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Perform Custom Dye Calibration on Applied Biosystems® Real-Time PCR Systems

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Overview

Purpose

Applied Biosystems® Real-Time PCR Systems can be used to run assays designed with custom dyes. Custom dyes must fall within the excitation and emission wavelength ranges for your system. Use the following procedure to identify the optimal concentration of custom dye(s) for system calibration.

Note: Refer to the Maintenance Guide for your system for information on the supported wavelength ranges of the system.

Compatible systems

This procedure is compatible with the following Applied Biosystems® Real-Time PCR Systems and replaces the custom dye calibration procedure shown in the Maintenance Guide for the system.

System	Publication	Pub. no.
QuantStudio® 6 Flex Real-Time PCR System or QuantStudio® 7 Flex Real-Time PCR System	*QuantStudio® 6 and 7 Flex Real-Time PCR Systems Maintenance and Administration Guide*	MAN0007992
QuantStudio® 12K Flex Real-Time PCR System	*Applied Biosystems® QuantStudio® 12K Flex Real-Time PCR System Maintenance and Administration Guide*	4470689
ViiA™ 7 Real -Time PCR System	*Applied Biosystems® ViiA™ 7 Real -Time PCR System: Calibration, Maintenance, Networking, and Security User Guide*	4442661

Note: Refer to the Maintenance Guide for your system for detailed instructions on using other features of the system.

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About the dye calibration data

The result of a dye calibration is a collection of spectral profiles that represent the fluorescence signature of each dye standard. Each profile consists of a set of spectra that correspond to the fluorescence collected from the wells of the calibration plate. Following the calibration of each dye, the software "extracts" a spectral profile for each dye. The software plots the resulting data for each profile in a graph of fluorescence versus filter. When the software extracts the dye calibration data, it evaluates the fluorescence signal generated by each well in terms of the collective spectra for the entire calibration plate. During each experiment run, the software receives data in the form of a raw spectra signal for each well. The software then determines the contribution of the fluorescence dye(s) in each well by comparing the raw spectra to the calibration spectra.

Workflow: Before you use custom dyes

Before using custom dyes with your system, you must follow this workflow:

START

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Dilute the custom dye to an optimal concentration[1]

▼

Prepare a custom dye dilution plate

▼

Run the dilution plate as an experiment

▼

Determine the optimal dye concentration

▼

Calibrate the custom dye[2]

▼

Create a custom dye plate

▼

Add the custom dye to the software

▼

Perform a dye calibration

▼

FINISH

[1] First time only for each custom dye.
[2] For each custom dye.

Dilute the custom dye to an optimal concentration

Custom dye dilution guidelines

Use the following guidelines to prepare a dilution series for each custom dye:

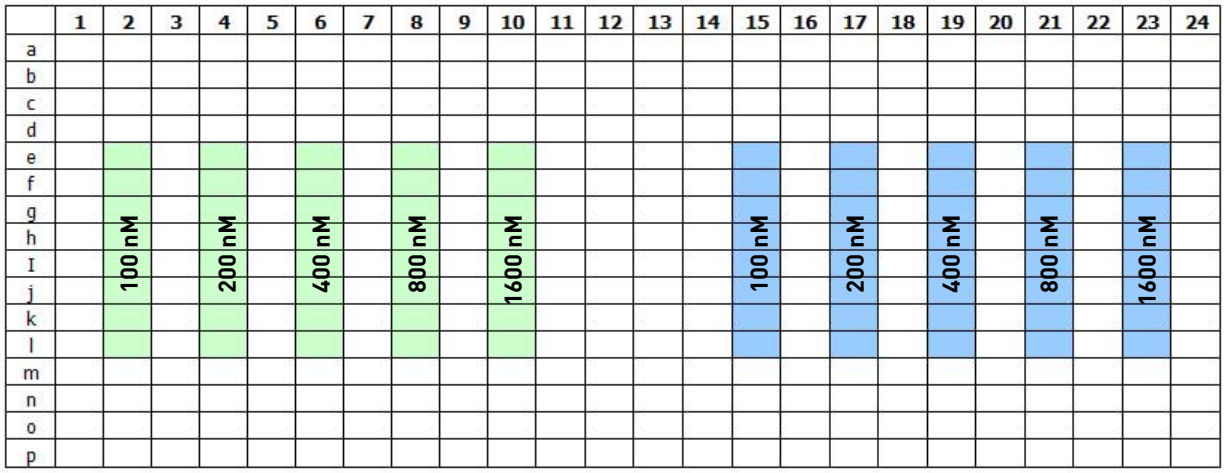
- Target several dye concentrations within a range of 100–2000 nM.
- Choose a 2- or 3-fold difference in dilution points.
- Use a recommended volume range of 10–20 µL/well for 96-well and 384-well plates.
- Dilute the dye in buffer that is compatible with your master mix. Refer to the Maintenance Guide for your system for Background Plate ordering information for use with Life Technologies™ dyes or for detailed instructions for preparing a background plate using the buffer provided with your custom dye.
- *(Intercalating dyes only)* Add the appropriate amount of amplified PCR product to generate fluorescence.

Prepare a custom dye dilution plate

IMPORTANT! Wear powder-free gloves while creating the dye plate.

Prepare and load the custom dye dilution plate:

1. Prepare a dilution series of the custom dye following the dilution guidelines.
2. Pipet the appropriate volume of the dilution series to the center of a 96/384-well plate, then seal the plate.
   A full plate is not needed, as shown in the following example (100, 200, 400, 800, and 1600 nM in replicate wells of a 384-well plate):



3. Vortex the plate for 5 seconds, then centrifuge it for 2 minutes at < 1500 rpm. Confirm that the liquid in each well of the plate is at the bottom of the well. If not, centrifuge the plate again at a higher rpm and for a longer period of time.
4. Load the plate into the instrument.

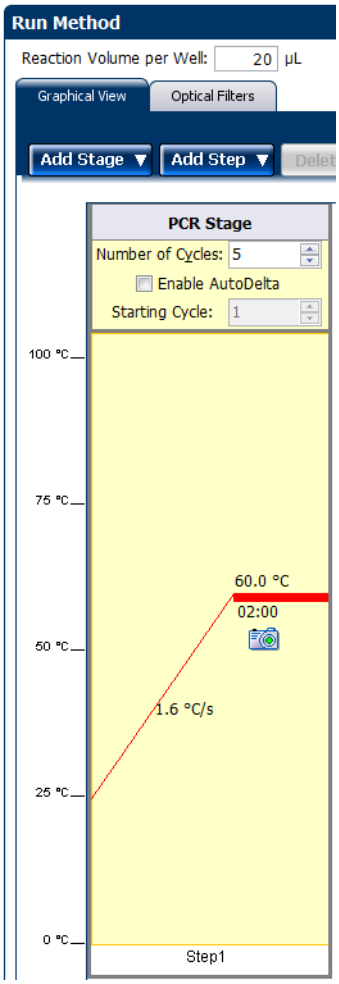
Run the dilution plate as an experiment

Run the custom dye dilution plate as a new experiment:

1. On the Home screen of your system software, create a new **Genotyping** experiment and select to include **Amplification**.

2. Enter the required experiment properties, then add the dilution series information to the appropriate wells in the plate layout.

3. Edit the run method to include a cycling stage that ramps to 60°C with a 2 minute hold. Make sure to enter the appropriate reaction volume and select the filters of interest.
   For example:



4. Save the experiment and start the run.

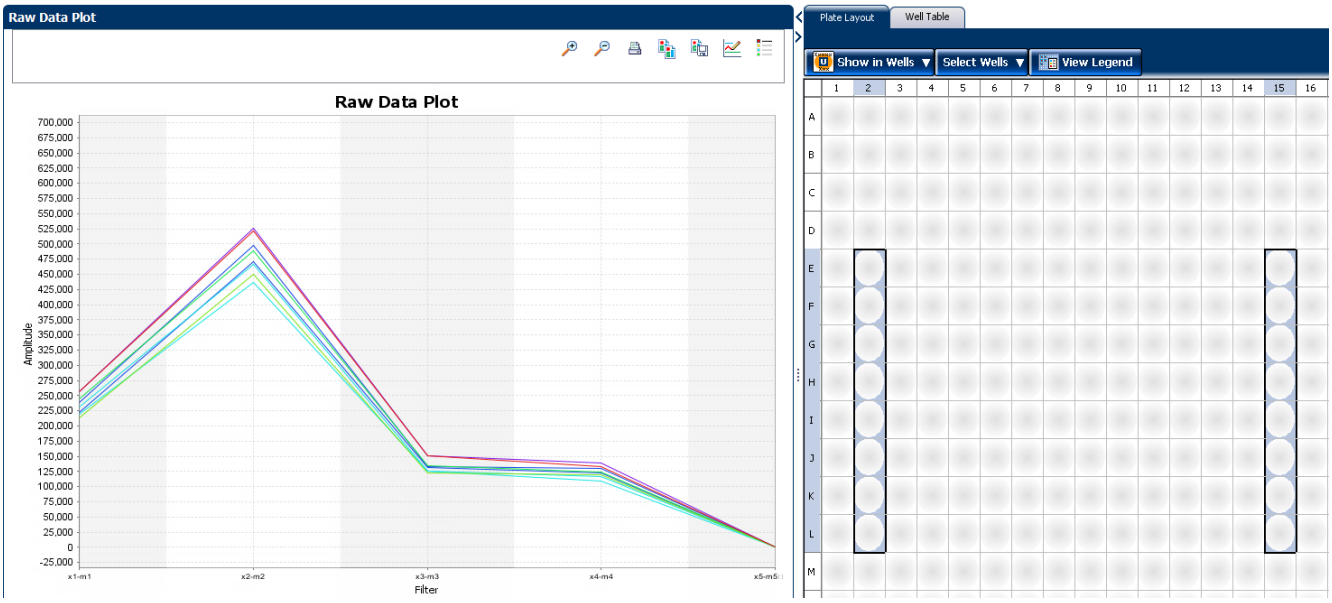
Determine the optimal dye concentration

Review the run results and select the dilution to calibrate:

1. When the run is complete, review the dye signal data:
   a. In the Analysis area of your system software, select the **Raw Data Plot**.
      This plot displays the raw fluorescent signal of each dye as detected through each emission filter, for individual wells and at individual cycles over the duration of the PCR run.

b. From the plot Options, drag the **Show Cycle** slider to the last cycle in the PCR run.

c. For each replicate population of dilutions, select the wells in the Plate Layout tab to view in the plot.
For example:



d. Examine the raw data and identify the well(s) yielding signals according the ranges shown in the following table, then select the lowest (optimal) dye concentration that falls within the acceptable signal range:

Plate type	Acceptable signal range
384-well	400,000–1,200,000
96-well	1,400,000–4,300,000

Note: You can also export the raw data and average for the various concentrations.

2. Unload the plate from the instrument and discard the plate.

WARNING! PHYSICAL INJURY HAZARD. During system operation, the plate can reach 100°C. Allow the plate to cool to room temperature before removing.

Calibrate the custom dye

Create a custom dye plate

IMPORTANT! Wear powder-free gloves while creating the dye plate.

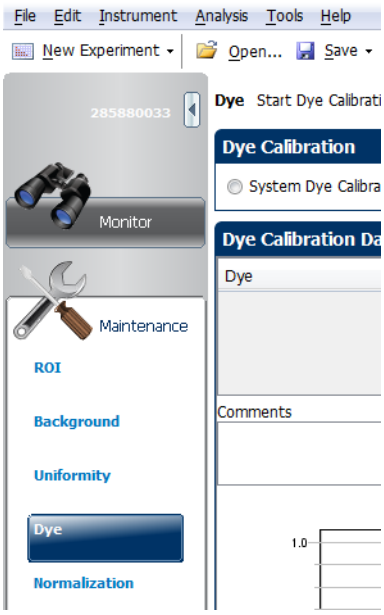
Create a full plate of the custom dye diluted to the optimal concentration:

1. Dilute the custom dye to the optimal concentration in buffer. Prepare adequate volume, using a recommended volume range of 10-20 µL/well for 96-well and 384-well plates.
2. Pipet the appropriate volume of the diluted custom dye to all wells of an optical reaction plate, then seal the plate.
3. Vortex the plate for 5 seconds, then centrifuge it for 2 minutes at < 1500 rpm. Confirm that the liquid in each well of the plate is at the bottom of the well. If not, centrifuge the plate again at a higher rpm and for a longer period of time.

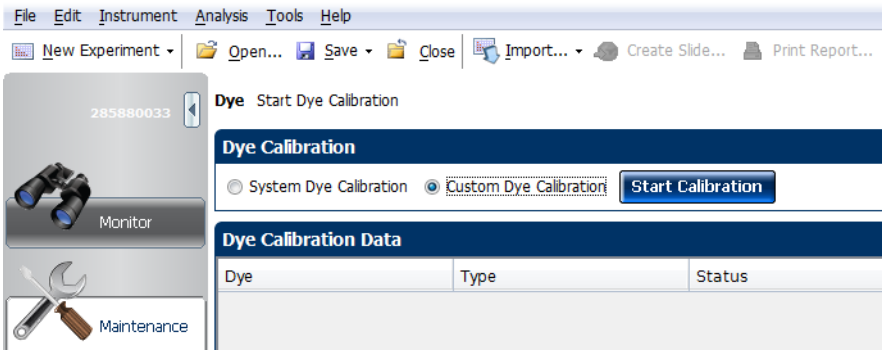
Add the custom dye to the software

1. On the Home screen of your system software, select to view the **Instrument Console**.
2. In the Instrument Console, select the icon for your instrument, then click **Manage Instrument**.

   Note: If the Manage Instrument button is inactive, select your instrument from the list of instruments on the network, then click **Add to My Instruments**.
3. In the navigation pane, click **Maintenance**, then click **Dye**.
   For example:

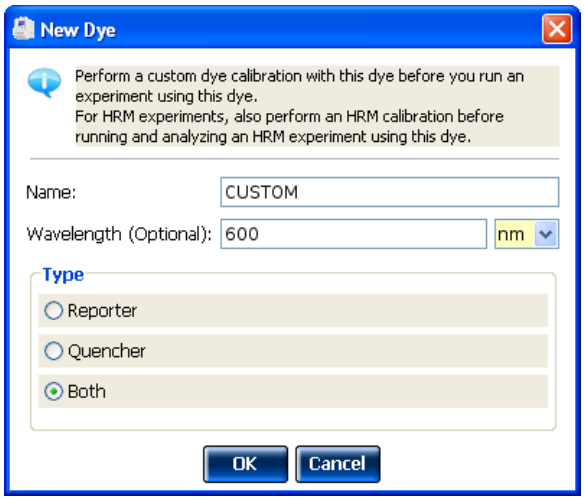


4. In the Dye Calibration screen, select **Custom Dye Calibration**, then click **Start Calibration**.
   For example:



5. Select the dye to calibrate. You may either:
   - select an existing custom dye from the list – skip step 6.
   - add a new custom dye – go to step 6.

6. To add a custom dye:
   a. In the Dye window, click **New Dye**.
   b. In the Dye Library dialog box, click **New**.
   c. Complete the New Dye dialog box, then click **OK**.
      For example:



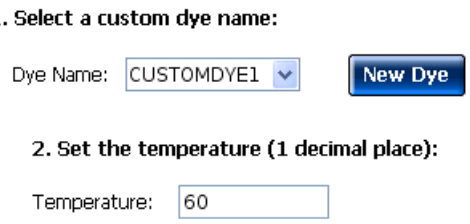
Setting	Action
Name	Enter a name for the custom dye.
Wavelength (Optional)	Enter the wavelength at which the dye fluoresces (the emission wavelength). **Note:** The value you enter is for reference only. The software does not use the entered value to calculate results.
Type	Select: • **Reporter** if the dye works in conjunction with a quencher dye to report an increase of PCR product. • **Quencher** if the dye suppresses the fluorescence of a reporter dye until amplification of PCR product. • **Both** if the dye reports an increase of PCR product without the aid of a quencher dye.

d. Click **Close** to exit the Dye Library.

Perform a dye calibration

1. Load your custom dye plate into the instrument, then start the calibration in the Dye window:

   a. In the SETUP screen, select the custom dye to calibrate and enter the temperature setting for the calibration.
   For example:

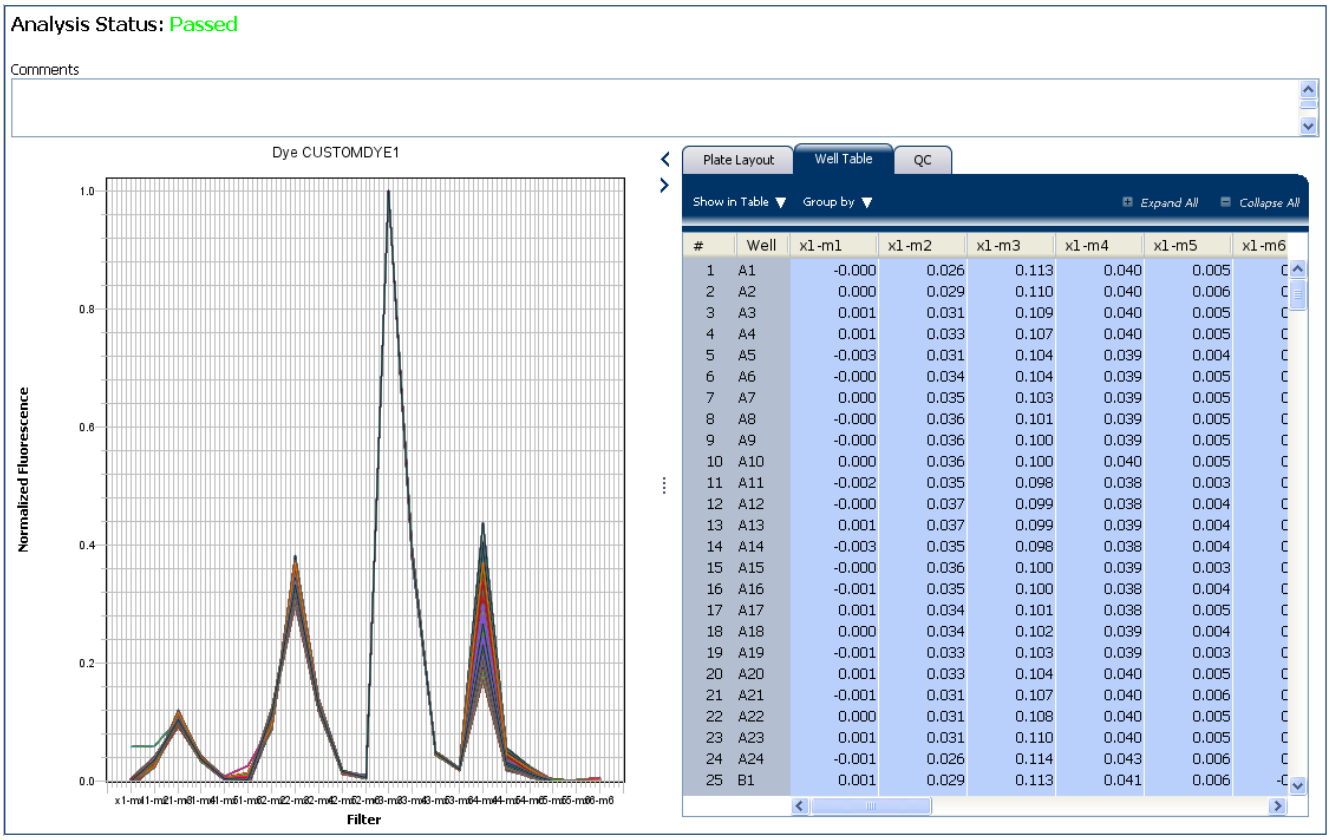


   Set the temperature to match the temperature at which you intend to collect data. For example, the temperature for all Life Technologies™ system dyes is 60°C because data collection for TaqMan® reagents occurs during the 60°C extension step of the PCR.

   b. When prompted in the SETUP screens, click **Next** and select the check box to verify the dye calibration plate has been loaded.

   c. In the RUN screen, click **START RUN**.
   When the run is complete the software displays the analysis results.

Dye spectra are generally acceptable if they peak within the same filter as their group, but diverge slightly at other wavelengths. For example:



2. Review the analysis status (success) of the calibration:

Analysis status	Description	Action
Passed	The run produced viable calibration data.	Save the calibration without reviewing the results: 1. *(Optional)* Enter any comments you have in the **Comments** field. 2. Click **Next**. 3. Click **Finish**, then follow the prompts to save the calibration results to the system.
Caution	The run produced data that did not pass the quality check (QC).	Select wells in the plate layout and review the spectra for irregularities, then troubleshoot the calibration results as described in " Dye calibration troubleshooting" on page 10.
Failed	The run did not produce data or the data it collected is unusable.	Troubleshoot the calibration results to determine the cause of the failure as described in " Dye calibration troubleshooting" on page 10.

3. Unload the plate from the instrument and discard the plate.

WARNING! PHYSICAL INJURY HAZARD. During system operation, the plate can reach 100°C. Allow the plate to cool to room temperature before removing.

Dye calibration troubleshooting

Observation	Possible cause	Recommended action
Dye calibration passed but the spectra did not peak in the correct filters.	Plate was not run in the correct orientation.	Confirm that the plate was run in the correct orientation. If it was not, repeat the calibration with the plate in the correct orientation.

One or more raw spectra are at or below the detectable threshold for the calibration.	• Dye calibration plate was centrifuged insufficiently.
	• Dye calibration plate contains old or insufficient reagents.
		WARNING! PHYSICAL INJURY HAZARD. During system operation, the plate can reach 100°C. Allow the plate to cool to room temperature before removing.
		1. Unload the system and view the wells of the calibration plate. If the liquid in the wells of the consumable is not:
		  • At the bottom of the wells, centrifuge the plate for a longer time, then repeat the calibration.
		  • Equivalent in volume, the plate is not sealed and the reagents have evaporated. Discard the consumable and run another.
		2. If the dye calibration plate appears to be normal, discard it and run another.
		3. If the problem persists, contact Technical Support.

	Dye may not be present at a sufficient concentration.	Create another custom dye plate using the next dye concentration greater than the optimal concentration, then perform the calibration again.
One or more raw spectra exceed the maximum limit for the instrument.	Fluorescent contaminants are on the sample block(s) or dye calibration plate.	Verify that contaminants are not present by performing a background calibration. If the background calibration does not show sample block contamination, the dye calibration plate may be contaminated. Discard the dye calibration plate and run another plate. **Note:** Refer to the Maintenance Guide for your system for detailed instructions on performing a background calibration.
	Dye may be too concentrated.	Create another plate but decrease the concentration of the dye that exceeds the detectable limit.

Observation	Possible cause	Recommended action
Spectra contain peaks in more than one filter.	Fluorescent contaminants are on the sample block(s) or dye calibration plate.	Verify that contaminants are not present by performing a background calibration. If the background calibration does not show sample block contamination, the dye calibration plate may be contaminated. Discard the dye calibration plate and run another plate. **Note:** Refer to the Maintenance Guide for your system for detailed instructions on performing a background calibration.
Instrument did not complete the calibration run.	Multiple possible causes.	1. Repeat the calibration. 2. If the problem persists, contact Technical Support.

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