Forensic Biology Section

Operation of QuantStudio 5

1. Scope

To provide instructions for the operation of the QuantStudio 5 Real-Time PCR System.

The QuantStudio 5 Real-Time PCR System uses fluorescent-based polymerase chain reaction (PCR) reagents to perform quantitative detection of target nucleic acid sequences, qualitative detection of targets, and qualitative analysis of the PCR product (post-PCR melt curve analysis).

The instrument is integrated with the QuantStudio Design and Analysis Software to design experiments or to analyze data.

2. Safety

During instrument operation, the sample block temperature can reach 100°C. Allow it to cool to room temperature before handling.

3. Instrumentation

- QuantStudio 5 (QS5) Real-Time PCR instrument (APPLIED BIOSYSTEMS).
- Applied Biosystems instrument-related consumables.
- Centrifuge with plate adapter.

4. Quality Assurance

4.1. The QS5 instrument should be performance tested by running samples from the NIST SRM #2372 "Human DNA Quantitation Standard" at the following times:

- Once annually.
- After extensive service is performed on the unit.
- If there is reason to question the precision of the instrument based on the standards and controls.
- Note: in the internal validation of PowerQuant (PQ), the autosomal values for NIST SRM 2372a standards A, B, and C were 7% to 12% higher than the mid-point values on the SRM's Certificate of Analysis. This will be considered during review of the data obtained for performance checks~~Note: at the time of the internal validation, the PQ values for NIST SRM standards were higher than the values listed on the Certificate of Analysis provided by NIST. This will be considered during review of the data obtained for the performance tests~~.

4.2. The background calibration plate can be performed as needed to check for block contamination. Note: Performing a background calibration does NOT invalidate any other calibration.

4.3. If contamination is detected, the sample block will need to be decontaminated. For Decontamination Instructions, see the "QuantStudio 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide".

5. Procedural Notes:

5.1. The ROI/Uniformity and background calibration plates are run by the service engineer during the annual Performance Maintenance (PM) visit. The software uses the ROI/Uniformity calibration data to map the increase in fluorescence to the plate wells during subsequent runs by capturing a plate image for each optical filter. The software also uses the ROI/Uniformity calibration data to

evaluate well-to-well consistency of the signals. **Note: Performing a ROI/Uniformity calibration invalidates all other calibrations.**

5.2. Once an ROI/Uniformity calibration is initiated, the instrument automatically prompts for calibrations to be performed in this order:

5.2.1. Perform a ROI/Uniformity calibration (r~~R~~outinely performed during the PM visit).

5.2.2. Perform a Background calibration (perform any time that the ROI/Uniformity calibration is current).

5.2.3. Perform Dye calibrations.

- Custom Dye calibration plates need to be run after the ROI/Uniformity and background calibration plates have been run, but may be run any time that the ROI/Uniformity and Background calibrations are current.

5.3. The RNase P Plate should be run as needed, to confirm instrument performance.

6. Background Calibration

6.1. Remove the background calibration plate from the freezer, thaw the plate in its packaging for approximately 30 minutes. Use the plate within 2 hours of thawing. Note: Keep the plate protected from light until you perform the calibration.

6.2. Remove the calibration plate from its packaging and retain the packaging. Do NOT remove the optical film. Vortex the plate for 5 seconds, then centrifuge for 2 minutes at 750 to 1000 x g.

6.3. Confirm that the liquid in each well is at the bottom of the well and free from bubbles.

- IMPORTANT! Keep the bottom of the plate clean. Fluids and other contaminants on the bottom of the plate can contaminate the sample block and cause abnormally high background signal.

6.4. In the Home screen select: "Settings" → "Maintenance and Service" → "Calibrations" → "Custom" → "Background".

6.5. Follow the instructions on the screen to start the calibration.

6.6. Load the plate and select "Start".

6.7. When the run is complete and the screen displays "Calibration Complete", touch "View Results" to check the calibration status. The calibration status will be displayed as "Passed" or "Failed".

6.8. Return the plate to its original packaging. Note: Each calibration plate can be used up to 3 times if the plate is:

- Stored in its packing sleeve at –15 to –25°C.
- Used within 6 months after opening.
- Used before the plate's expiration date.

7. Custom Dye Calibration

7.1. Thaw the five ~~PowerQuant~~PQ Calibration Standards and ~~PowerQuant~~PQ Calibration Buffer from the ~~PowerQuant~~PQ Calibration Kit. Note: -The Calibration Buffer needs to be thawed ~~for~~

at least 2-3 ~~hrs~~hours. ~~. Alternatively,~~ Frozen Calibration Buffer can also be thawed in ~~it may be transferred to~~ the refrigerator overnight~~.the night before.~~

7.2. Vortex each PowerQuant Calibration Standard and ~~PowerQuant~~PQ Calibration Buffer for 10 seconds to mix (~~. D~~do ~~not~~ NOT centrifuge the ~~PowerQuant~~PQ Calibration Standards after mixing.)

7.3. Dilute each ~~PowerQuant~~PQ Calibration Standard 100-fold in ~~PowerQuant~~PQ Calibration Buffer in ~~a~~ separate tubes as described below:

Reagent	FAM	CFG540	TMR	Q670	CXR
PQ Calibration Standard, **FAM**	**22 µl**	--	--	--	--
PQ Calibration Standard, **CFG540**	--	**22 µl**	--	--	--
PQ Calibration Standard, **TMR**	--	--	**22 µl**	--	--
PQ Calibration Standard, **Q670**	--	--	--	**22 µl**	--
PQ Calibration Standard, **CXR**	--	--	--	--	**22 µl**
PQ Calibration **Buffer**	**2,178 µl**	**2,178 µl**	**2,178 µl**	**2,178 µl**	**2,178 µl**
Total Volume	**2,200 µl**	**2,200 µl**	**2,200 µl**	**2,200 µl**	**2,200 µl**

7.4. Vortex each diluted PQ Calibration Standard for 10 seconds to mix (do NOT centrifuge the standards after mixing).

~~7.3.~~

Reagent	FAM	CFG540	TMR	Q670	CXR
PowerQuant® Calibration Standard, FAM	22µl	–	–	–	–
PowerQuant® Calibration Standard, CFG540	–	22µl	–	–	–
PowerQuant® Calibration Standard, TMR	–	–	22µl	–	–
PowerQuant® Calibration Standard, Q670	–	–	–	22µl	–
PowerQuant® Calibration Standard, CXR	–	–	–	–	22µl
PowerQuant® Calibration Buffer	2,178µl	2,178µl	2,178µl	2,178µl	2,178µl
Total volume	2,200µl	2,200µl	2,200µl	2,200µl	2,200µl

~~7.4. Vortex each diluted PowerQuant Calibration Standard for 10 seconds to mix. DO NOT centrifuge the standards after mixing.~~

7.5. ~~Reserve a~~Using **five** (5) separate MicroAmp optical 96-well reaction plates, label the side skirt of ~~for~~ each plate with one of the ~~PowerQuant~~ PQ Calibration Standard ~~. Label the side of the plate skirt with the~~ custom dye names (~~ie.~~ PQ_-FAM, PQ_-CFG540, PQ_-TMR, PQ_-Q670, and PQ_-CXR). ~~Note:~~ Wearing gloves, ~~and~~ handle the plate by the edges, and ~~. A~~avoid touching the plate wells and ~~MicroAmp~~ optical adhesive film.

~~7.6.~~ Dispense 20µl of one diluted ~~PowerQuant~~PQ Calibration Standard into all 96 wells of the ~~associated plate~~plate labeled with that dye name. Repeat this ~~this step~~ for each PQ ~~of the other PowerQuant~~ Calibration Standard~~. s.~~

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~~7.7.~~7.6. ~~S~~sealing each plate with MicroAmp optical adhesive film, and p~~. P~~rotect the plates from light exposure~~. to light.~~

~~7.8.~~7.7. Centrifuge each plate briefly.

~~7.9.~~7.8. Enter the "Settings" menu on the QS5 home screen. Select the "Maintenance and Service" option on the subsequent screen.

- Select "~~"~~Calibrations" → "Custom" → "Custom Dye"~~."~~.
- Choose the appropriate dye name for the plate being loaded into the instrument (~~:~~ "PQ_FAM", "PQ_CFG540", "PQ_TMR", "PQ_Q670" ~~and~~ or "PQ_CXR")~~.~~.
- Confirm that "Reporter" is selected as the Type for the dye.
- Select "Update".
- Enter "60°C" for the calibration temperature.

~~7.10.~~7.9. Open the instrument drawer, l~~L~~oad the appropriate dye calibration plate, and press "Start"~~. onto the instrument.~~

~~7.11.~~7.10. ~~Press "Start". E~~Each dye calibration takes approximately 3 minutes.

~~7.12.~~7.11. When complete, remove the plate ~~Unload the next plate~~ and repeat the calibration process for each plate containing the various ~~PowerQuant~~ calibration standard dyes~~. plates.~~

~~7.13.~~7.12. After calibration, store the Custom Dye calibration plates ~~protected from light~~ at –20°C for up to 4 months (wrapped in aluminum foil to protect from light). Re-use the plates ~~when re-analysis~~if recalibration is necessary.

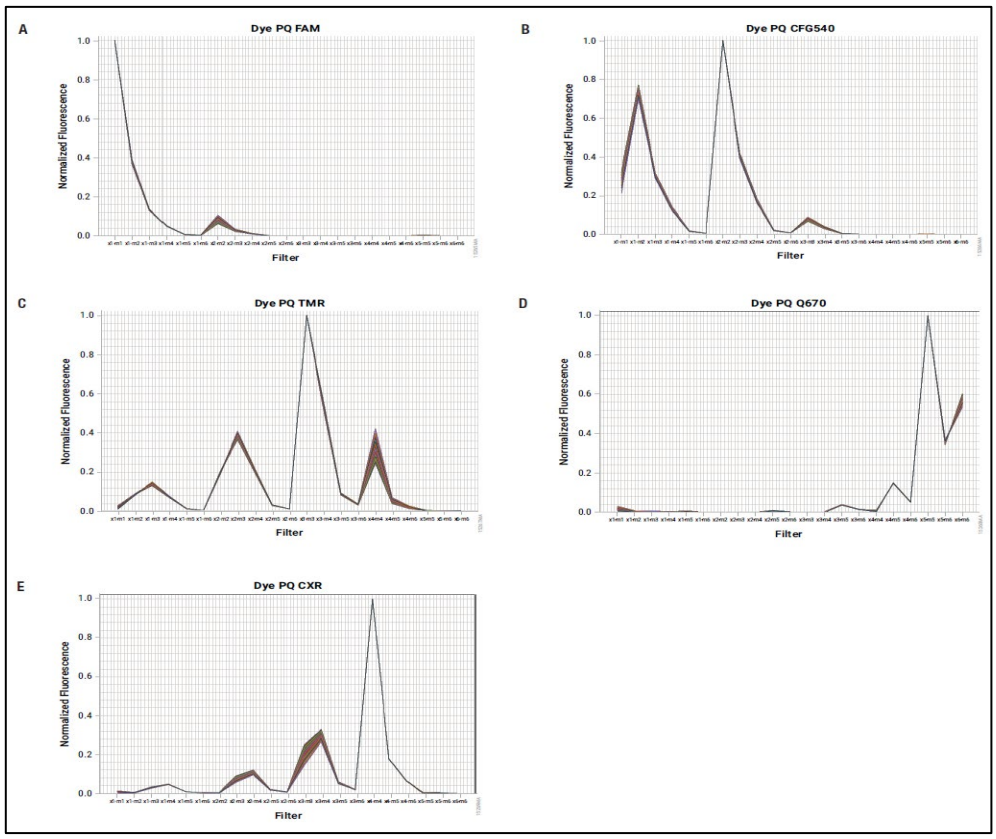
8. Evaluating the PowerQuant Dye Calibration Spectra

8.1. On the QS5 screen, select "View Results" → "Details".

8.2. The calibration spectra will be displayed.

8.3. Review the dye spectrum plot for each calibration run. See examples of passing calibration spectra for each of the ~~PowerQuant~~PQ Dyes below.

- Select "Accept Results" to confirm that the calibration result is acceptable. A second confirmation will appear in which you will have to "Accept Results" again. This action saves the calibration data.
- Select "Reject Results" if the results are unacceptable and test the calibration plate again.
- Note: For calibration troubleshooting refer to the Troubleshoot Calibration Failure section in Appendix A of the QS5 Real-Time PCR Instrument User Guide for Human Identification.



9. Instrument Verification Using the RNase P Plate

9.1. The RNase P plate contains the reagents necessary for the detection and quantitation of genomic copies of the human RNase P gene. ~~Each~~ Every well contains PCR master mix, RNase P primers, FAM dye labeled probe, and a known concentration of human genomic DNA template.

9.2. Remove the RNase P plate from the freezer and thaw the plate in its packaging for approximately 5 minutes. Use the plate within 30 minutes of thawing.

9.3. Remove the calibration plate from its packaging and retain the packaging. Do NOT remove the optical film.

9.4. Vortex the plate for 5 seconds, then centrifuge for 2 minutes at 750 to 1000 x g.

9.5. Confirm that the liquid in each well is at the bottom of the well and free from bubbles.

- IMPORTANT! Keep the bottom of the plate clean. Fluids ~~and other~~or contaminants on the bottom of the plate can ~~contaminate the sample block and~~ cause an abnormally high background signal.

9.6. In the Home screen select~~:~~ "Settings" → "Maintenance and Service" → "RNase P Verification".

9.7. Load the plate and select "Start".

9.8. When the run is complete, touch "View Results" to confirm the status of the run. Touch "Accept Results" to save the results to the instrument, or "Reject Results" to delete the RNase P ~~verification~~ results.

- IMPORTANT: Each RNase P plate can only be used once.

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10. Installing Promega PowerQuant Analysis Software

10.1. Navigate to H:\Crimelab\DNA\Validation\PowerQuant and CWD work\PQ on QuantStudio5\PQ PowerQuant Tools\PowerQuant Assays Installer v4.8.0.0 folder and double-click on the "PowerQuant Assays Installer v4.8.0.0.exe" application. Note: When installing the software, the person logged into the computer must have administrative rights to the computer.

10.2. When the application launches, choose "I accept the agreement" for the end-user license agreement and then click "Next".

10.3. Click "Next" when asked about administrator rights.

10.4. Choose "No, Keep t~~T~~he Existing Database" and click "Next".

10.5. Click "Install". The files will be extracted, and the program will be installed in the C:\Program Files (x86)\Promega\PowerQuant folder.

10.6. Click "OK" and ignore the warning message about folder permission.

10.7. Click "Finish" to complete the PowerQuant Assays installation. A shortcut to the program will appear on the desktop.

11. Creating Default Plate Map Template

11.1. Double-click on the PowerQuant Assays shortcut ( ) to launch the PQ analysis program.

11.2. Click on the "Configuration Tools" tab.

11.3. Click on "Configuration Settings". This will open as a separate window.

- An "Administrator Login" window opens. Enter the **Administrator Username** as **"pqadmin"**, the **Password** as **"Promega"**, and click "OK". The configuration settings can be viewed (but not changed) by clicking "OK" without entering the above username and password.
- The Concentration Units should be "ng/ul".
- The Well Naming Convention should be "A1, A2, A3…".
- Highlight all and then deselect the following sheets: Settings, Standard Curve, Table Results, Sample Name Map, IPC Map, or Imported Data sheets. This will hide all highlighted sheets. Any sheet with "Normalization" or "Norm" should be hidden. If preferred, additional sheets may be hidden.
- Click "Save" and then close the window.

11.4. Click on "Design Plate Map". This will open as a separate window.

11.4.1. Set the "Standard Curve Type":

- Use the dropdown menu and select "Imported"
- Click "Yes" when asked if it okay to clear the form

11.4.2. Set the "Sample Type":

- Highlight the first column of the Sample Type Plate Map, from the Sample Type dropdown menu, select "STANDARD" and click "Apply".
- Highlight the first two cells in the second column of the Sample Type Plate Map, from the Sample Type dropdown menu, select "NEGATIVE" and click "Apply".
- Highlight the remaining cells of the Sample Type Plate Map, from the Sample Type dropdown menu, select "UNKNOWN" and click "Apply".

11.4.3. Set the "Sample Concentration":

- In the first column of the Sample Concentration grid, type 50, 50, 2, 2, 0.08, 0.08, 0.0032, and 0.0032. These are the ng/ul concentrations of the standard curve (each done in duplicate) down the first column of wells.

11.4.4. Click "Save Plate Map" and name the template "PQ Template" (or some variation) in the top box and click "OK". If a template with that name already exists and you want to overwrite it, click "Yes" when asked "template name already exists; OK to Overwrite?".

11.5. The new template will now appear in the "Existing Plate Maps" pull-down list. Click the close "X" in the upper right corner to exit the Plate Designer.

11.6. Follow instructions for creating a custom sort list saved in the folder H:\Crimelab\DNA\Validation\Promega PowerQuant validation\PQ Assay Analysis software v4.8.0.0

12. Exporting Analyzed Data from the QuantStudio Design and Analysis Software

12.1. Confirm that all wells containing data for export are highlighted in the plate map. Navigate to the "Export" tab.

12.2. Specify an appropriate export file name. Use the "Browse" button to choose a file location and click the "Export" button. Export the file to the desktop folder containing run folders for each analyst.

12.3. Use a USB drive to transfer the export the run to the relevant user's data folder on the H Drive (H:\CrimeLab\DNA\QuantStudio\run data) for analysis and archiving.