Spectral Calibration of QuantStudio Instruments

PURPOSE

The purpose of this document is to provide guidance for the recalibration of spectral dyes on QuantStudio Real-Time PCR Systems. The following instructions are meant to be used with the QuantStudio Real-Time PCR Software v1.7.2, Design & Analysis Software 2.6, and the latest instrument firmware.

BACKGROUND

TaqMan qPCR probes covalently bind a fluorescent dye and a quencher molecule on either end of a target sequence. The quencher dampens fluorescence while held in close proximity to the fluorophore. During PCR, the probe is degraded by the 3' exonuclease activity of DNA polymerase. The fluorophore is separated from the quencher and PCR amplification can be detected by the increase in fluorescence.

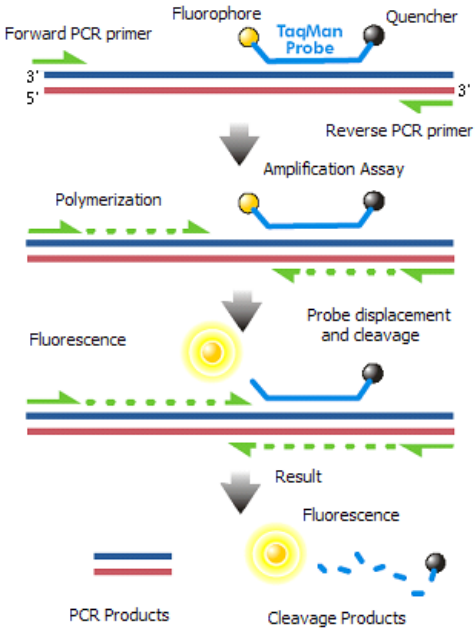

Figure 1: TaqMan qPCR chemistry.

Multiplex qPCR is made possible by associating a different color dye with each target. Each dye absorbs and emits light, and the wavelength of maximum absorption and emission depends on the color. QuantStudio instruments measure fluorescent intensity at a range of wavelengths by using a set of 5 or 6 filters. It is possible to discriminate which fluorescent dye(s) are present based on which wavelength(s) produce the brightest signal(s).

Dye	Excitation λmax (nm)	Emission λmax (nm)	Calibrator	Filter	Color
FAM	495	520	Calibration Plate 1	x1-m1	Blue
VIC / HEX	538	555	Calibration Plate 1	x2-m2	Green
ROX	588	608	Calibration Plate 1	x4-m4	Orange
CalFluor610	590	610	Custom Dye	x4-m4	
Texas Red	596	613	Custom Dye	x4-m4	
Cy5	648	668	Calibration Plate 3	x5-m5	Red
Cy5.5	685	706	Custom Dye	x6-m6	Deep-Red
Quasar705	690	705	Custom Dye	x6-m6	

Figure 2: Dyes, wavelengths, and QuantStudio filters.

Each dye emits a spectrum of light rather than a single wavelength. **Bleedthrough** is caused when a dye becomes bright enough to create a false positive signal in another channel or color. **Crosstalk** is when a well or capillary becomes bright enough to create a false positive signal in an adjacent well or capillary.

Bleedthrough can be mitigated through spectral calibration of the instrument. During calibration, the fluorescence emitted by each dye is measured using each filter. Later on, when fluorescence is detected in an experiment, this calibration data can be used to correct for and prevent bleedthrough into other channels.

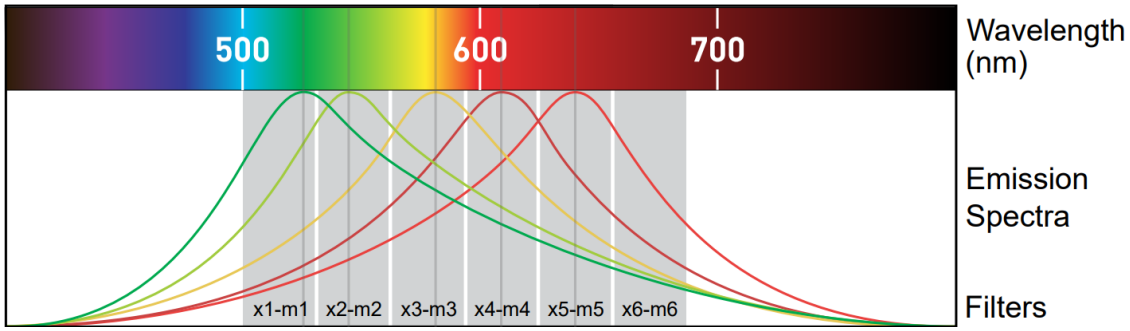

Figure 3: Fluorophore Emission Spectra (ref. 2)

Fluorescent dyes are divided into two categories: Standard (or system dyes) and Non-Standard (or custom). Calibration materials for standard dyes are readily available through ThermoFisher Scientific and calibration may be performed by the manufacturer as part of installation and routine preventive maintenance. Calibration for non-standard dyes may not be performed by the manufacturer during installation or PM, and calibration materials may or may not be available through ThermoFisher Scientific. This document contains some information about obtaining calibration materials. Contact PCRAssays.com for additional support.

REAGENTS / SUPPLIES

Note that the following items are to be used with QuantStudio Fast (0.1 mL) blocks. Similar products exist for 0.2 mL, 384 well, and OpenArray blocks, contact your ThermoFisher Scientific sales representative.

- https://www.thermofisher.com/order/catalog/product/4346906
- https://www.thermofisher.com/order/catalog/product/4311971
- https://www.thermofisher.com/order/catalog/product/4432426
- https://www.thermofisher.com/order/catalog/product/A26336
- https://www.thermofisher.com/order/catalog/product/A26337
- https://www.thermofisher.com/order/catalog/product/A26340
- https://www.thermofisher.com/order/catalog/product/A45218?SID=srch-srp-A45218
- https://www.biosearchtech.com/products/pcr-kits-and-reagents/dye-calibration-standards/cal-fluor174-red-610-t10-calibration-standard
- Low-EDTA TE (1X), pH 8.0, Quality Biological (VWR Cat #: 10128-588)
- Water, Molecular Biology Grade (VWR Cat #: VWRL0201-0500)

EQUIPMENT

- Freezer
- Pipettes
- Plate Centrifuge

PROCEDURE

Review Calibration Data

1. Power on the QuantStudio instrument and launch the QuantStudio Real-Time PCR Software.
2. On the computer, open **Instrument > Instrument Console** and connect to the instrument by double clicking the serial number under "**My Instruments**".
3. Review existing calibrations in the "Information" section as shown below. Note that the "**Block Type**" is listed. Calibration data is block specific. If multiple blocks are used on the same instrument, each block must be calibrated separately.

Calibrations Include:

ROI (Region of Interest) - The ROI calibration determines the physical of each well in the block. This information is necessary for all subsequent calibrations. Note that creating a new ROI calibration will invalidate every other calibration.

Background - Measures any residual fluorescence in the system by using a blank plate. It is recommended to run this calibration regularly to ensure there is no unwanted signal.

Uniformity - Measures the consistency of fluorescent intensity across the plate. Note that creating a new uniformity calibration will invalidate each of the dye calibrations for that block.

Dye - Each dye has its own spectral calibration. Using the latest firmware versions, the Standard Dyes are calibrated using a series of 3 plates that can be acquired through ThermoFisher Scientific. In most cases, custom dyes will be calibrated by obtaining a portion of the requisite fluorophore, diluting to an appropriate concentration, and distributing into the necessary plate format.

RNase P - Is a systems test that uses a consumable, single-use, premade PCR plate (available from ThermoFisher Scientific) to perform qPCR using the instrument. This test is optional.

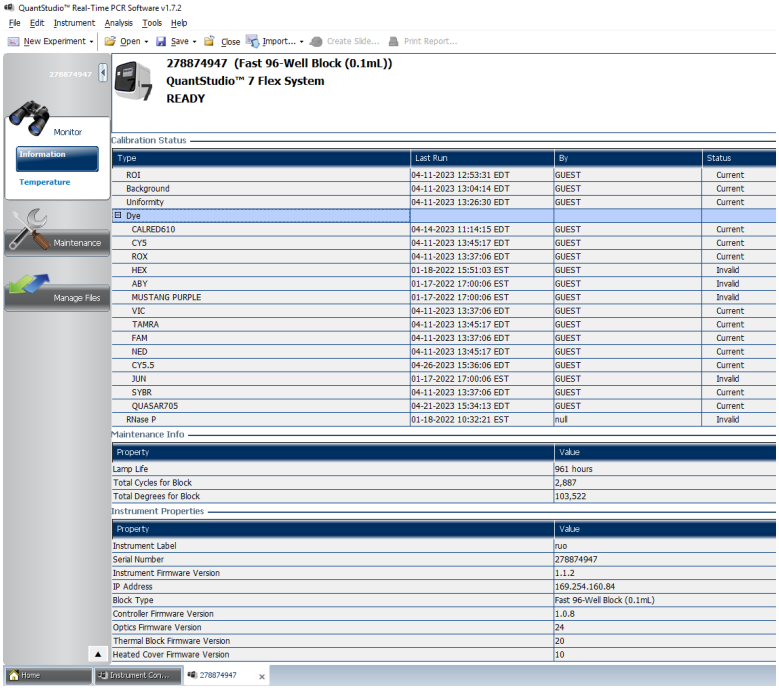

Figure 4: How to view the available dyes on the QuantStudio 7 instrument.

Calibrating Custom Dyes

1. If not already current, perform ROI, Background, Uniformity, and Standard Dye calibrations for the instrument. These calibrations are typically performed by the manufacturer during installation or routine preventive maintenance, but instructions can also be found in the instrument user manual.
2. Obtain a portion of the custom dye(s) for calibration. Plates for some block and dye combinations may be available through ThermoFisher Scientific (https://www.thermofisher.com/order/catalog/product/A45218?SID=srch-srp-A45218). A variety of calibration standards can be sourced from Biosearch Technologies, including CAL Fluor Red 610 (https://www.biosearchtech.com/products/pcr-kits-and-reagents/dye-calibration-standards/cal-fluor174-red-610-t10-calibration-standard). Dye calibration standards can be formulated with the fluorophore covalently attached to an oligo-dT (10-mer) (to better mimic the signal from a fluorescent probe) and synthesized by vendors such as IDT (http://www.idtdna.com). If having difficulty sourcing calibration materials for custom dyes, contact http://PCRAssays.com for additional support.
3. Rehydrate, dilute, and homogenize the fluorophore. Ideal concentrations typically range from 150 - 800 nM, but depend on the calibration standard and instrument. In most cases, a wide range of concentration will be acceptable, there is no ideal.
4. Distribute the calibration material into whichever plate type is being calibrated, seal tightly with optical film, and centrifuge. Make sure to fill every well. QuantStudio instruments record calibration data by well location. PCRassays.com recommends 20 microliter PCR volumes, so this may be most appropriate, but larger or smaller volumes may be necessary based on the block type.
5. In the QuantStudio Real-Time PCR Software, navigate to the instrument console and open the **Maintenance** section for the instrument being calibrated. Advance to the **Dye** calibrations and select **Custom Dye** calibration. Select the requisite **Dye Name** from the drop-down menu OR add a **New Dye** if it is not in the list.

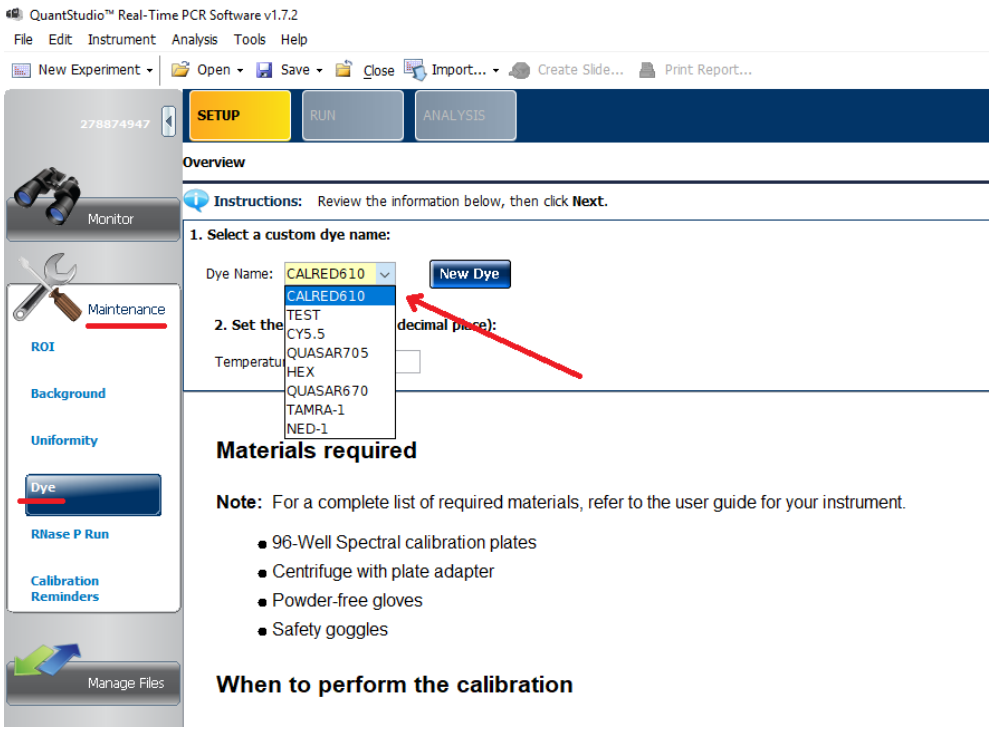

Figure 5: Starting the calibration. Choose the dye you want to calibrate or select "New Dye"

6. Load the calibration plate onto the instrument and advance through the prompts. After the calibration is started, the instrument will run for approximately 5 minutes before reporting a result. Provided that the calibration passes, make sure advance through prompts and **Save** the result.

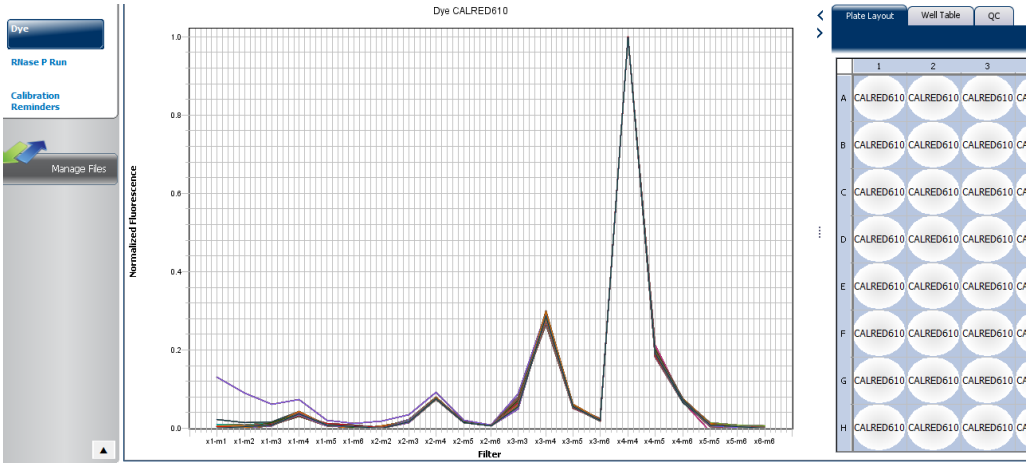

Figure 6: Calibration results.

Ensure filters are enabled

When creating a QuantStudio template file to be used in future experiments, it may be necessary to ensure that all filters are enabled. By default, it's not possible to see whether any filters have been disabled in a template file. Note that this is a template specific setting.

1. Open the QuantStudio Real-Time PCR Software and navigate to **Tools > Preferences**. Select the **Defaults** tab and ensure that the "Show optical filters for run method" box is checked.

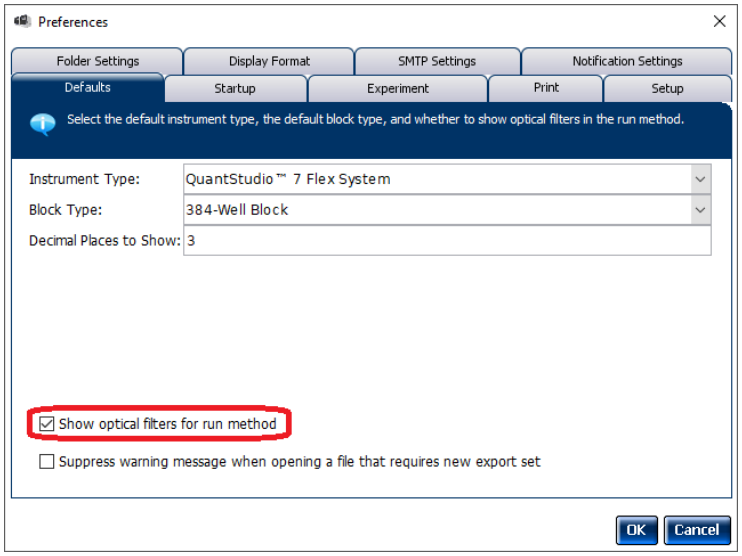

Figure 7: Show optical filters.

2. Open the template file and navigate to the Run Method. Click on the Optical Filters tab and ensure that all filters are enabled as shown here. Select File>Save to save the template file.

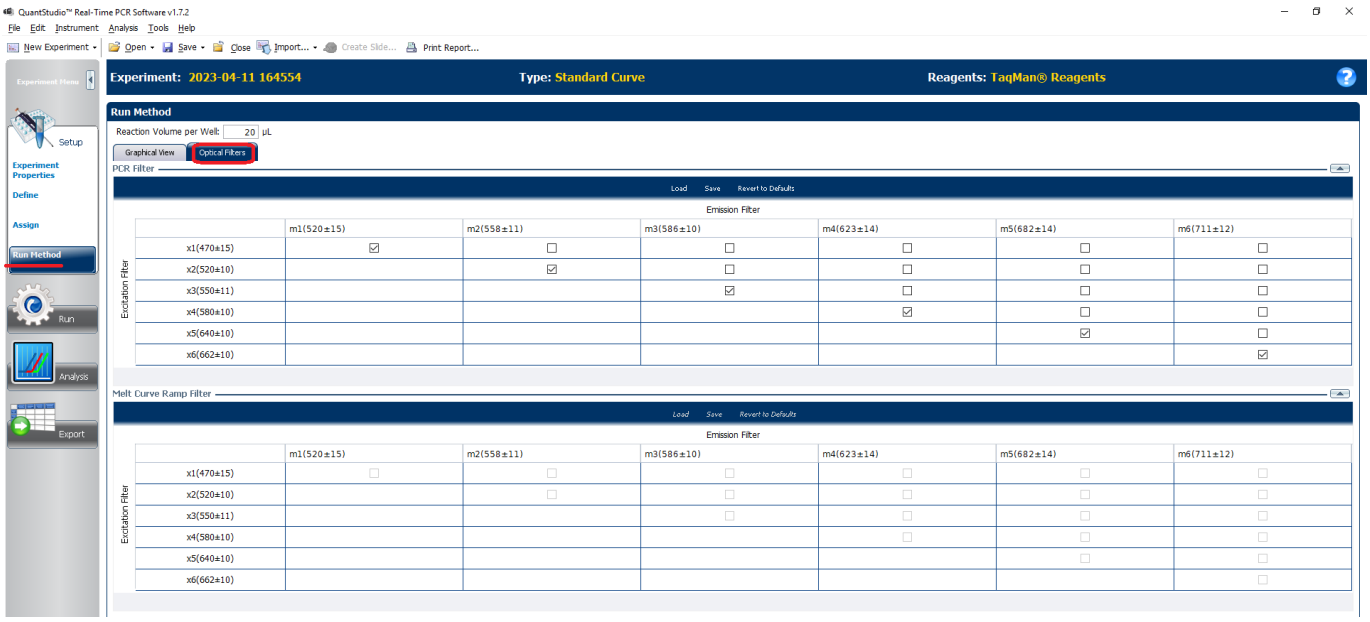

Figure 8: Enable all filters (select "Optical Filters" tab, and then check the boxes as shown).

REFERENCES

1. https://assets.fishersci.com/TFS-Assets/LSG/manuals/MAN0010407_QuantStudio_3_5_InstallUseMaint_UG.pdf
2. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0018045_QS_6Pro_7Pro_UG.pdf
3. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0018832_QS12K_Flex_v1.5_MaintAdmin_UG.pdf

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