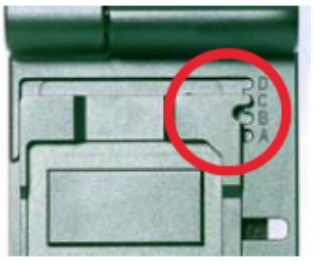


5.7 **WITNESS:** Have a witness verify the ***entire*** input amp tube labels, and the output tube top names and dilution factor as they appear on the plate loading screen in LIMS. The load plate screen in LIMS should show the samples loaded three in a row.



5.8 Open a new Agilent DNA chip. Make sure it has not expired.

5.9 Pipette 9 uL of gel-dye mix into the bottom of the well marked **G**. Make sure there are no bubbles; if any, use a 1 uL pipette tip to remove them. This can also be accomplished by tapping the chip gently against the benchtop.



5.10 Place the chip in the priming station. 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").

5.11 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.**

5.12 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.

5.13 Record the priming station's number in the comment section of the quantitation Performed By tab in LIMS.

5.14 Pipette 9 µL of gel-dye mix into the two wells marked **G**. Make sure there are no bubbles; if any, use a 1 µL tip to remove them. This can also be accomplished by tapping the chip gently against the benchtop.

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

5.16 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.

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

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

5.19 Run chips within 5 minutes.

5.20 Click on the START button. The run will begin to pre-heat and should take approximately 35-40 minutes for a full chip.

5.21 Record the instrument usage in LIMS. Fill in the batch description as the Purpose and "DNA 1000" as the Program.

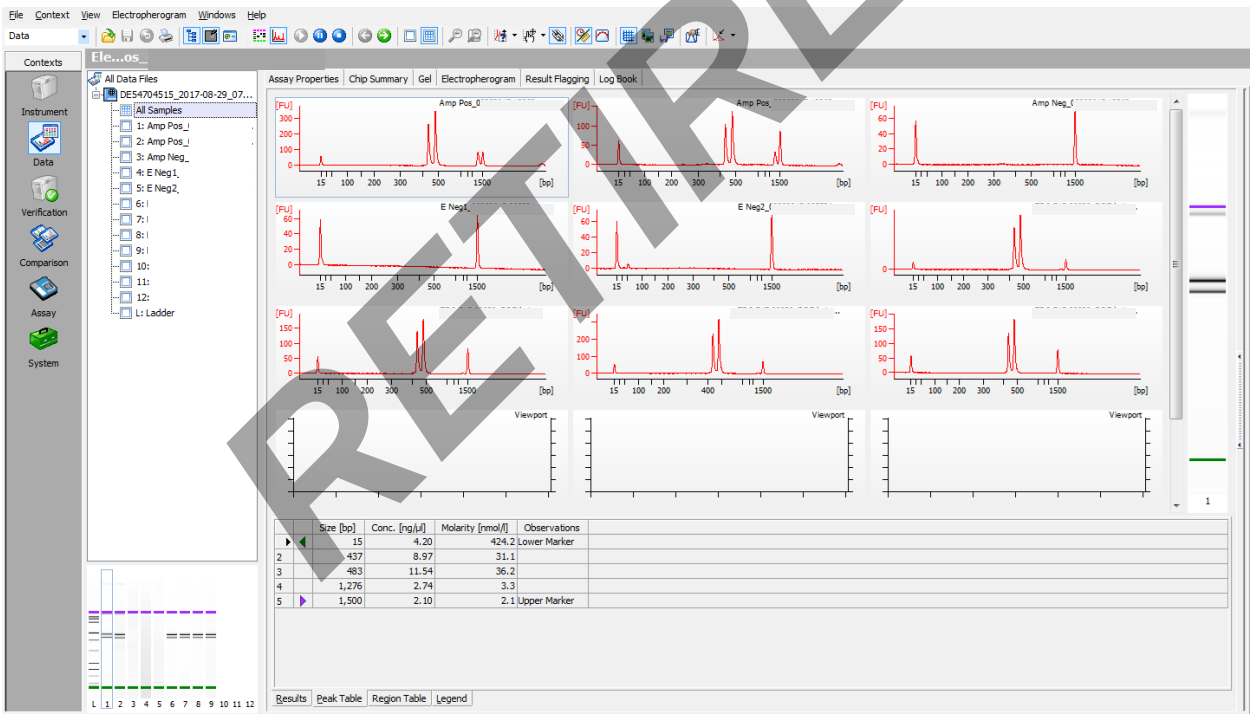
5.22 Record the run name taken off the instrument, in the LIMS QC Batch Parameters. Follow the format of: year-month-date_hour-minute-second (e.g. 2020-10-22_13-13-26). Update the test batch description and plate name using the instrument date and time (e.g. Q102220-1313). In addition, if a Quantification Negative is run, update the Quantification Negative sample date and time with the instrument date and time (e.g. QuantNegative_10222020.131326) in both the LIMS input and output tables.

5.22.1 *Note:* the ladder sample will process first. It is a good idea to monitor this sample to make sure the upper 1500 bp and lower 15 bp markers come out.

5.23 After the run, remove the sample chip. The electrodes need to be cleaned with the clear electrode cleaner chip within 5 minutes after the run is done. To do this, begin by filling one of the large wells with 350 uL of deionized water. Place the cleaner chip in the Agilent 2100 Bioanalyzer and close the lid for approximately 10 seconds. Open the lid, remove the electrode cleaner chip, and let the pin set dry for another 10 seconds, then close the lid. Drain and dry the electrode cleaner chip.

6 Data collection, analysis, electronic filing

6.1 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 troubleshooting).



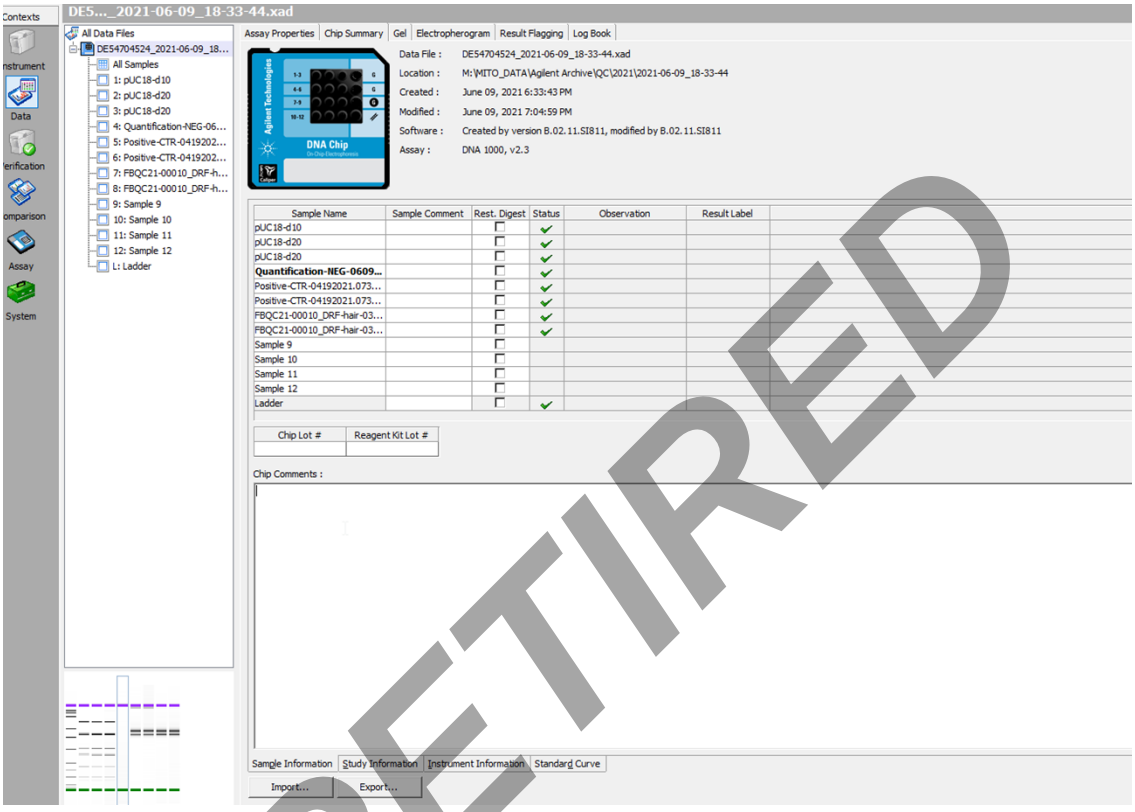
6.2 If there is a quantitation negative control included in the run, the date and time for the sample need to be updated at this time. Use the Agilent instrument date and time for this entry (e.g. QuantNegative_10222020.131326).

6.3 Click on the Data icon and then the run header.

6.4 At the bottom of the window, select the Sample Information tab.

6.5 Double click on the Quantification NEG sample in the first column so that it is editable.

6.6 Add in the instrument run date and time.



6.7 2100 Expert Software

6.7.1 On the Agilent computer, click on the "Data" folder on the desktop, click on the corresponding date and then copy and paste the run file into the following directory:

6.7.2 L:\FB\Instrument to LIMS\Agilent

6.7.3 On a networked computer, open M:\Mito_Data\AgilentArchive\[Year] and create a new folder with the run date and time.

6.7.4 Open L:\FB\Instrument to LIMS\Agilent and copy the run file into the newly created folder in the M: drive.

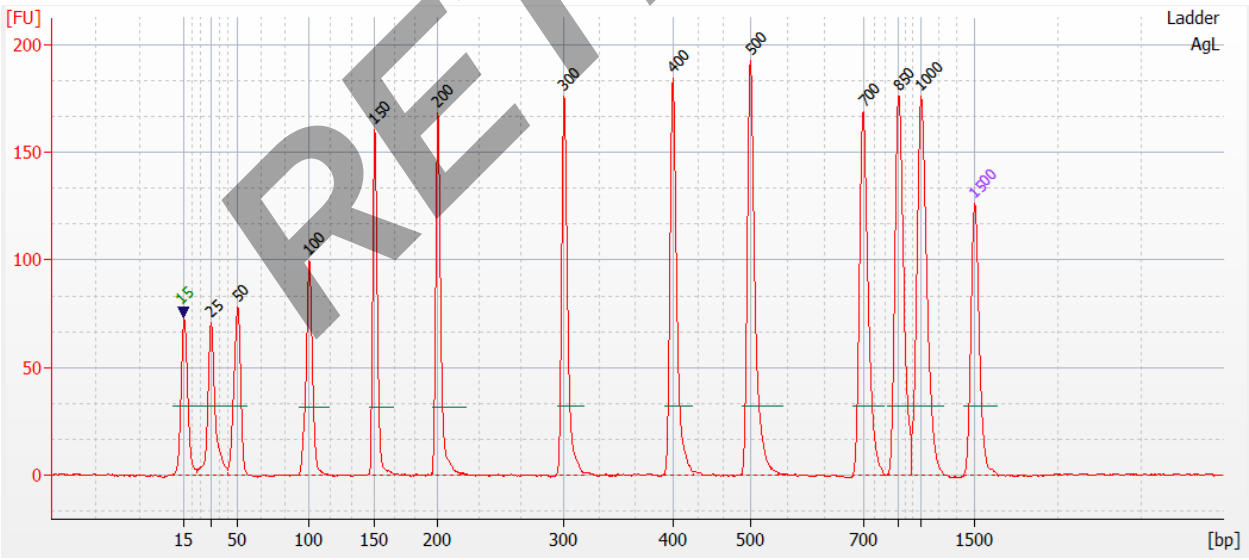
6.7.5 Open your desktop Agilent software and click on the "Data" button on the sidebar. Select "File", "Open", and select the run previously copied to the M: drive.

7 Analysis

7.1 Ladder:

7.1.1 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



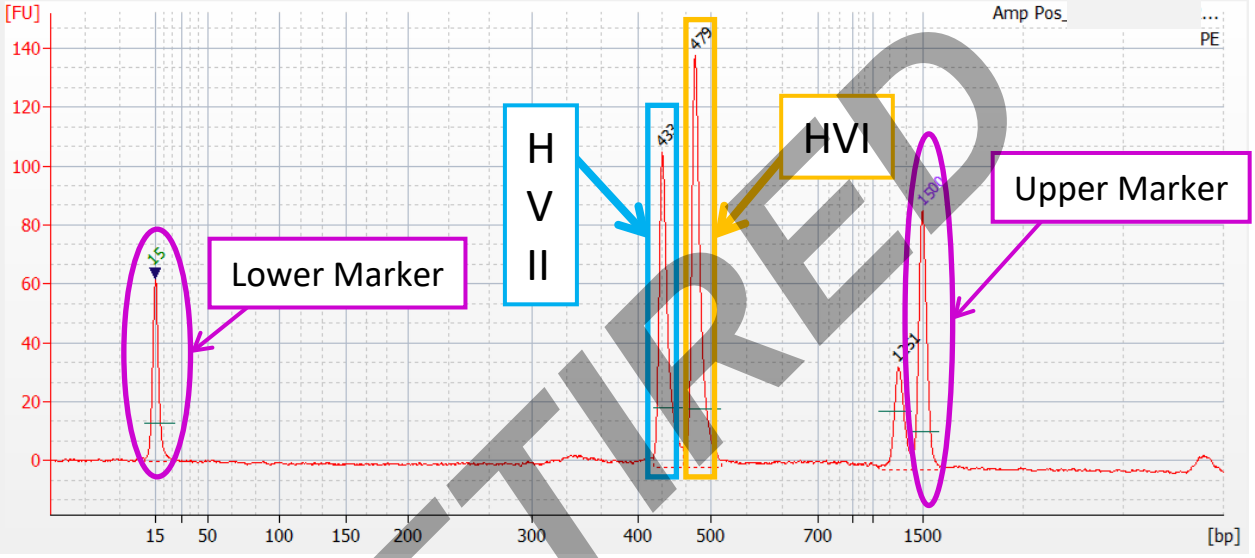
7.1.2 **Note:** Any additional peaks observed in the ladder that appear to be shoulder peaks or spikes may be manually edited (see manual editing section below).

7.2 Controls and Samples:

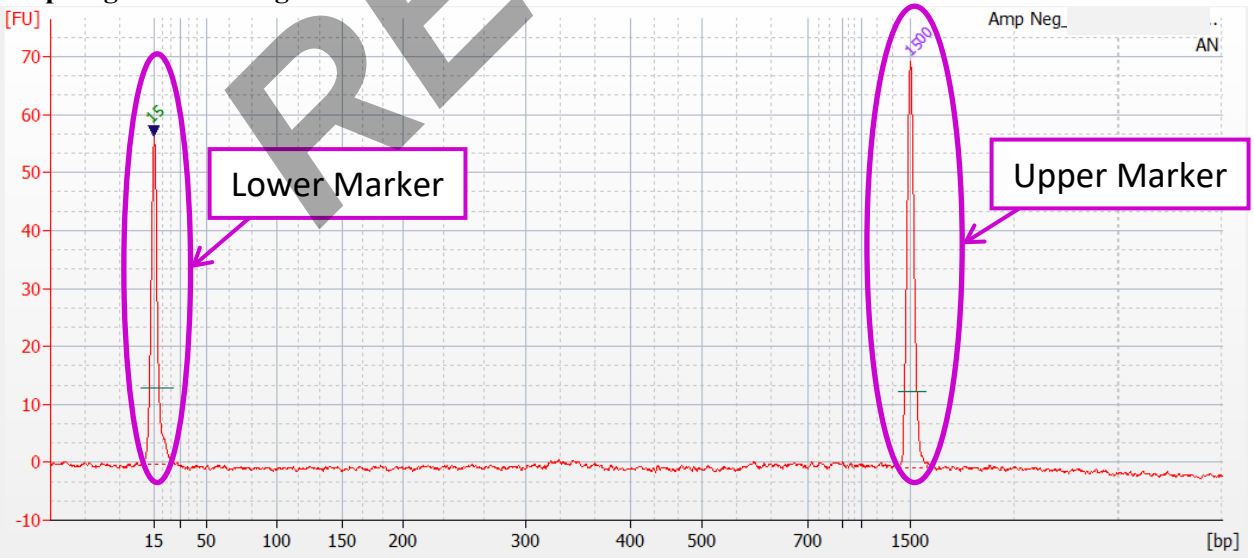
7.2.1 Click on the individual sample on the sample list. All samples, including negative controls, should have the lower marker (~15 bp) and upper marker (~1500 bp).

7.2.2 The positive control and sample lanes should show two peaks, indicating the HVII and HVI amplified products (around 400-500 bp) for samples amplified with HVI and HVII multiplex primers.

Amp Positive and Samples



Amp Negative and Negative Controls



7.3 Manual Editing:

7.3.1 While manually editing peaks using the Agilent software, also open the run in LIMS and concurrently record these edits in the LIMS data entry table. Save these entries prior to importing the edited Agilent data into LIMS.

7.3.2 Agilent Editing Codes:

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

7.3.3 Indicate if editing is necessary for any sample, including the ladder, in the "Editing needed" column in the LIMS data entry table. If a sample is edited, enter the location in bp followed by one of the symbols from the table above in the "Edits" column in LIMS.

7.3.4 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. Be sure to do this before making other edits, as resetting the marker will change the size of other peaks detected.

7.3.5 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", and add or remove peak at that position (bp).

7.3.6 Length heteroplasmy may cause the HVI and HVII peaks to be severely unbalanced, resulting in several smaller amplified product peaks which should be manually edited out. Be sure to record all edits within the LIMS run documentation.

7.3.6.1 If potential heteroplasmy is seen in either HVI or HVII, manually remove all peaks at the heteroplasmic region, including the main peak, and change its value from "0" to "INC" in the LIMS data entry table.

7.3.6.2 If potential heteroplasmy is observed in both HVI and HVII, keep the two highest peaks and manually remove all other peaks.

7.3.7 If peaks are observed but not called in negative controls, attempt to call the peak(s) to confirm if the peak is above or below the threshold. If above the threshold, manually add the peak and document this edit in LIMS. Proceed to follow the Guidelines for Negative controls section in the Interpretation Guidelines protocol. If below the threshold, enter the peaks as "0" in the LIMS data entry table and make the following documentation in LIMS:

- "Peak(s) is/are present in [sample name] at the HVI [and/or] HVII region(s) that is/are below the calling threshold (<0.5 ng/uL). Therefore, the extraction/amplification negative passes. [Initial] and [date]"

7.3.8 If peaks are observed but not called in a sample, attempt to manually call the peak(s) to confirm if it is above or below the threshold. If above the threshold, manually add the peak and document this edit in LIMS. If below the threshold, enter the peak as "INC" in the LIMs data entry table only if one of the peaks is below the threshold; enter the peaks as "0" if both peaks are below the threshold. In addition, make the following documentation in LIMS:

- "Peak(s) is/are present in [sample name] at the HVI [and/or] HVII region(s) that is/are below the calling threshold (<0.5 ng/uL). [Initial] and [date]"

7.3.9 Once editing is completed, select "File" and "Save as". Add an underscore followed by the analyst's initials at the end of the run name. Save the revised file in the same folder as the original.

7.4 Create a PDF

7.4.1 Go to the "File" menu and select "Print". When the Print window opens, select "Run Summary", "Electropherograms", and "Results Table". Choose "all wells" if the chip was full, or fill in the well numbers of partially filled chips. Select "Include Ladder", one per page, and PDF. Click on "…" to select the drive. Select the appropriate folder on the network. Click save.

7.4.2 Check that the PDF and .xad files are present in M:\MITO DATA\AgilentArchive\yyyy\yyyy-mm-dd-hh-mm-ss

7.5 Export edited data to the network

7.5.1 Go to the "File" menu and select "Export". When the export window opens, make sure only the box for "Result Tables" is selected.

7.5.2 Save the exported file in the following directory: L:\FB\Instrument to LIMS\Agilent\[Instrument#]

7.6 Import edited data into LIMS

7.6.1 In the LIMS test batch, go to the data entry table for the output samples and click "Import Instrument Data".

7.6.2 Click "Browse" and select the appropriate .csv file.

7.6.3 Click save and the data entry table will automatically populate.

7.6.4 Make sure all mandatory fields in the LIMS data entry table have been completed. This should have been done during the "Manual Editing" step above. The "Vol, used" and "Interpretation" columns will be completed in the following section.

8 LIMS Data Entry:

8.1 Fill the "Interpretation" column following the guidelines in the table below.

8.1.1 If only one dilution (e.g., d1) is used for a sample, only the top section applies for the interpretation.

8.1.2 If two dilutions are used in the run(e.g., d2 and d5), initially follow the top section to assess each separately. Determine if the concentration value will be used for further interpretation. Use the second section of the table guidelines to then evaluate which dilution concentration will be pushed for further testing.

one dilution			
HVII	**HVI**	**Comments**	**Interpretation**
[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				
Concentration value in range for further interpretation?		**Comparison of two concentrations**		**Interpretation**
dilution A	[0.5-20]	dilution A within ± 2.5 X dilution B	use lower value (e.g. dil. A < dil. B)	**USE**
dilution B	[0.5-20]			-
dilution A	[0.5-20]	dilution A outside ± 2.5 X dilution B	Rerun	**RQ**
dilution B	[0.5-20]		Rerun	-
dilution A	[0.5-20]	n/a	use for further testing	**USE**
dilution B	INC		n/a	-
dilution A	INC	n/a	n/a	-
dilution B	[0.5-20]		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	**-**
appropriate dilution example 1				
dilution d2	<0.5	n/a	Rerun at appropriate dilution	**RQ, d1**
dilution d5	<0.5		n/a	**-**
appropriate dilution example 2				
dilution 2	>20	n/a	Rerun at appropriate dilution	**RQ, d10 and d100**
dilution 5	>20		n/a	**-**

INC = sample inconclusive
USE = concentration will be used for further testing
RQ = sample will be re-quantified
dx = sample will be re-quantified at dilution x

8.2 The "Vol, Used" column in the LIMS data entry table refers to the volume aliquoted of the original amplified sample. Assign a "Volume used" only to the samples whose interpretation was "Use" (note that if two dilutions of a sample were prepared, then the "Volume used" for the sample designated as "Use" should be the *combined* volume used to prepare both dilutions of the sample). The "Volume used" for all other samples should be "0".

8.3 Run Interpretation and additional sample interpretation guidelines

- If the ladder fails (e.g., discrete bands) or upper or lower markers are not present (e.g., can be edited) the run fails. Select "fail" as the "Ladder Result." All samples must be requantified, indicate as such in the appropriate Interpretation cell as indicated above.

  If one or both markers in a sample are not called, the sample run is inconclusive, and the sample will have to be requantified if (i) only one sample dilution was run, or (ii) both sample dilutions had marker(s) not called

- If a sample peak concentration value is out of range, manually change it to INC after the data has been imported into the LIMS data entry page.

9 Review

9.1 The reviewer will review the documentation, as well as any comments based on the parameters as described above in the LIMS Data Entry section.

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

10 Reruns

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

11 Troubleshooting

11.1 For troubleshooting, refer to the Agilent 2100 Bioanalyzer Maintenance and Troubleshooting Guide archived in MITO_DATA folder.