Manual MPS Library Quantitation with PowerSeq® Quant MS

1 Purpose

1.1 To determine the DNA concentrations of the purified libraries in order to establish the appropriate dilution schemes for normalization.

2 LIMS Processing

2.1 Refer to the LIMS Process Manual for the general test batch processing protocol.

2.2 This quantitation batch requires that the samples are loaded onto a plate in LIMS.

2.2.1 Within the test batch, select all the output samples and click *Load Plate.*

2.2.2 Fill in the "Plate Name" field (mandatory).

2.2.3 Adding samples to the plate:

2.2.3.1 From the list on the left side of the load plate screen, select all standards and the NTC.

2.2.3.2 With well A1 highlighted, click *Plate Load Algorithm*.

2.2.3.3 In the window that opens, click the hyperlink.

2.2.3.4 Next, from the list on the left side of the load plate screen, select all unknown samples.

2.2.3.5 Click well A3 to load samples.

2.2.3.6 Again, select all unknown samples, from the list on the left side on the load plate screen.

2.2.3.7 Click well A7 to load samples.

2.2.4 Click *Save* to complete the plate.

2.2.5 Click *Download Instrument* to generate the 7500 import file.

2.3 The file generated for instrument import should be modified before import on the 7500.

2.3.1 Open the Microsoft Excel application.

2.3.2 Within Excel, open the import file generated. Click *Next* twice, followed by *Finish* to open the file.

2.3.3 In cells B34 and B46, type "**'2 pM**" (apostrophe followed by 2 pM).

2.3.4 To modify the file for import onto the 7500, click *Save As*. The file type should be "text (tab delimited)".

3 Preparation

3.1 Locate the PowerSeq® Quant test batch for your run in LIMS.

3.2 Retrieve the following reagents and allow them to equilibrate to room temperature.

PowerSeq® Quant MS 2x qPCR Master Mix
PowerSeq® Quant MS 10x Primer Mix
PowerSeq® Quant MS DNA Standard (200 pM)
PowerSeq® Quant MS 1x Library Dilution Buffer
PowerSeq® Quant MS Amplification Grade Water

Note: Once thawed PowerSeq® Quant MS 1x Library Dilution Buffer may be stored at 4°C for up to 3 months.

3.3 Log all reagent lot numbers in LIMS as appropriate.

3.4 Vortex the reagents thoroughly. It is suggested to vortex three times for 10 seconds each time. Centrifuge briefly.

3.5 Retrieve a reagent reservoir.

3.6 Retrieve the purified library plate to be quantified and allow to equilibrate to room temperature.

3.7 Vortex the library plate on a plate mixer for 1 minute at 1000 rpm and then centrifuge the plate at 1000 rpm for 1 minute.

3.8 Prepare standards:

3.8.1 Label five (5) - 2 mL Sarstedt screw cap tubes with the standard concentrations (20 pM, 2 pM, 0.2 pM, 0.02 pM, and NTC) on both the cap and the side of the tube.

3.8.2 Pipette 45 µL of PowerSeq® Quant MS 1x Dilution Buffer into each labelled DNA standard tube.

- Pipette 5 µL of the 200 pM DNA standard into the 20 pM tube. Vortex for 10 seconds and centrifuge briefly.
- Pipette 5 µL of the 20 pM standard into the 2 pM tube. Vortex for 10 seconds and centrifuge briefly.
- Pipette 5 µL of the 2 pM standard into the 0.2 pM tube. Vortex for 10 seconds and centrifuge briefly.
- Pipette 5 µL of the 0.2 pM standard into the 0.02 pM tube. Vortex and centrifuge briefly.

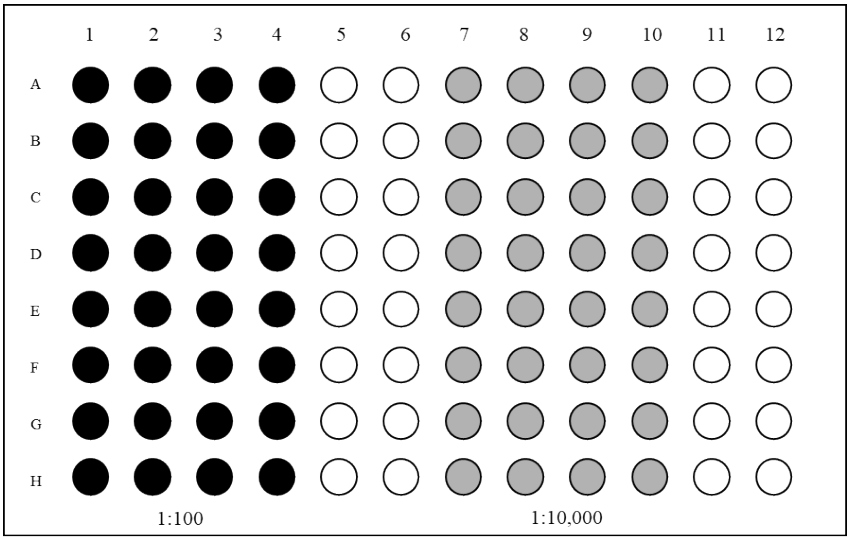
3.8.3 Standards may be stored in at 4°C and used for up to three (3) months. If storing, record your initials and the date on the tubes.

3.9 Create 1:10,000 dilutions of libraries:

3.9.1 The following instructions are for the creation of 1:10,000 dilutions during the initial quantification of the purified or normalized libraries).

3.9.2 If quantifying a smaller number of libraries, the dilution described in the steps below may be performed in 1.5 mL microcentrifuge tubes using a single channel pipet.

3.9.3 Label a new 96-well plate.
Columns 1-4 will be used to create 1:100 dilutions of columns 2-5 from the purified library plate.
Columns 7-10 will be used to create the final 1:10,000 dilutions from the columns 1-4.



3.9.4 Pour approximately 15 mL of 1x Dilution Buffer into the reagent reservoir.

3.9.5 Remove the seal from the library plate.

3.9.6 Using a multi-channel pipette, add 2 µL of the purified library plate into the appropriate wells of the plate.

3.9.7 Add 198 µL of PowerSeq® Quant MS 1x Dilution Buffer into each well.

3.9.8 Seal the plate, vortex on plate mixer at 1000 rpm for 1 minute and then centrifuge at 1000 rpm for 1 minute. This creates a 1:100 dilution.

3.9.9 Transfer 2 µL of the 1:100 library dilutions into the appropriate 1:10,000 column.

3.9.10 Add 198 µL of PowerSeq® Quant MS 1x Dilution Buffer into each well.

3.9.11 Seal the plate, vortex on a plate mixer at 1000 rpm for 1 minute and centrifuge at 1000 rpm for 1 minute. This creates the final, 1:10,000 dilution.

3.9.12 Reseal the purified libraries plate using a BioRad Microseal B film and return to -20°C freezer.

Note: Unpooled, purified libraries may be stored for up to six months at -20° C.

3.10 If a dilution factor other than 1:10,000 is necessary, follow the instructions below.

3.10.1 1:1000 dilution:

- Transfer 2 µL of the 1:100 library dilutions into a new column. You may use a new plate, if needed.
- Add 18 µL of PowerSeq® Quant MS 1x Dilution Buffer into each well to complete 1:1000 dilution.
- Seal the plate, vortex on a plate mixer at 1000 rpm for 1 minute and centrifuge at 1000 rpm for 1 minute.

3.10.2 1:100,000 dilution:

- Transfer 2 µL of the 1:10,000 library dilutions into a new column. You may use a new plate, if needed.
- Add 18 µL of PowerSeq® Quant MS 1x Dilution Buffer into each well to complete 1:100,000 dilution.
- Seal the plate, vortex on a plate mixer at 1000 rpm for 1 minute and centrifuge at 1000 rpm for 1 minute.

3.11 Prepare reaction mix:

3.11.1 Create a reaction mix by adding the appropriate volumes of each of the following reagents in the amounts listed in LIMS. Reaction mix should be created in a 2 mL Sarstedt screw cap tube. Vortex for 10 seconds to mix.

Reagent	Per Reaction
2x qPCR Master Mix	10.0 µL
10x Primer mix	2.0 µL
Water	4.0 µL

4 Procedure

4.1 Obtain a new Applied Biosystems® MicroAmp® Optical 96-Well Reaction Plate.

4.2 Add 16.0 µL of reaction mix to each well in use according to the plate layout for your run.

4.3 Add 4 µL of the Quant MS standards, NTC, and the 1:10,000 dilution of the libraries according to the plate layout in LIMS.

4.4 Seal the dilution plate with optical adhesive film and store at 4°C until the quantitation run is complete. If rework must be performed, these dilutions may be used.

4.5 Seal the quantitation plate with optical adhesive film, use a straight edge or tube opener to secure the seal.

4.6 Centrifuge for 1 minute at 3000 rpm.

4.7 Ensure that there are no bubbles in the reaction wells before placing the plate on the 7500.

5 Software Operations

5.1 Turn on the Applied BioSystems® 7500 Real-Time PCR System. Allow time for the instrument to warm up.

5.2 Press the tray door to open and place the plate on the instrument. Be sure the plate is correctly aligned when position A12 is in the top right corner of the tray.

5.3 Close the tray door by pushing the depressed imprint on the right side of the tray. Do not push from the center.

5.4 Double click the icon for the HID Real-Time PCR Analysis Software.

5.5 Click the *Custom Assay* icon.

5.6 Click the down arrow for *New Experiment.*

5.7 Click From Template.

5.8 Open the “PSQuant MS” template.

5.9 Inside the Experiment Menu on the left side of the screen, click *Setup » Experiment Properties*.

5.10 Enter the run name into the field labeled Experiment Name.

5.11 To import samples, click *File » Import*. Locate file in the LIMS file share folder. Click *Start Import.*

5.12 Plate set-up imported successfully » click *OK.*

5.13 Check the top header and ensure the following:

- **Experiment Name:** Current Run Name
- **Type:** Standard Curve
- **Reagents:** SYBR® Green Reagents

7500 **PSQuant MS**	The PSQuant MS file is as follows: Soak at 95°C for 2 minutes 30 cycles: Denature at 95°C for 15 seconds Anneal at 60°C for 15 seconds Extend at 72°C for 45 seconds – fluorescent collection

5.14 Click *Start Run*. The run time is ~1:15 hour.

5.15 Once the run is complete, discard the plate and turn the instrument off.

6 Exporting Results

6.1 Open the HID Real-Time PCR Analysis Software on the desktop, if needed.

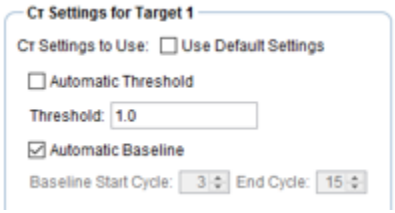
6.2 Enter custom assay mode.

6.3 If the assay that needs analysis is not currently open, click *File* » *Open*. Navigate to desired file, select the file, and click *Open*.

6.4 In the *Experiment Menu* located on the left side of the screen, click *Analysis*.

6.5 In the *Analysis* tab on the top right side of the screen, click *Analysis Settings* » *CT Settings*.

6.6 Verify the settings below and click *Cancel.*



6.7 Click *Analyze*.

6.8 After analysis, results can be exported. Click *View Plate Layout » Highlight All Wells*.

6.9 Located on the top toolbar, click *Export.*

6.9.1 Select data to export » *Results.*

6.9.2 Select one file or separate files » *One File.*

6.9.3 Ensure the file name is correct.

6.9.4 In the Custom Export tab, check the data is exporting columns (A1, B1, etc.)

6.9.5 Click *Start Export*.

6.10 With all wells still highlighted, click *Print Report* located on the top toolbar. Select *All Report Types.*

6.11 Click *Print* and choose to save as a *.PDF*. Ensure the correct run name is listed. Add "*reports* to the end of the file name.

6.12 Save file in appropriate LIMS folder and click *Save*.

6.13 Transfer the raw data .EDS files from the instrument PC to the Forensic Biology network drive.

7 Data Analysis

7.1 Use the standard curve to determine the concentration of your unknown samples. The concentrations of sample replicates will be averaged.

7.2 Use the reports generated to interpret the results for each assay.

7.2.1 Using the standard curve reports, ensure the following parameters are met for the slope, Y-intercept and R^2 value. All three parameters must fall within the quality criteria for the quantitation to pass.

- Standard slope must be between –3.2 and –3.8
- R^2 values must be ≥ 0.980
- The Y-Intercept value must be between 12.5 and 18.5

7.2.2 Record the QC batch parameters. Make sure to release and save all data stored in the QC batch parameters tab.

7.2.3 The reports should be attached to the test batch in LIMS.

7.3 Navigate to the data entry page in LIMS.

7.4 Account for the 1:10,000 dilution factor by typing "1" into the dilution column for all your standards and typing "0.0001" into the dilution column for your unknown samples. If your samples were diluted to a value other than 1:10,000, modify the value entered in the dilution column accordingly.

7.5 Import the data into the LIMS test batch.

7.5.1 Mandatory fields for nM concentration and the average nM concentration should be manually filled in with the corrected nM concentration for each standard and the NTC.

7.5.2 Evaluate the NTC. The library quant must quant at 0 pM for the quantitation batch to pass. The status of NTC must be recorded in LIMS.

7.5.3 The NTC will only be quantified once per run. Remove the replicate quantitation row from the data entry screen.

7.5.4 Extraction negative controls and amplification negative controls are not evaluated for pass/fail during this library quantification procedure.

7.6 Sample interpretations

7.6.1 Samples whose dilutions are reported outside the dynamic range of 0.2 nM to 200 nM, should be scheduled for requant at a dilution whose quantitation value would be expected to fall within that range.

7.6.2 The difference between replicate concentrations of the same sample must fall within 60% of each other. Replicates whose quantitation values fall outside of this range should be requanted.

$$Percent\ Difference\ Formula\ (\%D) = \frac{|n_1 - n_2|}{\frac{n_1 + n_2}{2}} \times 100$$

7.7 When evaluating normalized libraries, concentrations should fall within 2.5 nM and 8 nM. If library concentrations fall outside of this range, consult a supervisor before you proceed.

7.8 Push Concentration. The pushed concentration for each library will be the average of the quantitation values of the two replicates. Concentrations for normalized libraries should not be pushed.

7.8.1 If a library was quantified at different dilution factors and both fall within the dynamic range, the concentration with the least significant dilution should be pushed. If only one concentration falls within the dynamic range, this passing concentration should be pushed.

7.9 Record the results of your analysis in the interpretation column of the data entry page.