Custom Dye Calibration of Applied Biosystems™ Real-Time PCR Instruments

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About this guide

This document describes custom dye calibration procedures for:

- Interchangeable-block instruments (see "Compatible instruments" on page 2).
- Plate formats of 96-well Fast (0.1-mL), 96-well Standard (0.2-mL), and 384-well.

Custom dyes overview

Applied Biosystems™ real-time PCR instruments and systems can run assays designed with custom dyes. Custom dyes include:

- Dyes that are not manufactured by Thermo Fisher Scientific.
- Dyes or formulations of dyes that are not system dyes for the instrument.

To use a custom dye on the instrument, review the following requirements:

- Ensure that the custom dye excites and emits within the supported wavelength ranges for the instrument.
- Select a custom dye that does not overlap with other dyes used in the run.
- The custom dye must be attached to the 5' end of a short DNA oligonucleotide consisting of the first two bases of the probe sequence without a quencher at the 3' end.
- Calibrate the instrument for the custom dye.

Note: See the instrument maintenance guide for information about the following:

- Supported wavelength ranges for the instrument
- Descriptions of dyes (including their wavelengths) that are considered system dyes

About dye calibration

The software uses calibration data to characterize and distinguish the individual contribution of each dye in the total fluorescence signals collected by the instrument.

The software extracts a spectral profile for each dye standard, then produces a set of spectral profiles plotted as fluorescence versus filter.

The pass criteria for a dye calibration is that the dye spectra peak within the same filter as their group.

Compatible instruments

The procedures in this guide are compatible with the following interchangeable-block real-time PCR instruments or systems. These procedures replace the custom dye calibration described in the user guides for the following instruments or systems:

Instrument or system	User guide	Pub. No.
ViiA™ 7 Real-Time PCR System	*Applied Biosystems™ ViiA™ 7 Real-Time PCR System User Guide: Calibration, Maintenance, Networking, and Security*	4442661
QuantStudio™ 6 Flex Real-Time PCR System	*QuantStudio™ 6 and 7 Flex Real-Time PCR Systems Maintenance and Administration Guide*	4489821
QuantStudio™ 7 Flex Real-Time PCR System		
QuantStudio™ 12K Flex Real-Time PCR System	*QuantStudio™ 12K Flex Real-Time PCR System Maintenance and Administration Guide*	4470689
QuantStudio™ Dx Real-Time PCR Instrument[1]	User guide or getting started guide for the QuantStudio™ Test Development Software	—[2]

[1] In this document, for the QuantStudio™ Dx Real-Time PCR Instrument, the term 'desktop software' refers to the QuantStudio™ Test Development Software. QuantStudio™ Test Development Software is for Research Use Only.

[2] See the user guide that corresponds to your version of QuantStudio™ Test Development Software.

Note: Some of the listed compatible instruments can support TaqMan® Array Cards. For custom dye calibration procedures for array cards, contact your Thermo Fisher Scientific service representative.

Workflow: Custom dye calibration

For each custom dye, determine the optimal dye concentration. Use this concentration to prepare all subsequent dye calibration plates.

Use a dilution series to determine an optimal custom dye concentration

Prepare a custom dye dilution plate (page 4)

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Run the dilution plate as an experiment (page 5)

▼

Determine the optimal dye concentration (page 6)

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Calibrate the custom dye using the optimal concentration

Create a custom dye calibration plate (page 7)

▼

Add a new custom dye to the desktop software (page 7)

▼

Perform a custom dye calibration (page 8)

Use a dilution series to determine an optimal custom dye concentration

Custom dye dilution guidelines

Prepare a dilution series for each custom dye.

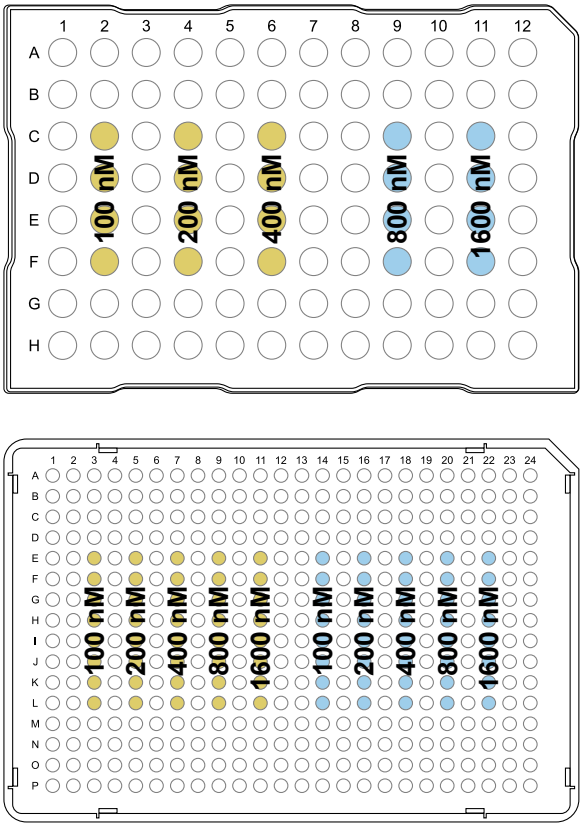
- Target several dye concentrations within a range of 100–2,000 nM.
- Choose a 2- or 3-fold difference in dilution points.
- Dispense an appropriate volume for the plate format.
  - 96-well Standard plate: 20 µL per well
  - 96-well Fast plate: 10 µL per well
  - 384-well plate: 10 µL per well
- Dilute the dye in buffer compatible with your master mix.
- *(Intercalating dyes only)* Add the appropriate amount of amplified PCR product to generate fluorescence.

Prepare a custom dye dilution plate

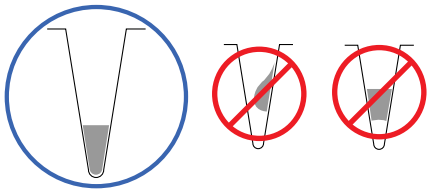
IMPORTANT! Wear powder-free gloves throughout the procedure.

1. Prepare a 2- or 3-fold dilution series of the custom dye.
2. Dispense aliquots of each dilution into the center of a reaction plate, then seal the plate.

   A full plate is not needed. See the following figure for suggested replicates.



3. Vortex the plate for 5 seconds, then centrifuge at 750 to 1,000 × *g* for 2 minutes.
4. Confirm that the liquid in each well is at the bottom of the well and free of bubbles. If it is not, centrifuge the plate again.



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 an abnormally high background signal.

Run the dilution plate as an experiment

1. Load the plate into the instrument.

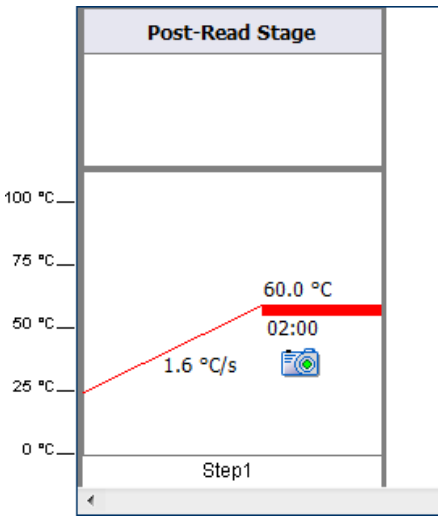
   IMPORTANT! The instrument should be used by trained operators who have been warned of the moving parts hazard.

2. Set up a genotyping experiment in the desktop software.
   a. In the **Home** tab, select **New Experiment** ▸ **Experiment Setup**.

   b. In the **Properties** screen:
      - Select **Genotyping** as the experiment type.
      - At the bottom of the screen:
        - Deselect both **Pre-PCR Read** and **Amplification**.
        - Ensure that **Post-PCR Read** is selected (default).
      - *(Optional)* Enter or edit other experiment properties as needed.

   c. In the **Define** and **Assign** screens, enter the dilution series information for the plate wells.

   d. In the **Run Method** screen:
      - Enter the appropriate reaction volume.
      - Set the hold time to 2 minutes.
      - Set the hold temperature to the detection temperature used in your experiments.
        For TaqMan® Assays, we recommend 60°C.
      - Ensure the correct filter for the custom dye is selected. If you do not know the correct filter settings, select all the available filter combinations.



3. Save the experiment, then start the run.
4. When the run ends, the EDS file automatically transfers to the desktop software.
5. Unload the plate from the instrument.

   CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the plate temperature can reach 100°C. Allow it to cool to room temperature before handling.

   Note: If the instrument does not eject the tray arm, contact Support.

Determine the optimal dye concentration

Review the dye signal data and select the dilution to use for dye calibrate.

1. In the left menu pane, select **Analysis ▸ Raw Data Plot**.
   This plot displays the raw fluorescence signal of each optical filter, for individual wells.

2. For each replicate population of dilutions, select the wells in the plate layout to view in the plot.

3. Examine the raw data to identify the wells yielding signals according to the ranges shown in the following table.

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

[1] Signal range for the optical filter where the dye is brightest.

4. *(Optional)* Export the raw data, then calculate the average fluorescence value for each concentration.

5. Select the lowest (optimal) dye concentration that falls within the acceptable signal range.

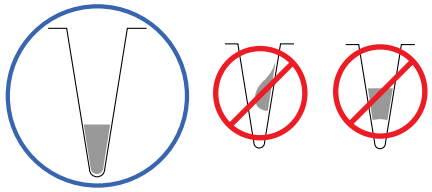
Calibrate the custom dye

Create a custom dye calibration 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 an adequate volume, using an appropriate volume for the plate format.
   - 96-well Standard plate: 20 µL per well
   - 96-well Fast plate: 10 µL per well
   - 384-well plate: 10 µL per well
2. Pipet the appropriate volume of the diluted custom dye to the plate wells.
3. Seal the plate.
4. Vortex the plate for 5 seconds, then centrifuge at 750 to 1,000 × *g* for 2 minutes.
5. Confirm that the liquid in each well is at the bottom of the well and free of bubbles. If it is not, centrifuge the plate again.



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 an abnormally high background signal.

Add a new custom dye to the desktop software

1. In the desktop software **Home** tab, click **Instrument Console**.
2. Select the instrument, then click **Manage Instrument**.

   Note: If **Manage Instrument** is inactive, add your instrument (see the instrument maintenance guide).
3. In the left menu pane, select **Maintenance** ▸ **Dye**.
4. Select **Custom Dye Calibration**, then click **Start Calibration**.
5. Click **New Dye**, then in the **Dye Library** dialog box, click **New**.

6. Enter the custom dye information (see the following table), then click **OK** ▸ **Close**.

Field/option	Action
Name	Enter a name for the custom dye. IMPORTANT! • Do not use a system dye name for a custom dye name. • Dye names are spacing- and case-sensitive and cannot contain special characters.
Wavelength	Enter the wavelength at which the dye fluoresces.
Type	Select: • **Reporter**—The dye works in conjunction with a quencher dye to report an increase of PCR product. • **Quencher**—The dye suppresses the fluorescence of a reporter dye until amplification of PCR product. • **Both**—The dye can be used as a reporter or quencher dye.

Perform a custom dye calibration

1. Load the plate into the instrument.

   IMPORTANT! The instrument should be used by trained operators who have been warned of the moving parts hazard.

2. In the desktop software **Home** tab, click **Instrument Console**.

3. Select the instrument, then click **Manage Instrument**.

   Note: If **Manage Instrument** is inactive, add your instrument (see the instrument maintenance guide).

4. In the left menu pane, select **Maintenance** ▸ **Dye**.

5. Select **Custom Dye Calibration**, then click **Start Calibration**.

6. Select a custom dye from the dropdown list.

7. Enter the calibration temperature.

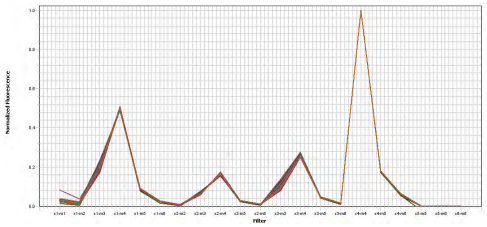
   Note: The calibration temperature is the temperature at which the software will collect data. For TaqMan® reagents, we recommend using 60°C.

8. In the **Reagent Information** pane:
   a. Enter the reagent information for the plate.
   b. Select the checkbox to confirm that you loaded the plate and entered the reagent information.
   c. Click **Next**.

9. Click **Start Run**.

10. When the run is complete, review the analysis status and dye spectra. Confirm that the dye signal peaked in the correct filter.

Analysis status	Action
Passed	Click **Next**.
Caution	See "Troubleshoot calibration failure" on page 10.



Pass criteria—Signals from each well follow a uniform trend, and the dye peaks at the correct filter.

11. Click **Finish**, then follow the prompts to save the calibration results.

12. Unload the plate from the instrument.

CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the plate temperature can reach 100°C. Allow it to cool to room temperature before handling.

Note: If the instrument does not eject the tray arm, contact Support.

Troubleshoot calibration failure

Observation	Possible cause	Recommended action
Calibration result is **Caution**	The plate was improperly prepared.	Ensure the following: • The correct plate was used for the calibration performed. • The plate was properly centrifuged. • The plate was properly sealed. • Consistent pipetting from well-to-well
	The plate is damaged or contaminated.	Check for damage, improper plate seal, or contamination.
High fluorescence signal in individual wells	Signals that exceed the limit of normal fluorescence may indicate fluorescent contaminants on the plate or the sample block.	See the instrument maintenance guide for information about identifying contamination.
High fluorescence signal across the plate *(Custom dye calibrations only)*	The dye concentration used to create the custom dye plate is too high.	Create a new custom dye plate using the next dye concentration lower than the optimal concentration, then perform the calibration again.
	A different temperature was used for instrument calibration than was used for determining the optimal dye concentration.	Ensure that the temperatures are consistent.
Low fluorescence signal across the plate *(Custom dye calibrations only)*	A different temperature was used for instrument calibration than was used for determining the optimal dye concentration.	Ensure that the temperatures are consistent.
Unexpected peaks or peaks exceed maximum limit • Spectra contain peaks that are detected in more than one filter. • One or more raw spectra exceed the maximum limit for the instrument.	Fluorescent contaminants on the sample block or on the plate.	Perform a background calibration to detect sample block contamination. If the background calibration does not detect contamination, the dye plate may be contaminated.
	(Custom dye calibrations only) Dye may be too concentrated.	Prepare a new custom dye calibration plate but decrease the concentration of the dye that exceeds the detectable limit.
One or more raw spectra are at or below the detectable threshold for the calibration	• The dye plate was centrifuged insufficiently. • The dye plate contains old or insufficient reagents.	Examine the wells of the plate. • Liquid in the wells is not at the bottom of the wells—Centrifuge the plate for a longer time, then repeat the calibration. • Liquid in the wells are equivalent in volume—The plate is improperly sealed and the reagents have evaporated. Discard the plate, then prepare and run a new plate.
		Examine the plate and the plate wells. If the plate appears to be normal, discard the plate, then prepare and run a new plate.
		If the problem persists, contact Support.

Observation	Possible cause	Recommended action
One or more raw spectra are at or below the detectable threshold for the calibration	*(Custom dye calibrations only)* Dye may not be present at a sufficient concentration.	Create a new custom dye plate using the next dye concentration greater than the optimal concentration, then perform the calibration again.
Dye calibration passed, but the spectra did not peak in the correct filters	The ROI calibration or background calibration is invalid.	See the instrument maintenance guide for instructions on performing instrument calibrations.

Documentation and support

Customer and technical support

Visit **thermofisher.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

Symbols that may be displayed on the instrument, in the software, or in this guide

Symbol	Description	Symbol	Description
	MANUFACTURER		DATE OF MANUFACTURE
REF	CATALOG NUMBER	SN	SERIAL NUMBER
	CONSULT INSTRUCTIONS FOR USE		CAUTION, CONSULT ACCOMPANYING DOCUMENTS

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The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN001765

Revision	Date	Description
B.0	18 April 2018	• Update branding, general formatting, and images. • Streamline content and phrasing. • Clarify custom dye overview and software setup for determining optimal custom dye concentration. • Add applicable instruments, troubleshooting items, and recommended volumes for plate formats.
A.0	27 August 2014	New document.