QuantStudio™ Design and Analysis Desktop Software

USER GUIDE

Getting started with design and analysis of experiments in the desktop software v1.5.x

for use with:
QuantStudio™ 1 Real-Time PCR System
QuantStudio™ 3 Real-Time PCR System
QuantStudio™ 5 Real-Time PCR System

Publication Number MAN0010408
Revision C.0

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For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

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Revision	Date	Description
C.0	19 October 2018	Updated to add QuantStudio™ 1 Real-Time PCR System information.
B.0	December 2015	Updates include: • Combination of define and assign functions into a single **Plate** tab • Display of VeriFlex™ Zones on plate layout • Real-time data monitoring in the **Run** tab • Security, Audit, and E-Signature (SAE) features • Implementation of locked workflow • Selection of an instrument before starting a run • Ability to select multiple targets in the results view • Various minor changes to the user interface
A.0	April 2015	New document.

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Product description

The QuantStudio™ Design and Analysis Desktop Software allows the user to open, run, and analyze experiments generated with QuantStudio™ 1 Real-Time PCR System, QuantStudio™ 3 Real-Time PCR System, and QuantStudio™ 5 Real-Time PCR System. The software also allows you to set up experiments, send experiments to the instrument, collect data, and analyze the collected data.

Features in the QuantStudio™ Design and Analysis Desktop Software

Actions	Unlocked template	Locked template[1,2]
Properties tab		
Edit experiment name; enter / scan plate barcode; enter user name	✓	✓
Select instrument / block type, experiment type, chemistry, run mode	✓	—
Enter reagent information (chemistry details)	✓	✓
Method tab		
Edit the thermal protocol, reaction volume, optical filter selection	✓	—
Plate tab		
Quick Setup subtab		
Define plate attributes	✓	—
Define or assign samples	✓	✓
Define or assign targets or SNP assays	✓	—
Advanced Setup subtab		
Define samples	✓	✓
Assign samples	✓	✓
Define targets or SNP assays	✓	—
Assign targets or SNP assays	✓	✓
Run tab		
Start and monitor a run in progress	✓	✓
View time remaining and plots[3]	✓	✓
Results tab		
Review run results (analyzed run data)	✓	✓
Configure analysis settings	✓	—
Export tab		
Select export options for run data and run results (analyzed run data)	✓	✓
Export run data[3]; export template settings	✓	✓

[1] If you enter the password for a locked template, all listed actions are available.

[2] Always save a backup *unlocked* version of a template before saving it as a locked template.

[3] This feature is also available from the instrument touchscreen.

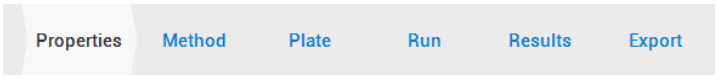
Experiment types

Purpose	Description
Standard curve experiment	
Determines absolute target quantity in samples.	1. The software measures amplification of the target in a standard dilution series and in test samples. 2. The software generates a standard curve using data from the standard dilution series. 3. The software uses the standard curve to interpolate the absolute quantity of target in the test samples.
Relative standard curve experiment	
Determines relative target quantity in samples.	1. The software measures amplification of the target of interest and of an endogenous control target in a standard dilution series, in a reference (calibrator) sample, and in test samples. The endogenous control is a target that is expressed equally in all samples; examples of endogenous controls are ß-actin, GAPDH, and 18S ribosomal RNA. The software can algorithmically incorporate multiple endogenous control targets in relative quantification calculations. The reference sample is used as the basis for relative quantification results (or 1× sample). For example, in a study of drug effects on gene expression, an untreated control is an appropriate reference sample. 2. The software generates standard curves for the target of interest and the endogenous control using data from the corresponding standard dilution series. 3. The software uses the standard curves to interpolate the quantities of the target of interest and the endogenous control in each sample. The target quantity in each sample is then normalized to the sample's endogenous control quantity. 4. To determine the relative quantity of the target in test samples, the software divides the normalized target quantity in the sample by the normalized target quantity in the reference sample.
Comparative C_t ($\Delta\Delta C_t$) experiment	
Determines relative target quantity in samples.	1. The software measures amplification of the target of interest and of an endogenous control target in a reference (calibrator) sample and in test samples. The endogenous control is a target that is expressed equally in all samples; examples of endogenous controls are ß-actin, GAPDH, and 18S ribosomal RNA. The software can algorithmically incorporate multiple endogenous control targets in relative quantification calculations. The reference sample is used as the basis for relative quantification results (or 1× sample). For example, in a study of drug effects on gene expression, an untreated control is an appropriate reference sample. 2. The measurements for the target of interest are normalized to the endogenous control. 3. To determine the relative quantity of the target in test samples, the software compares the normalized ΔC_q (ΔC_t or ΔC_{rt}) for the sample to the normalized ΔC_q (ΔC_t or ΔC_{rt}) for the reference sample.

Purpose	Description
Genotyping experiment	
Detects single nucleotide polymorphism (SNP) variants of a target nucleic acid sequence.	Genotyping experiments use preformulated TaqMan® SNP Genotyping Assays that include the following components: • Two sequence-specific primers for amplification of sequences containing the SNP of interest • Two allele-specific TaqMan® probes for Allele 1 and Allele 2 1. The software normalizes the fluorescence of the reporter dyes to the fluorescence of the passive reference dye in each well. 2. The software plots the normalized reporter dye signal of each sample well on an Allelic Discrimination Plot, which contrasts the reporter dye intensities of the allele-specific probes. 3. The software algorithmically clusters the sample data, and assigns a genotype call to the samples of each cluster according to its position on the plot.
Presence/absence experiment	
Determines the presence or absence of a target nucleic acid sequence in a sample.	The software calls the target present or absent based on an algorithmically determined call threshold. (The call threshold is different from the C_t threshold; the C_t threshold is not used to make calls.)
Melt curve experiment	
Determines the melting temperature (T_m) of the amplification products of a PCR that used intercalating dyes.	In the software, melt curve analysis is included in the default run method for any experiment type that uses intercalating dyes. 1. The software plots a melt curve based on the fluorescence of the dye with respect to change in temperature. 2. Using the melt curve, the software calculates the melting temperature (T_m).

Workflow overview

Use the tabs across the top of the screen to navigate the workflow in the QuantStudio™ desktop software.



Create a template (EDT file)

▼

Set up template parameters (properties, method, plate and well details)

▼

Configure analysis settings

▼

Run the EDT file on the instrument

▼

Review run results (EDS file)

2 General procedures to set up and run experiments

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Set global desktop software preferences

1. In the menu bar, select **Tools** ▸ **Preferences**.
2. Set preferences in each tab.

Tab	Options	Action
Defaults	**Decimal Places to Show**	Enter the number of significant figures in exported results.
	Language [if available]	Choose a language for the software from the dropdown menu.
Experiment	**Baseline Start Cycle** and **Baseline End Cycle**	Enter the first and last cycles to be used to calculate the baseline for runs that include amplification.
	Auto Analysis	Select to perform auto analysis at the end of each run.
	Auto Save	Select to save changes at the end of each run.
Print	**Disable color when printing the Well Table**	Select to disable color printing.
Export	**Use Last File Location** or **Use Default Folder**	Select where to export results. Click **Browse**, then navigate to and select a default location.
Display Format	**Date Format**, **Time Format**, and **Decimal Point Format**	Select the display formats. These formats are also used in the export or import of data.

3. Click **Save**.

Workflow: Set up and run an experiment

Set up a template (page 15)

Create or open a template (page 16)

Enter template properties (page 16)

Confirm or edit the run method and optical filter selection (page 17)

Assign plate and well attributes (page 18)

Save a template file (page 20)

▼

Prepare reactions (page 21)

▼

Start and monitor a run (page 22)

For post-run procedures, see the following sections:

- Chapter 3, "General procedures to review results"
- "Export experiments or results" on page 40

Set up a template

This section describes the general procedures to set up a template in the desktop software. For setup information for a specific experiment type, see the following sections.

- "Set up a standard curve experiment in the software" on page 43
- "Set up a relative standard curve experiment in the software" on page 51
- "Set up a comparative Ct experiment in the software" on page 53
- "Set up a genotyping experiment in the software" on page 61
- "Set up the presence/absence experiment in the software" on page 69
- "Set up a melt curve experiment in the software" on page 76
- "Set up a custom experiment in the software" on page 84

You can also use features of the desktop software to more easily set up some or all of an experiment. For example, you can set up an experiment using one of the following strategies.

- Use desktop software libraries to set up samples, targets or SNP assays, run methods, and analysis settings (see "Sample, target, and SNP assay libraries" on page 96).
- Import experiment parameters from external files or templates (see "Experiment setup using libraries, external files, and templates" on page 24).

Create or open a template

Create a new template or open an existing template in the **Home** screen.

- In the **New Experiment** pane, perform one of the following tasks to create a new template.

To	Action
Create a template without preexisting settings	Click **Create New Experiment**.
Create a template from a system template	1. Select **Create New Experiment** ▸ **Template**. 2. Navigate to and select the desired file, then click **Open**. System template files are installed with the software in: <*drive*>:\Program Files (x86)\Applied BioSystems\QuantStudio Design & Analysis Software\templates, where <*drive*> is the drive on which the software is installed.

- In the **Open Existing Experiment** pane, perform the following tasks to open an existing template.
  a. Click **Open**.
  b. Navigate to and select the desired file, then click **Open**.

Enter template properties

1. Click the **Properties** tab to open and edit the experiment properties.
2. (*Optional*) In the **Name** field, modify the file name.
   The **Name** field determines two file names:
   - The initial EDT file name.

     Note: *After* the initial EDT file save, modifying the **Name** field does not update the EDT file name. To change the EDT file name *after* the initial save, select **Save** ▸ **Save As.**
   - The default file name for the EDS file created during an instrument run.
3. (*Optional*) Click the **Barcode** field, then scan or enter a plate barcode.
4. (*Optional*) Enter information in the **User name** and the **Comments** fields, if applicable.
5. Select an **Instrument type**, **Block type**, **Experiment type**, **Chemistry** (reagents), and **Run Mode** (Fast or Standard cycling) from the dropdown lists.

   Note: The experiment type defines the available options for the template setup. For more information on the parameters defined in each experiment type, see "Experiment definitions" on page 20.
6. (*Optional*) Click **Manage chemistry details** (see "Enter reagent information").
7. (*Optional*) Click **Save** or select **Save** ▸ **Save as**.

Enter reagent information

1. In the **Properties** tab, click **Manage chemistry details**.
2. Click ⊞ **Add**.
3. Enter the reagent type, name, part number, lot number, and expiration date.
4. (*Optional*) Add a custom attribute for a reagent.
   a. Click ➕ in the table header to add a column for a custom attribute.
   b. Click the **Custom Attribute** column header, then enter a new attribute.
   c. Select a cell in the **Custom Attribute** column, then enter its information.
   d. (*Optional*) Click ✕ in the header to delete a custom attribute from the table.
5. (*Optional*) Click ✕ to delete a reagent from the table.
6. Click **Close**.

Scan a barcode using the optional barcode scanner

The instrument is compatible with an optional Handheld Barcode Scanner (Cat. No. 4488442, purchased separately). The barcode scanner reads Code 128 (alphanumeric), which supports 128 ASCII character barcodes.

1. Click the **Barcode** field.
2. Hold the scanner 20–30 cm away from a plate or container label and aim at the center of the barcode, then press the trigger.
3. Slowly move the scanning beam across the barcode until the scanner emits a high-pitched tone.

When the scanner scans a barcode, it automatically transmits the following information:

- Transmits the alphanumeric equivalent of the barcode to the barcode field.
- Transmits other reagent information (Lot #, Part #, Expiration Date, etc.)

For more information about the hand-held barcode scanner, see the user documentation provided with the barcode scanner.

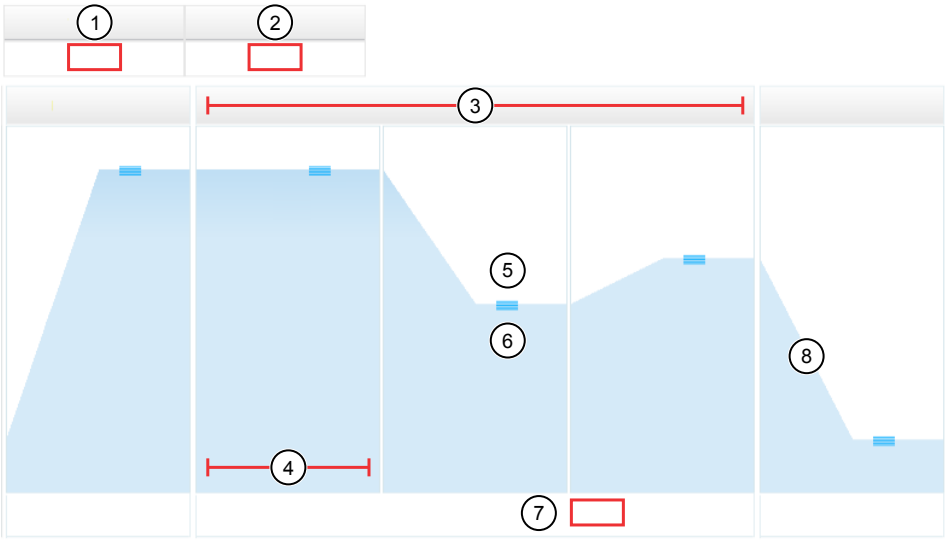
Confirm or edit the run method and optical filter selection

In the **Method** tab, perform the follow tasks if needed.

- (*Optional*) Adjust the reaction volume.
- (*Optional*) Edit the default run method (thermal protocol).
  – The default run method is optimized for TaqMan® assays and a broad range of other reagents.
  – To edit the default run method, see “Adjust method parameters“ on page 88.

- (*Optional*) Edit the default optical filter selection (see "Select optical filters" on page 90).
  - The default optical filter selection is for factory-calibrated (system) dyes.
  - For more information about instrument supported dyes and their calibration and optical filter selection, see *QuantStudio™ 1 Real-Time PCR System Installation, Use, and Maintenance Guide* (Pub. No. MAN0017853) or *QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide* (Pub. No. MAN0010407).

Method elements



① Reaction volume
② Heated cover temperature
③ Stage of thermal protocol
④ Step within a stage
⑤ Step temperature
⑥ Step hold time
⑦ Number of cycles
⑧ Temperature ramp rate

Assign plate and well attributes using the Quick Setup subtab

Note: This section provides general procedures to set up the plate.

- For detailed procedures to set up a plate, see Appendix D, "Detailed procedures to set up plate / well details and libraries".
- For specific instructions for each experiment type, see the corresponding chapter in this guide.

1. In the **Plate** tab, select plate wells in the **Plate Layout** or the **Well Table**. For more information, see "Select plate wells" on page 19.
2. Click **Quick Setup**.

3. Assign the well attributes for the selected wells.
   - Into the text fields, enter the sample and target or SNP assay names.
   - From the dropdown lists, select a defined sample and target or SNP assay.
     For more information about defining or importing samples and targets or SNP assays, see "Define and assign well attributes (Advanced Setup subtab) " on page 93.
   - Click **Advanced Setup**, then change the default selections for the reporter and quencher dyes and for tasks where applicable. For more information, see "Assign a task to wells" on page 93.
4. (*Optional*) Enter comments for the selected wells.
5. In the **Plate Attributes** pane, select a **Passive Reference** from the dropdown list.

Select plate wells

- Select plate wells in the **Plate Layout**.

To	Action
Select a single well	Click a well in the plate
Select multiple wells	Click-drag in the plate
Select contiguous wells	Shift-click wells in the plate
Select non-contiguous wells	Ctrl-click wells in the plate
Select a column of wells	Click a column header
Select all wells	Click the top-left corner of the plate grid
Select a block of wells	Click a well to define a corner, then Shift-click another well on the opposite corner
Deselect a single well	Ctrl-click the selected well

- Select plate wells in the **Well Table**.

To	Action
Select a single well	Click a row in the table
Select multiple wells	Click-drag in the table
Select contiguous wells	Shift-click rows in the table
Select non-contiguous wells	Ctrl-click rows in the table
Deselect a single well	Ctrl-click the selected row

Experiment definitions

The parameters that you can define vary by experiment type.

Experiment Type	Targets	SNP Assays	Samples	Biological Replicate Groups	Passive Reference	Reference and Endogenous Controls
Standard Curve	✓		✓	✓	✓	
Relative Standard Curve	✓		✓	✓	✓	✓
Comparative C_t	✓		✓	✓	✓	✓
Melt Curve	✓		✓		✓	
Genotyping		✓	✓		✓	
Presence / Absence	✓		✓		✓	
Custom	✓		✓	✓	✓	

Save a template file

Save a template file as an unlocked template

Note: You cannot save a locked template as an unlocked template.

- Save the template with the same EDT file name.
  - In any tab, click **Save**.
  - In the menu bar, select **File** ▸ **Save**.
- Save the template with a new EDT file name.

  Note: You cannot save a locked template with a new file name.
  - In any tab, select **Save** ▸ **Save as**.
  - In the menu bar, select **File** ▸ **Save As**.

Save a template file as a locked template

IMPORTANT! Always save a backup *unlocked* version of a template before saving it as a locked template.

1. In the menu bar, select **File** ▸ **Save As a Locked Template**.
2. Enter and confirm a password, then click **OK** to continue saving the file.

   Note: The password is required to open the template with unlimited editing options. Without the password, a locked template can still be opened but with limited editing options.

   Note: Record the password because lost passwords cannot be recovered.

Prepare reactions

See instrument user guide for information about compatible reagents and required materials for PCR reactions.

Follow the instructions provided by the manufacturer to prepare reactions.

Follow the other guidelines described in this section.

Good laboratory practices for PCR and RT-PCR

- Wear clean gloves and a clean lab coat.
  - Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples.
- Change gloves if you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA decontamination solution.

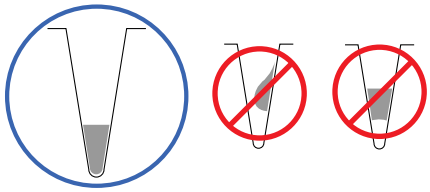
Guidelines for handling samples and reagents

- Use calibrated pipettors and aerosol-resistant tips.
- Prepare the reaction mixes according to the recommendations that are provided by the manufacturer of the master mixes and assay mixes.
- Include excess volume in calculations to account for the loss that occurs during reagent transfers.
- Use TE buffer or water to dilute samples and standards.
- Use care when diluting samples and standards. Mistakes or inaccuracies in making the dilutions affect data accuracy.
- Keep the dilutions and assay mix frozen and protected from light until use. Excessive exposure to light can affect the fluorescent probes or dyes.
- Perform the following tasks before each use:
  - Mix the master mix thoroughly by swirling the bottle.
  - Resuspend the assay mix by vortexing, then centrifuge the tube briefly.
  - Thaw any frozen samples by placing them on ice. When thawed, resuspend the samples by vortexing, then centrifuge the tubes briefly.

Guidelines for setting up the reactions in the plates or tubes

- Use good laboratory practices for PCR and RT-PCR. For more information, see “Good laboratory practices for PCR and RT-PCR“ on page 21.
- Ensure that the arrangement of the PCR reactions matches the plate layout displayed in the software.

- 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.



- Ensure that plates or tubes are properly sealed.
- Keep the reaction plate or tubes at 4°C and protected from light until you are ready to load the plate into the instrument.
- 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.
- If necessary, use a permanent marker or pen to mark a tube and the side of a plate. Do not use fluorescent markers.

Start and monitor a run

IMPORTANT! Before loading a reaction-filled plate into the instrument, review the detailed procedures in "Load and unload the plate in the instrument" on page 81.

1. Go to an instrument connected to the computer that is running the desktop software.
2. Load the plate into the instrument.

   CAUTION! *(QuantStudio™ 3 Real-Time PCR System and QuantStudio™ 5 Real-Time PCR System only)* The instrument should be used by trained operators who have been warned of the moving parts hazard.

3. Open a template (EDT file) in the desktop software.
4. (*Optional*) In the **Export** tab, select **Auto Export** to export run results automatically after the run ends.
5. In the **Method** tab and the **Plate** tab, review the template parameters and setup.
6. In the **Run** tab, select the instrument to use from the **START RUN** dropdown list.
7. Accept or edit the default name for the EDS file, then click **Save**.

   Note: EDS files contain the run data and results. The system creates the EDS default file name for the EDS file from the **Experiment Name** in the **Properties** tab.

8. During the instrument run, monitor the run:
   - In the **Run** tab of the desktop software.
   - In the instrument touchscreen.
9. 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: *(QuantStudio™ 3 Real-Time PCR System and QuantStudio™ 5 Real-Time PCR System only)* If the instrument does not eject the plate, contact Support.

Note: If the connection between the instrument and the desktop software is interrupted during the run, the instrument still completes the run. However, the run data (EDS file) must be transferred from the instrument to the desktop software using a USB drive or a network drive.

View real-time run information in the desktop software

You can view real-time run information in the **Run** tab of the desktop software.

- In the **Run** tab, view the time remaining for the run and the run status.
- In the **Run** tab, in the **Amplification Plot** subtab, view real-time data and plots.
  - Click **View** to select the data that are displayed in each well.
  - Select wells in the plate layout to highlight respective curves in the plot.
  - Select curves in the plot to highlight respective wells in the plate layout.

  Note: For melt curve experiments, you can only monitor the melt curve plot.

View real-time run information on the instrument touchscreen

You can view real-time run information in the instrument home screen.

- In the instrument home screen, view the block temperature, the time remaining for the run, and the run status.
- Touch › or swipe left once to view real-time run method information.
- Touch › or swipe left twice to view real-time data and plots.

View real-time data and plots on the instrument touchscreen

1. In the instrument home screen, during an instrument run, touch › or swipe left twice.
2. Touch **Well details**.
3. Touch **Samples**, **Targets**, or **Tasks** to select a graphical representation of each selection.
4. Touch **Close** to return to the home screen.

Adjust the display of real-time plots on the instrument touchscreen

1. In the instrument home screen, during an instrument run, touch › or swipe left twice to view real-time data and plots.
2. Touch **Zoom**.
3. Touch ⊕ or ⊖ to zoom in or out.
4. Touch the arrows to pan left, right, up, or down on the graph.
5. Touch **Close** to return to the default view.

View the Post-Run Summary in the desktop software

You can view a summary of the run after the run ends.

In the **Run** tab, click the **Post-Run Summary** tab to view a summary of the run, including the following information:

- **Experiment Name**
- **User Name**
- **Errors Encountered**
- **Instrument Serial Number** and **Instrument Name**
- **Start Time**, **Stop Time**, and **Run Duration**

Experiment setup using libraries, external files, and templates

The desktop software offers the following features so that you can more easily set up some or all of an experiment using libraries, external files, and templates.

- Use desktop software libraries to set up samples, targets or SNP assays, run methods, and analysis settings (see "Libraries overview" on page 96).
- Import some or all of an experiment setup from external files or templates (see the following table).

Table 1 Experiment setup from external files or templates

Option	Action	Setup information
Import sample information (define samples).	Import a sample definition file (see "Assign samples using a sample definition file" on page 85).	– Sample name – *(Optional)* Custom sample properties
Import samples, targets, and well assignments.	Import a plate setup file (see "Assign samples and targets using plate setup files" on page 86).	Plate setup information: – Well number – Sample name – Sample color – Target name – Dyes – *(Optional)* Other well information

Option	Action	Setup information
Set up the plate layout in a spreadsheet without saving to a special format. *or* Use a subset of the columns in a plate layout spreadsheet.	Copy-paste from an XLS file (see "Assign targets, samples, and biological replicate groups from an XLS file" on page 87).	Plate setup information: – Well number – Sample name – Biological Group – Target name – Task – Dyes – Quantity – Comments – *(Optional)* Other well information
Use a complete template setup from an existing EDT or EDS file.	Create a new template from an existing template or run results file (see "Create new EDT files using existing EDT and EDS files" on page 87).	– Plate setup information, as above – Reagent information – Thermal protocol – Analysis settings

3 General procedures to review results

- About the quantification cycle (Cq) 26
- Overview of the Results tab 27
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- Guidelines for viewing and analyzing results 29
- Assess results in the Amplification Plot 30
- Assess results in the Well Table view 35
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This section includes information about reviewing results and configuring analysis settings for all experiment types. For information about a specific experiment type, see the corresponding chapter in this guide.

For step-by-step instructions for results review procedures, see the desktop software Help.

About the quantification cycle (C_q)

Term	Name	Description
C_q	Quantification cycle	C_q is the general form for gene expression metrics. C_q values (both C_t and C_{rt}) are used as the primary input values for sample quantification experiments: absolute quantification (AQ) and relative quantification (RQ). C_t and C_{rt} are the algorithm-specific calculations of C_q.
C_t	Threshold cycle	The PCR cycle number at which the fluorescence signal meets the threshold in the amplification plot. C_t is the gene expression metric result when using the Baseline Threshold Algorithm.
C_{rt}	Relative threshold cycle	The PCR cycle number for the threshold calculated from the modeled amplification efficiency profile. C_{rt} is the gene expression metric result when using the Relative Threshold Algorithm.

Overview of the Results tab

Review and analyze run data in the **Results** tab. In the **Results** tab, two additional tools display at the right of the workflow bar.

- Click **Analyze** after omitting wells or changing the analysis settings.
- Click ⚙ to access analysis settings.

Note: The analysis settings and plots that are available vary by experiment type.

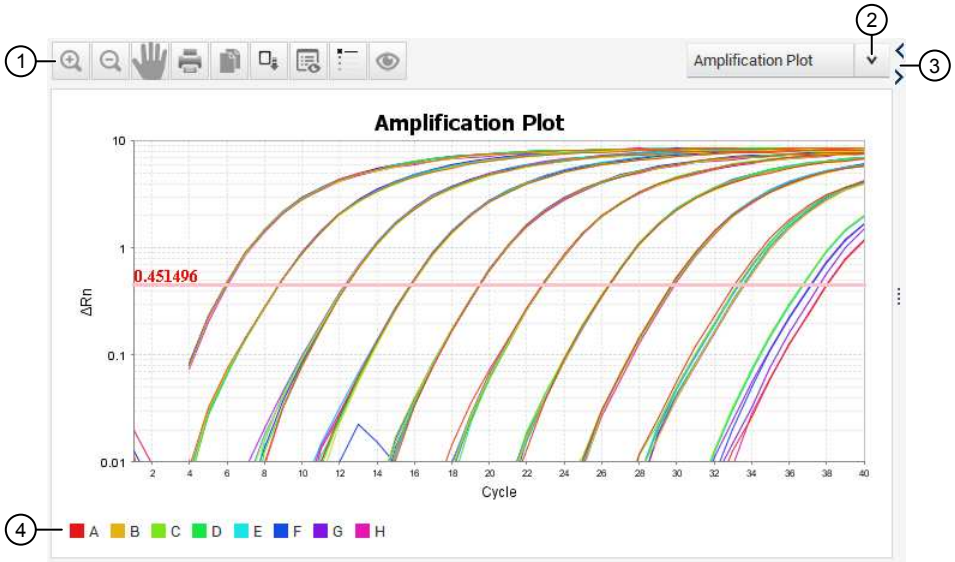


Figure 1 Plot pane

① Plot toolbar
② Plot selection list (varies by experiment)
③ Expand/contract the plot pane display
④ Plot legend

The Plot toolbar includes the following options:

- Zoom in and out
- Print or copy plot image
- Save plot as image file
- Configure plot properties
- Show/hide plot legend
- Configure plot settings

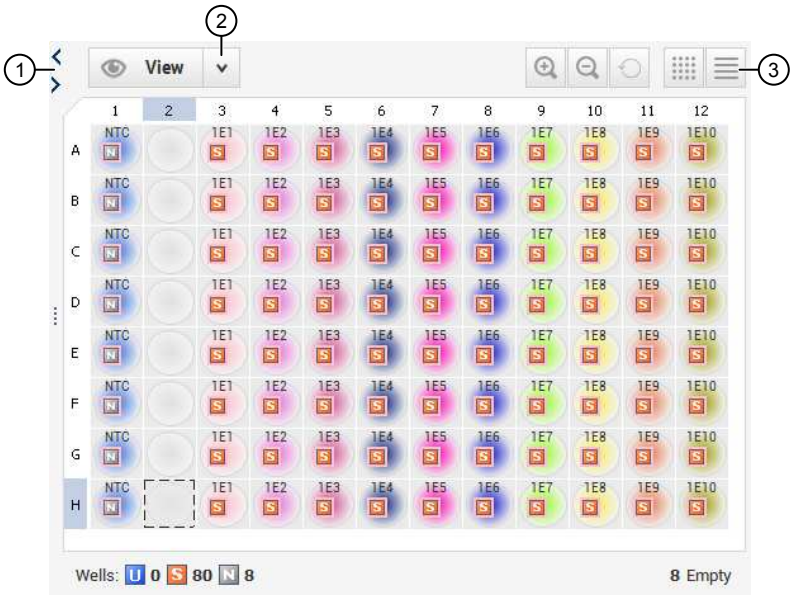


Figure 2 Plate Layout

① Expand/contract the **Plate Layout** display (layout is expanded in this figure)
② **View**: Select well properties to display
③ **Plate Layout** toolbar

The **Plate Layout** toolbar includes the following options:

- Zoom in and out
- Fit plate to window
- Display **Plate Layout**
- Display **Well Table**

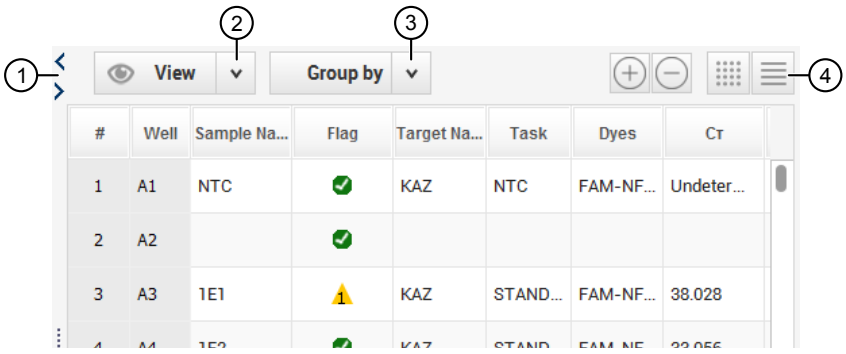


Figure 3 Well Table

① Expand/contract the **Well Table** display (table is expanded in this figure)
② **View**: Select well properties to display
③ **Group by**: Select a parameter by which to group well rows
④ **Well Table** toolbar

The **Well Table** toolbar includes the following options:

- Expand grouped rows
- Collapse grouped rows
- Display **Plate Layout**
- Display **Well Table**

Workflow: General procedures to review the run results

When a run is complete, the desktop software automatically analyzes the run data using the analysis settings that are specified during template development. The software then displays the run results in the **Results** tab.

View the Amplification Plot to confirm or correct threshold and baseline settings (page 30)

▼

Assess the experiment plot for the experiment
(for example, view the Allelic Discrimination Plot for genotyping experiments)

(see the corresponding chapter in this guide)

▼

Review data for outliers and *(optional)* omit wells (page 33)

▼

(Optional) View the Multicomponent Plot to review the dye signal profile (page 36)

▼

(Optional) View the Raw Data Plot to review the signal profile (page 37)

▼

(Optional) Review flags in the QC Summary (page 38)

▼

(Optional) Configure the analysis settings (page 100)

IMPORTANT! If you omit wells or configure the analysis settings, click **Analyze** to reanalyze the data.

Guidelines for viewing and analyzing results

- For information about adjusting the views in the **Results** tab, see the desktop software Help.
- To reanalyze the data, select all the wells in the **Plate Layout**, then click **Analyze**.
- To enable auto-analysis of data after a run, select **Tools** ▸ **Preferences** ▸ **Experiment**, then select **Auto Analysis**.

Assess results in the Amplification Plot

Amplification Plot overview

The Amplification Plot displays sample amplification as a function of cycle number or well. You can use the amplification plot to perform the following tasks:

- Confirm or correct baseline and threshold values.
- Identify outliers.
- Identify and examine abnormal amplification. Abnormal amplification can exhibit one of the following characteristics:
  - Increased fluorescence in negative control wells.
  - Absence of detectable fluorescence at an expected cycle.

    Note: If you notice abnormal amplification or a complete absence of fluorescence, see the instrument user guide for troubleshooting information.

Three plots are available. Some plots can be viewed as a linear or $\log_{10}$ graph.

Table 2 Amplification Plot types

Plot type	Description	Use to
ΔRn vs Cycle	ΔRn is the magnitude of normalized fluorescence signal, relative to the baseline fluorescence, generated by the reporter at each cycle during the PCR amplification.	• Identify and examine irregular amplification. • View threshold values for the run.
Rn vs Cycle	Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference.	• Identify and examine irregular amplification. • View baseline values for the run.
C_t vs Well	C_t is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot.	• Locate outlying amplification (outliers).

Assess the overall shape of the Amplification Plot curves

You can assess the overall shape of the Amplification Plot curves in the **Results** tab.

If no data are displayed in the **Results** tab, click **Analyze**.

1. In the **Results** tab, select **Amplification Plot** from the dropdown list.
2. Click to configure the plot, then make the following selections:
   - **Plot Type**: **ΔRn vs Cycle**
   - **Graph Type**: **Log**
   - **Plot Color**: **Target**, **Sample**, or **Well**

   The **Amplification Plot** is displayed for the selected wells in the **Plate Layout**.

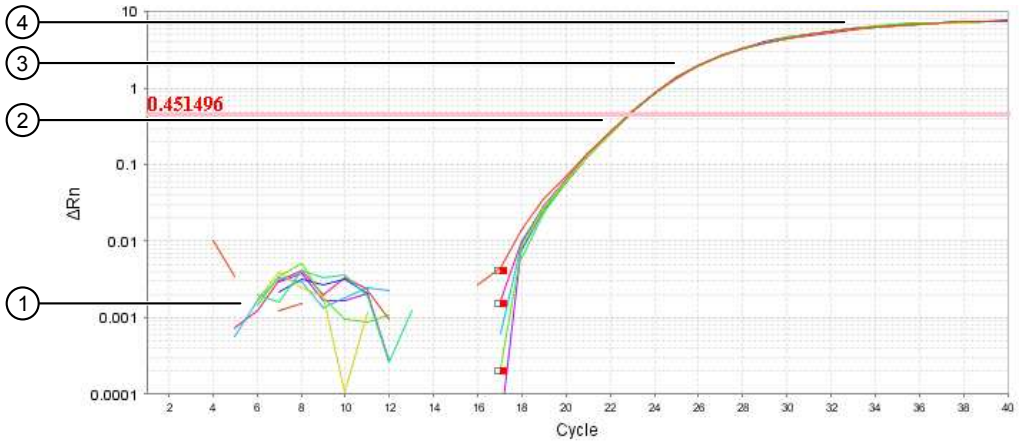


Figure 4 Typical Amplification Plot

A typical amplification curve has four distinct sections:

① Baseline
② Exponential (geometric) phase
③ Linear phase
④ Plateau phase

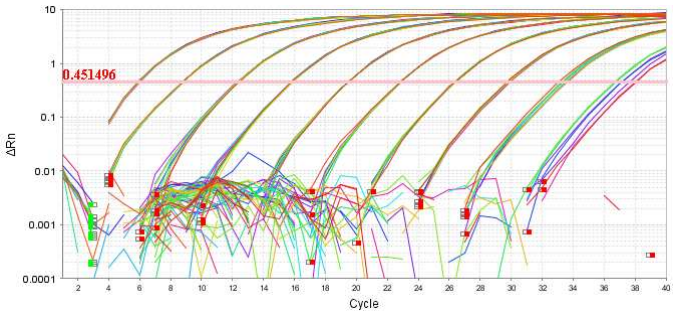
Confirm or correct threshold settings

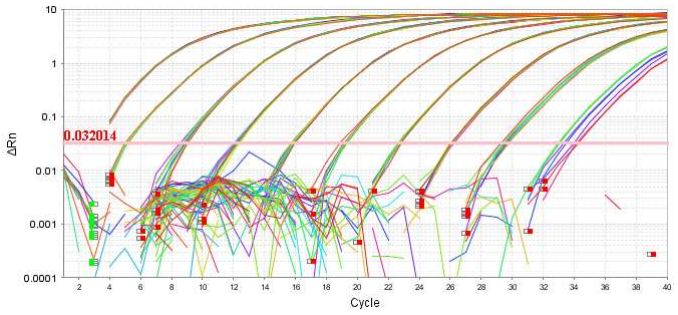
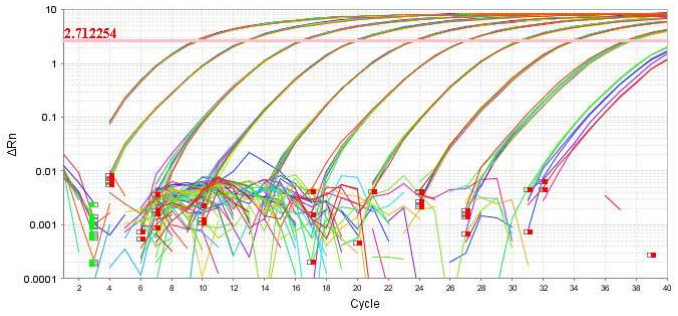
1. In the **Results** tab, select **Amplification Plot** from the dropdown list.
2. Click 👁 to configure the plot, then make the following selections:
   - **Plot Type**: **ΔRn vs Cycle**
   - **Graph Type**: **Log**
   - **Plot Color**: **Target**, **Sample**, or **Well**

   The **Amplification Plot** is displayed for the selected wells in the **Plate Layout**.
3. (*Optional*) Adjust the threshold.
   - Click-drag the threshold bar into the exponential phase of the curve.
   - Configure the C_t analysis settings (see "Ct settings overview" on page 101).

Table 3 Examples of threshold settings

Set the threshold in the exponential phase of the amplification curve. A threshold set above or below the optimum can increase the standard deviation of the replicate groups.

Threshold setting evaluation	Example
Threshold set correctly.	

Threshold setting evaluation	Example
Threshold set too low.	
Threshold set too high.	

Confirm or correct baseline settings

1. In the **Results** tab, select **Amplification Plot** from the dropdown list.
2. Click to configure the plot, then make the following selections:
   - **Plot Type**: **Rn vs Cycle**
   - **Graph Type**: **Linear**
   - **Plot Color**: **Well**
   - Select **Show: Baseline Start / Baseline End**

   Note: The start and end cycles are used to calculate the baseline.

   The **Amplification Plot** is displayed for the selected wells in the **Plate Layout**. The start ( ) and end ( ) cycles display for each well.
3. (*Optional*) Adjust the start and end cycle values for the baseline (see "Ct settings overview" on page 101).

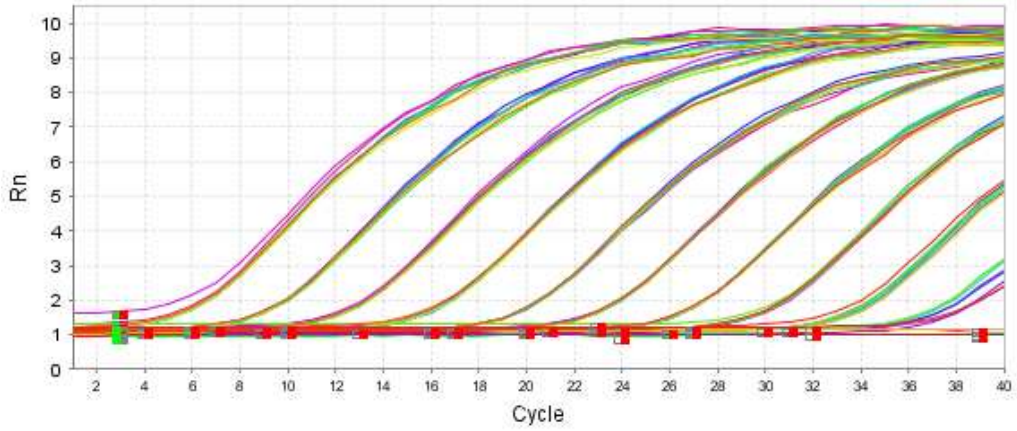


Figure 5 Example of correct baseline

Set the end cycle (■) a few cycles before the cycle number where significant fluorescence signal is detected.

Omit outliers from analysis

Outlier wells have C_q (C_t or C_{rt}) values that differ significantly from the average for the associated replicate wells. To ensure C_q (C_t or C_{rt}) precision, consider omitting the outliers from analysis.

1. In the **Results** tab, select **Amplification Plot** from the dropdown list.
2. Click 👁, then make the following selections to configure the plot:
   - **Plot Type**: **C_t vs Well**
   - **Graph Type**: **Linear**
   - **Plot Color**: **Well**

   The C_t values are displayed for the selected wells in the **Plate Layout**.
3. Click ☰ to examine the **Well Table** for outliers.
   a. Select **Group by ▸ Replicate**.
   b. Identify outliers in each replicate group.
      Outlier wells typically have one or more QC flags.
4. Omit outliers in either the ☰ **Well Table** or **Plate Layout** view.
   - In the ☰ **Well Table**, select **Omit** in outlier rows of the table.
   - In the **Plate Layout**, right-click a well, then select **Omit**.
5. Click **Analyze** to reanalyze the run data with any outliers removed.

Optimize display of negative controls in the Amplification Plot

1. In the **Results** tab, select **Amplification Plot** from the dropdown list.
2. Click to configure the plot, then make the following selections:
   - **Plot Type**: **ΔRn vs Cycle**
   - **Graph Type**: **Linear**
   - **Plot Color**: **Target**
   - Deselect **Show: Threshold**
   - Deselect **Show: Baseline Start / Baseline End**
3. In either the **Plate Layout** or **Well Table**, select the negative control wells (wells that should not have amplification for a particular target).
4. Click (configure plot properties), then select the **Y Axis** tab.
   a. Deselect **Auto-adjust range**.
   b. Enter **Minimum value** of –1.
   c. Enter **Maximum value** of 2.
   d. Click **Save**.

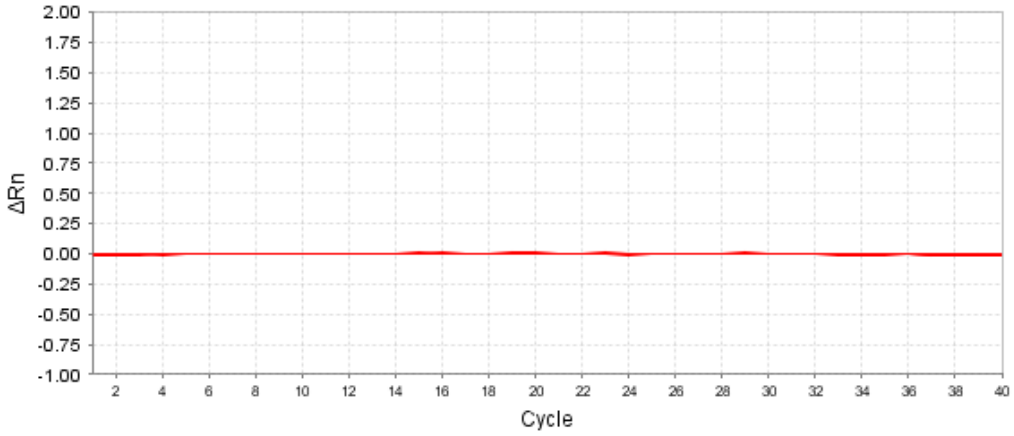


Figure 6 Example Amplification Plot of negative controls

The linear plot displays the Amplification Plot for negative controls as smooth lines. The expanded Y axis displays low levels of amplification.

Assess results in the Well Table view

Well Table overview

The ≣ **Well Table** displays data for each well in the reaction plate. The data that are displayed depend on the specific experiment type and can include the following information:

- Sample name, target name, task, and dyes
- Values that are specific to particular stage of the method
  For example: C_t or C_{rt}, normalized fluorescence (Rn), or melt temperature (T_m)
- Values that are specific to a particular experiment type
  For example: genotype calls, presence/absence calls, or quantities
- Omitted wells
- QC flags
- Comments

Group or sort the Well Table

Some of the possible options for grouping or sorting the ≣ **Well Table** are described in the following table. Available grouping categories depend on the specific experiment type and analysis settings.

Note: You can select multiple columns when sorting, but you can only make one selection for grouping rows.

Group category	Description	Notes
Replicate[1,2,3]	Grouped by replicate	• Examine the C_t or quantity values for each replicate group to assess the precision of C_t values.
Flag	Grouped as flagged and unflagged wells	• A flag indicates that the software found a potential error in the flagged well. • For more information about QC flags, see the desktop software Help.
C_t[1,2,4]	Grouped by C_t value	• C_t value < 8—There may be too much template in the reaction. • C_t value > 35—There may be a low amount of target in the reaction; for C_t values > 35, expect a higher standard deviation.
RQ[2]	Grouped by RQ value	• RQ value < 1—There is less relative target in the test sample as compared to the calibrator sample. • RQ value > 1—There is more relative target in the test sample as compared to the calibrator sample.
Call[4,5]	Grouped by genotype call or presence/absence call	—

Group category	Description	Notes
Tm1[3]	Grouped by T_m value	**Tm1** refers to the dominant peak. This grouping category is only applicable for the following experiment types: • Melt curve experiments • Any experiment with a melt curve data collection step (e.g., absolute standard curve)

[1] For standard curve experiments.
[2] For relative standard curve and comparative C_t ($\Delta\Delta C_t$) experiments.
[3] For melt curve experiments.
[4] For genotyping experiments.
[5] For presence/absence experiments.

Review the dye signal profile using the Multicomponent Plot

Multicomponent Plot overview

The Multicomponent Plot displays the complete spectral contribution of each dye over the duration of the PCR run.

Use the Multicomponent Plot to obtain the following information.

- Confirm that the signal from the passive reference dye remains unchanged throughout the run.
- Review reporter dye signal for spikes, dips, and/or sudden changes.
- Confirm that no amplification occurs in the negative control wells.

View and assess the Multicomponent Plot

You can view and assess the **Multicomponent Plot** in the **Results** tab.

If no data are displayed in the **Results** tab, click **Analyze**.

1. In the **Results** tab, select **Multicomponent Plot** from the dropdown list.
2. Click to configure the plot, then make the following selections:
   - **Plot Color**: **Dye**

   The **Multicomponent Plot** is displayed for the selected wells in the **Plate Layout**.
3. In the **Plate Layout**, select wells one at a time, then examine the **Multicomponent Plot** for the following plot characteristics.

Plot characteristic	Description
Passive reference dye	The passive reference dye fluorescence signal should remain relatively constant throughout the PCR process.
Reporter dye	The reporter dye fluorescence signal should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.

Plot characteristic	Description
Irregularities in the signal	Spikes, dips, and/or sudden changes in the fluorescence signal may have an impact on the data.
Negative control wells	The negative control wells should show no significant increase in fluorescence signal.

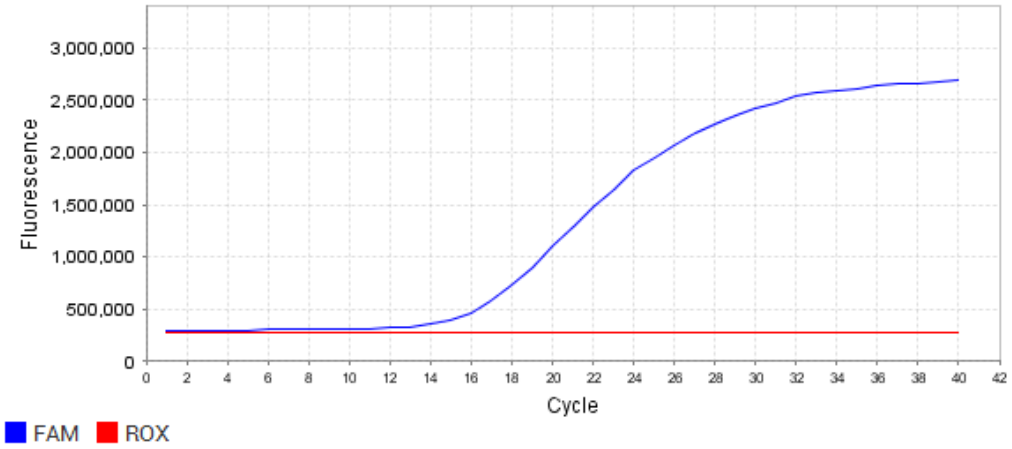


Figure 7 Example Multicomponent Plot (single well)

Review the signal profile using the Raw Data Plot

Raw Data Plot overview

The Raw Data Plot displays the raw fluorescence signal (not normalized) for each optical filter during each cycle of the real-time PCR.

View the Raw Data Plot to confirm a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View and assess the Raw Data Plot

You can view and assess the **Raw Data Plot** in the **Results** tab.

If no data are displayed in the **Results** tab, click **Analyze**.

1. In the **Results**, select **Raw Data Plot** from the dropdown list.
   The **Raw Data Plot** is diaplayed for the selected wells in the **Plate Layout**.
2. Click to display the **Show Cycle** scale.
3. Click-drag the **Show Cycle** pointer from cycle 1 to cycle 40, and confirm that each filter displays the characteristic signal increase.
   For more information on each filter set, see the instrument user guide (see Appendix F, "Documentation and support").

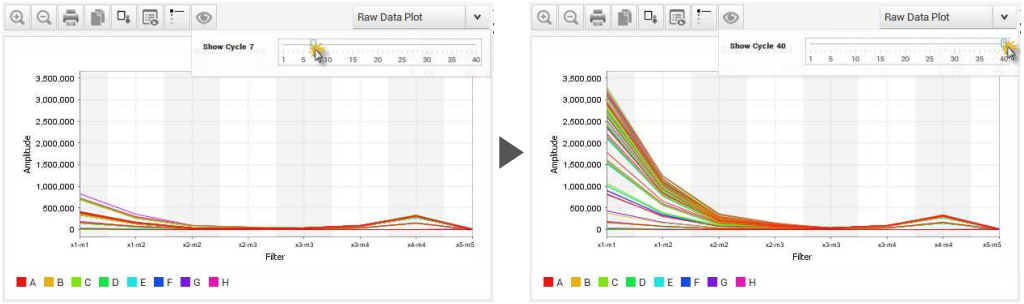


Figure 8 Example Raw Data Plot

Review the flags in the QC Summary

The **QC Summary** in the **Results** tab displays a list of the QC flags, including the flag frequency and location.

If no data are displayed in the **Results** tab, click **Analyze**.

1. In the **Results** tab, select **QC Summary** from the dropdown list.
2. Review the **Flag Details** table and the summary.
   The **Wells** column of the **Flag Details** table identifies wells that triggered a flag.
3. (*Optional*) In the **Flag Details** table, click each flag to display a brief description of the flag.
   For more information about the flag, see the desktop software Help.

View calibration results in the desktop software

Calibration results obtained on the instrument can be transferred via USB to the desktop software.

1. In the **Home** screen, click **Open**.
2. Navigate to and select the desired calibration EDS file.

If you are viewing calibration data files, **Calibration QC Status** is displayed to the right of the **Plate Layout** and **Well Table**.

Note: For more information about using the **Plate Layout** and **Well Table**, see Chapter 3, "General procedures to review results" .

Review ROI/Uniformity calibration results

1. In the **ROI** tab, select a **Filter Set** from the dropdown list to see the corresponding results.
2. In the **Uniformity** tab, use the **Plate Layout**, **Well Table**, and **Calibration QC Status** to review the run results.

Review Background calibration results

1. Select the plate wells in the **Plate Layout** or the **Well Table** to view the corresponding curves. For more information, see "Select plate wells" on page 19.
2. Review data in the **Well Table**.
   a. Review the results for each well in tabular format.
   b. Sort the wells according to well or normalized fluorescence with each filter.
   c. Select wells to review data in the analysis plot.
3. Click **Calibration QC Status** to review the quality of the calibration data.

Review Dye calibration results

1. Select a Dye row in the **Calibration** table to view the corresponding analysis data plot.
2. Select the plate wells in the **Plate Layout** or the **Well Table** to view the corresponding curves. For more information, see "Select plate wells" on page 19.
3. Review data in the **Well Table**.
   a. Review the results for each well in tabular format.
   b. Sort the wells according to well or normalized fluorescence with each filter.
   c. Select wells to review data in the analysis plot.
4. Click **Calibration QC Status** to review the quality of the calibration data.

Override the calibration data

Each EDS file contains the calibration data from the instrument on which it was run. You can use calibration data from another instrument for analysis of your run data.

- Calibration data must be from the same block type (96-well 0.2-mL block, 96-well 0.1-mL block, or 384-well block).
- Calibration data for a QuantStudio™ 1 Real-Time PCR Instrument run must be from a QuantStudio™ 1 Real-Time PCR Instrument run.
- Calibration data from a QuantStudio™ 5 Real-Time PCR Instrument run can override calibration data for a QuantStudio™ 3 Real-Time PCR Instrument run.
- Calibration data from a QuantStudio™ 3 Real-Time PCR Instrument run can *not* override calibration data for a QuantStudio™ 5 Real-Time PCR Instrument run.

1. Open the EDS file to recalibrate.
2. Select **Analysis ▸ Override Calibration ▸ Use Calibration From Another File**.
3. Navigate to, then select the EDS file containing the alternative calibration data.
4. Click **Open**.
5. (*Optional*) To revert to the original calibration data, select **Analysis ▸ Override Calibration ▸ Revert To Original Calibration**.

Export experiments or results

For step-by-step instructions for exporting experiments or results, see the desktop software Help.

Options for exporting run data and results

To	Action
Save a plot as an image file	Click
Print a plot	Click
Copy a plot to the clipboard	Click
Export run data and results	Click **Export**
Print the plate layout	Select **File** ▸ **Print**
Create slides	Select **File** ▸ **Send to PowerPoint**
Print a report	Select **File** ▸ **Print Report**

Export configurations

Data type	Description	File format
Plate setup files, for future experiments	Plate setup information For example, the well number, sample name and color, target name, dyes, and other reaction plate contents.	• XLS • XLSX • TXT
Analyzed data, for further analysis	QuantStudio™ format	• XLS • XLSX • TXT
	RDML (Real-Time PCR Data Markup Language) format Used for standard curve, relative standard curve, and comparative C_t experiments.	• RDML

4 Set up, run, and review standard curve experiments

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- Set up and run the PCR reactions 44
- Review results 44

Standard curve experiments

Overview

Use standard curve experiments to determine absolute target quantity in samples.

In a standard curve experiment, the software performs the following tasks.

1. The software measures amplification of the target in a standard dilution series and in test samples.
2. The software generates a standard curve using data from the standard dilution series.
3. The software uses the standard curve to interpolate the absolute quantity of target in the test samples.

Reaction types

Multiple targets can be assayed in a standard curve experiment, but each target requires its own standard curve.

Table 4 Reaction types for standard curve experiments

Reaction type (task)	Sample description
Standard	A sample that contains known or known relative quantities of the target • For known quantities—Quantify the target in the standard sample using an independent method. • For known relative quantities—Generate a relative dilution series of the target standards.

Reaction type (task)	Sample description
Unknown	Test sample
No-template control (NTC/ Negative Control)	Water or buffer No amplification of the target should occur in NTC wells.

- The precision of quantification experiments improves as the number of replicate reactions increases. Set up the number of replicates appropriate for the experiment.
- For accurate and precise efficiency measurements, set up the standard dilution series with at least five dilution points over a broad range of standard quantities, 4 to 6 logs (10^4- to 10^6-fold). A concentrated template, such as a plasmid or PCR product, is best for this purpose.

  A narrow range of standard quantities may be appropriate if the amount of standard is limited, the target is in low abundance, or the target is known to fall within a given range.

Compatible PCR options

Table 5 PCR options for standard curve experiments

Single- or multiplex PCR	PCR or RT-PCR[1]	Detection chemistry
Singleplex Multiplex	PCR 1-step RT-PCR 2-step RT-PCR	TaqMan® SYBR™ Green

[1] RT-PCR: reverse transcription-PCR

Set up a standard curve experiment in the software

1. In the **Home** screen, create or open a template.
   - In the **New Experiment** pane, click **Create New Experiment** to create a new template.
   - In the **Open Existing Experiment** pane, click **Open** to select and open an existing template.
2. In the **Properties** tab, enter the template information.
3. In the **Method** tab, adjust the reaction volume.
   For most experiments, the default run method is appropriate.
4. In the **Plate** tab (**Quick Setup**), assign plate attributes.
   a. In the **Plate Attributes** pane, select the **Passive Reference** from the dropdown list.
5. In the **Plate** tab (**Quick Setup**), define and assign well attributes.
   a. Select wells in the **Plate Layout** or the **Well Table**.
   b. Assign samples and targets to selected wells.
      - Enter new sample and target names in the text fields.
      - Select previously defined samples and targets from the dropdown lists.

      Note: New sample or target names entered in the **Quick Setup** subtab are automatically populated with default values for **Reporter** (FAM) and **Quencher** (NFQ-MGB) and assigned a task (U **Unknown**). Edit these values in the **Advanced Setup** subtab.
6. (*Optional*) In the **Plate** tab, set up standard dilutions (see "Define and set up standard dilutions" on page 94).
7. (*Optional*) In the **Plate** tab (**Advanced Setup**), assign tasks.
   a. Select wells in the **Plate Layout** or the **Well Table**.
   b. In the **Targets** table, select the checkbox of a target, then select a task from the dropdown list.

Reaction type	Task
Unknown (test sample)	U
No-template control	N

8. (*Optional*) In the **Plate** tab (**Advanced Setup**), define and assign biological replicate groups (see "Define and assign biological replicate groups" on page 95).

In the **Plate** tab (**Advanced Setup**), ensure the **Samples** table contains the following sample information:

- One sample name for each technical replicate group of an unknown sample
- (*Optional*) A standard sample for each target

Set up and run the PCR reactions

1. Assemble the PCR reactions. Follow the manufacturer's instructions for the reagents and follow the plate layout set up in the software (see "Prepare reactions" on page 21).
2. In the desktop software, open the appropriate template (EDT file).
3. Load the reaction plate into the instrument and start the run (see "Start and monitor a run" on page 22).

Review results

Workflow: Review standard curve experiments

View the Amplification Plot to confirm or correct threshold and baseline settings (page 30)

▼

Assess the Standard Curve Plot (page 46)

▼

Review data for outliers and *(optional)* omit wells (page 33)

▼

(Optional) View the Multicomponent Plot to review the dye signal profile (page 36)

▼

(Optional) View the Raw Data Plot to review the signal profile (page 37)

▼

(Optional) Review flags in the QC Summary (page 38)

▼

(Optional) Configure the analysis settings (page 47, page 100)

IMPORTANT! If you omit wells or configure the analysis settings, click **Analyze** to reanalyze the data.

Standard Curve Plot overview

The Standard Curve Plot displays the standard curve for samples designated as standards. The software calculates the quantity of an unknown target from the standard curve.

Table 6 Results or metrics to review in the Standard Curve Plot

Results or metrics	Description	Criteria for evaluation
Slope and amplification efficiency	The amplification efficiency is calculated using the slope of the regression line in the standard curve.	A slope close to –3.3 indicates optimal, 100% PCR amplification efficiency. Factors that affect amplification efficiency: • Improper design of the primer and probe • Range of standard quantities—For accurate and precise efficiency measurements, use a broad range of standard quantities, 5 to 6 logs (10^5- to 10^6-fold). • Number of standard replicates—For accurate efficiency measurements, include replicates to decrease the effects of pipetting inaccuracies. • PCR inhibitors—PCR inhibitors and contamination in the reaction can reduce amplification efficiency. • Other possible factors: – Component and properties of the reaction mix, such as salt content, DMSO, pH, etc. – Inaccurate sample or reagent pipetting – Improper analysis settings – Incorrect plate setup
R^2 value (correlation coefficient)	The R^2 value is a measure of the closeness of fit between the regression line and the individual C_q data points of the standard reactions.	• A value of 1.00 indicates a perfect fit between the regression line and the data points. • An R^2 value > 0.99 is desirable.
Error	The standard error of the slope of the regression line in the standard curve. The error can be used to calculate a confidence interval (CI) for the slope and therefore the amplification efficiency.	Acceptable value is determined by the experimental criteria.
C_t values	The threshold cycle (C_t) is the PCR cycle number at which the fluorescence level meets the threshold.	A C_t value > 8 and < 35 is desirable. • C_t value < 8—There may be too much template in the reaction. • C_t value > 35—There may be a low amount of target in the reaction; for C_t values > 35, expect a higher standard deviation.

View and assess the Standard Curve Plot

You can view and assess the Standard Curve Plot in the **Results** tab.

If no data are displayed in the **Results** tab, click **Analyze**.

1. Select **Standard Curve** from the dropdown list.
2. Click to configure the plot, then make the following selections:
   - **Target**: Select the target of interest
   - **Plot Color**: **Sample**, **Target**, or **Task**
   - Select all wells in the **Plate Layout**

   The Standard Curve Plot is displayed. The slope, R^2 value, amplification efficiency, and error are displayed below the plot.
3. Confirm that the slope, R^2 value, amplification efficiency, and error meet the experimental criteria.
4. Visually check that all unknown sample C_q (C_t or C_{rt}) values fall within the standard curve range.
5. In the **Well Table**, use the **Group By** dropdown list to confirm that the C_q (C_t or C_{rt}) values of all replicate samples meet the experimental criteria.

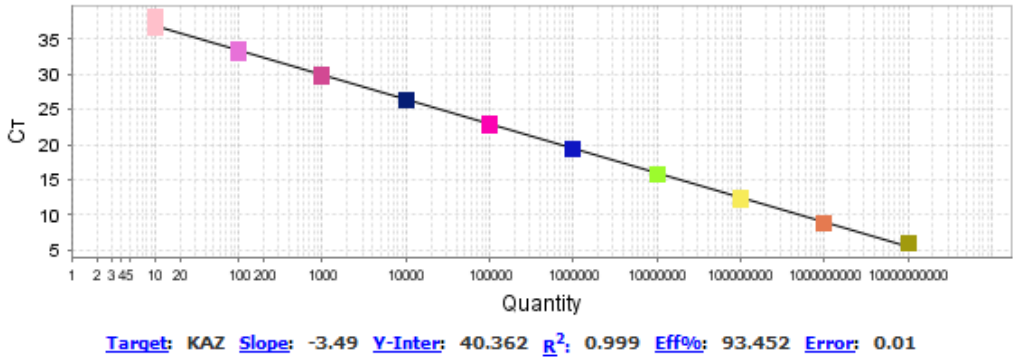

Figure 9 Example Standard Curve Plot

If the results do not meet the experimental criteria, troubleshoot using one of the following strategies:

- Omit wells, then reanalyze. For more information, see "Omit outliers from analysis" on page 33.
- Repeat the experiment, adjusting the template setup and analysis settings to improve results.

To learn more about the Standard Curve Plot, see "Standard Curve Plot overview" on page 45.

Standard curve settings overview

You can use the standard curve from another experiment and apply it to the current experiment. The two experiments must be from the same instrument type, block type, and run method.

To import an external standard curve, select ⚙ **Analysis Settings** ▸ **Standard Curve Settings**, then follow the instructions on the screen.

For step-by-step instructions for adjusting the standard curve settings, see the desktop software Help.

5 Set up, run, and review relative standard curve experiments and comparative C_t experiments

- Relative standard curve experiments 48
- Comparative Ct experiments 50
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- Set up a relative standard curve experiment in the software 51
- Set up a comparative Ct experiment in the software 53
- Set up and run the PCR reactions 54
- Review results 54

Relative standard curve experiments

Overview

Use relative standard curve experiments to determine relative target quantity in samples.

In a relative standard curve experiment, the software performs the following tasks.

1. The software measures amplification of the target of interest and of an endogenous control target in a standard dilution series, in a reference (calibrator) sample, and in test samples.

   The endogenous control is a target that is expressed equally in all samples; examples of endogenous controls are β-actin, GAPDH, and 18S ribosomal RNA. The software can algorithmically incorporate multiple endogenous control targets in relative quantification calculations.

   The reference sample is used as the basis for relative quantification results (or 1× sample). For example, in a study of drug effects on gene expression, an untreated control is an appropriate reference sample.
2. The software generates standard curves for the target of interest and the endogenous control using data from the corresponding standard dilution series.
3. The software uses the standard curves to interpolate the quantities of the target of interest and the endogenous control in each sample. The target quantity in each sample is then normalized to the sample's endogenous control quantity.
4. To determine the relative quantity of the target in test samples, the software divides the normalized target quantity in the sample by the normalized target quantity in the reference sample.

For a comparison of this method to the comparative C_t ($\Delta\Delta C_t$) method, see "Relative quantitation: relative standard curve vs. comparative Ct" on page 51.

Reaction types

Relative standard curve experiments include the following reaction types for the endogenous control target and each target of interest.

Table 7 Reaction types for relative standard curve experiments

Reaction type (task)	Sample description
Standard	A sample that contains known or known relative quantities of the target • For known quantities—Quantify the target in the standard sample using an independent method. • For known relative quantities—Generate a relative dilution series of the target standards.
Reference sample[1]	The sample that is used as the basis for relative quantification results
Unknown	Test or reference sample
No-template control (NTC/ Negative Control)	Water or buffer No amplification of the target should occur in NTC wells.

[1] To identify a sample as a reference sample, review the relative quantification settings.

- The precision of quantification experiments improves as the number of replicate reactions increases. Set up the number of replicates appropriate for the experiment.
- For accurate and precise efficiency measurements, set up the standard dilution series with at least five dilution points over a broad range of standard quantities, 4 to 6 logs (10^4- to 10^6-fold). A concentrated template, such as a plasmid or PCR product, is best for this purpose.
  A narrow range of standard quantities may be appropriate if the amount of standard is limited, the target is in low abundance, or the target is known to fall within a given range.

Compatible PCR options

Table 8 PCR options for relative standard curve experiments

Single- or multiplex PCR	PCR or RT-PCR[1]	Detection chemistry
Singleplex Multiplex	PCR 1-step RT-PCR 2-step RT-PCR	TaqMan® SYBR™ Green

[1] RT-PCR: reverse transcription PCR.

Comparative C_t experiments

Overview

Use comparative C_t ($\Delta\Delta C_t$) experiments to determine relative target quantity in samples.

In a comparative C_t experiment, the software performs the following tasks.

1. The software measures amplification of the target of interest and of an endogenous control target in a reference (calibrator) sample and in test samples.
   The endogenous control is a target that is expressed equally in all samples; examples of endogenous controls are β-actin, GAPDH, and 18S ribosomal RNA. The software can algorithmically incorporate multiple endogenous control targets in relative quantification calculations.
   The reference sample is used as the basis for relative quantification results (or 1× sample). For example, in a study of drug effects on gene expression, an untreated control is an appropriate reference sample.
2. The measurements for the target of interest are normalized to the endogenous control.
3. To determine the relative quantity of the target in test samples, the software compares the normalized ΔC_q (ΔC_t or ΔC_{rt}) for the sample to the normalized ΔC_q (ΔC_t or ΔC_{rt}) for the reference sample.

For a comparison of this method to the relative standard curve method, see "Relative quantitation: relative standard curve vs. comparative Ct" on page 51.

Reaction types

Comparative C_t experiments include the following reaction types for the endogenous control target and each target of interest.

Table 9 Reaction types for comparative C_t experiments

Reaction type (task)	Sample description
Reference sample[1]	The sample that is used as the basis for relative quantification results
Unknown	Test or reference sample
No-template control (NTC/ Negative Control)	Water or buffer No amplification of the target should occur in NTC wells.

[1] To identify a sample as a reference sample, review the relative quantification settings.

The precision of quantification experiments improves as the number of replicate reactions increases. Set up the number of replicates appropriate for the experiment.

Compatible PCR options

Table 10 PCR options for comparative C_t experiments

Single- or multiplex PCR	PCR or RT-PCR[1]	Detection chemistry
Singleplex Multiplex	PCR 1-step RT-PCR 2-step RT-PCR	TaqMan® SYBR™ Green

[1] RT-PCR: reverse transcription PCR

Relative quantitation: relative standard curve vs. comparative C_t

Use either relative standard curve experiments or comparative C_t experiments to determine the relative quantity of a target of interest in a test sample relative to a reference sample. Relative quantitation experiments are commonly used for the following applications.

- Comparison of expression levels of a gene in different tissues.
- Comparison of expression levels of a gene in a treated sample vs. an untreated sample.
- Comparison of expression levels of a gene of interest in different genetic backgrounds.
- Analysis of the gene expression changes over time under specific treatment conditions.

Table 11 Comparison of relative standard curve experiments and comparative C_t experiments

Characteristic	Relative standard curve	Comparative C_t
Typical use	Best for assays that have suboptimal PCR efficiency.	Best for high-throughput measurements of relative gene expression of many genes in many samples.
Advantage	Requires the least amount of validation because the PCR efficiencies of the target and endogenous control do not need to be equivalent.	• Relative levels of target in samples can be determined without the use of a standard curve, if the PCR efficiencies of the target and endogenous control are relatively equivalent. • Reduced reagent usage. • More space available in the reaction plate.
Limitation	A standard curve must be constructed for each target, which requires more reagents and more space in the reaction plate.	• Suboptimal (low PCR efficiency) assays may produce inaccurate results. • Before you use the comparative C_t method, we recommend that you determine that the PCR efficiencies for the target assay and the endogenous control assay are approximately equal.

Set up a relative standard curve experiment in the software

1. In the **Home** screen, create or open a template.
   - In the **New Experiment** pane, click **Create New Experiment** to create a new template.
   - In the **Open Existing Experiment** pane, click **Open** to select and open an existing template.
2. In the **Properties** tab, enter the template information.

3. In the **Method** tab, adjust the reaction volume.
   For most experiments, the default run method is appropriate.

4. In the **Plate** tab (**Quick Setup**), assign plate attributes.
   a. In the **Plate Attributes** pane, select a **Passive Reference**, **Reference Sample**, and **Endogenous Control** from the dropdown lists.

5. In the **Plate** tab (**Quick Setup**), define and assign well attributes.
   a. Select wells in the **Plate Layout** or the **Well Table**.

   b. Assign samples and targets to selected wells.
      - Enter new sample and target names in the text fields.
      - Select previously defined samples and targets from the dropdown lists.

      Note: New sample or target names entered in the **Quick Setup** subtab are automatically populated with default values for **Reporter** (FAM) and **Quencher** (NFQ-MGB) and assigned a task (U **Unknown**). Edit these values in the **Advanced Setup** subtab.

6. In the **Plate** tab, set up standard dilutions (see "Define and set up standard dilutions" on page 94).

7. (*Optional*) In the **Plate** tab (**Advanced Setup**), assign tasks.
   a. Select wells in the **Plate Layout** or the **Well Table**.

   b. In the **Targets** table, select the checkbox of a target, then select a task from the dropdown list.

Reaction type	Task
Unknown (test sample)	U
No-template control	N

8. (*Optional*) In the **Plate** tab (**Advanced Setup**), define and assign biological replicate groups (see "Define and assign biological replicate groups" on page 95).

The targets of interest and the endogenous control target should each have wells that are assigned with standard dilutions, unknown, and no-template-control tasks, and corresponding samples.

In the **Plate** tab (**Advanced Setup**), ensure the **Samples** table contains the following samples.

- Unknown samples
- Reference sample
- (*Optional*) For a dilution of target standards, each dilution step for each endogenous control target and target of interest has its own sample name.

Set up a comparative C_t experiment in the software

1. In the **Home** screen, create or open a template.
   - In the **New Experiment** pane, click **Create New Experiment** to create a new template.
   - In the **Open Existing Experiment** pane, click **Open** to select and open an existing template.
2. In the **Properties** tab, enter the template information.
3. In the **Method** tab, adjust the reaction volume.
   For most experiments, the default run method is appropriate.
4. In the **Plate** tab (**Quick Setup**), assign plate attributes.
   a. In the **Plate Attributes** pane, select a **Passive Reference**, **Reference Sample**, and **Endogenous Control** from the dropdown lists.
5. In the **Plate** tab (**Quick Setup**), define and assign well attributes.
   a. Select wells in the **Plate Layout** or the **Well Table**.
   b. Assign samples and targets to selected wells.
      - Enter new sample and target names in the text fields.
      - Select previously defined samples and targets from the dropdown lists.

      Note: New sample or target names entered in the **Quick Setup** subtab are automatically populated with default values for **Reporter** (FAM) and **Quencher** (NFQ-MGB) and assigned a task (U **Unknown**). Edit these values in the **Advanced Setup** subtab.
6. (*Optional*) In the **Plate** tab (**Advanced Setup**), assign tasks.
   a. Select wells in the **Plate Layout** or the **Well Table**.
   b. In the **Targets** table, select the checkbox of a target, then select a task from the dropdown list.

Reaction type	Task
Unknown (test sample)	U
No-template control	N

7. (*Optional*) In the **Plate** tab (**Advanced Setup**), define and assign biological replicate groups (see "Define and assign biological replicate groups" on page 95).

The targets of interest and the endogenous control target should each have wells assigned with unknown and no-template-control tasks, and corresponding samples.

In the **Plate** tab (**Advanced Setup**), ensure the **Samples** table contains:

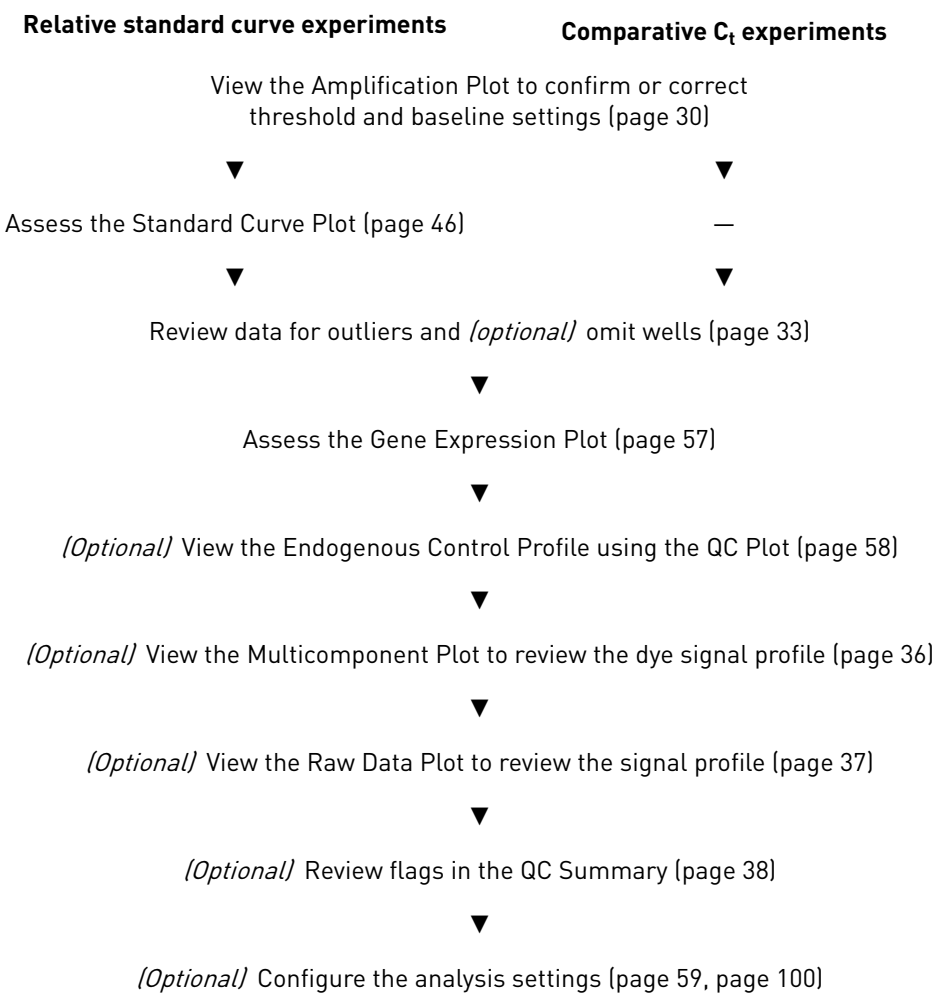
- Unknown samples
- Reference sample

Set up and run the PCR reactions

1. Assemble the PCR reactions. Follow the manufacturer's instructions for the reagents and follow the plate layout set up in the software (see "Prepare reactions" on page 21).
2. In the desktop software, open the appropriate template (EDT file).
3. Load the reaction plate into the instrument and start the run (see "Start and monitor a run" on page 22).

Review results

Workflow: Review relative standard curve and comparative C_t experiments



IMPORTANT! If you omit wells or configure the analysis settings, click **Analyze** to reanalyze the data.

View and assess the Standard Curve Plot

This section only applies to relative standard curve experiments.

Standard Curve Plot overview

The Standard Curve Plot displays the standard curve for samples designated as standards. The software calculates the quantity of an unknown target from the standard curve.

Table 12 Results or metrics to review in the Standard Curve Plot

Results or metrics	Description	Criteria for evaluation
Slope and amplification efficiency	The amplification efficiency is calculated using the slope of the regression line in the standard curve.	A slope close to –3.3 indicates optimal, 100% PCR amplification efficiency. Factors that affect amplification efficiency: • Improper design of the primer and probe • Range of standard quantities—For accurate and precise efficiency measurements, use a broad range of standard quantities, 5 to 6 logs (10^5- to 10^6-fold). • Number of standard replicates—For accurate efficiency measurements, include replicates to decrease the effects of pipetting inaccuracies. • PCR inhibitors—PCR inhibitors and contamination in the reaction can reduce amplification efficiency. • Other possible factors: – Component and properties of the reaction mix, such as salt content, DMSO, pH, etc. – Inaccurate sample or reagent pipetting – Improper analysis settings – Incorrect plate setup
R^2 value (correlation coefficient)	The R^2 value is a measure of the closeness of fit between the regression line and the individual C_q data points of the standard reactions.	• A value of 1.00 indicates a perfect fit between the regression line and the data points. • An R^2 value > 0.99 is desirable.
Error	The standard error of the slope of the regression line in the standard curve. The error can be used to calculate a confidence interval (CI) for the slope and therefore the amplification efficiency.	Acceptable value is determined by the experimental criteria.
C_t values	The threshold cycle (C_t) is the PCR cycle number at which the fluorescence level meets the threshold.	A C_t value > 8 and < 35 is desirable. • C_t value < 8—There may be too much template in the reaction. • C_t value > 35—There may be a low amount of target in the reaction; for C_t values > 35, expect a higher standard deviation.

View and assess the Standard Curve Plot

You can view and assess the Standard Curve Plot in the **Results** tab.

If no data are displayed in the **Results** tab, click **Analyze**.

1. Select **Standard Curve** from the dropdown list.
2. Click to configure the plot, then make the following selections:
   - **Target**: Select the target of interest
   - **Plot Color**: **Sample**, **Target**, or **Task**
   - Select all wells in the **Plate Layout**

   The Standard Curve Plot is displayed. The slope, R^2 value, amplification efficiency, and error are displayed below the plot.
3. Confirm that the slope, R^2 value, amplification efficiency, and error meet the experimental criteria.
4. Visually check that all unknown sample C_q (C_t or C_{rt}) values fall within the standard curve range.
5. In the **Well Table**, use the **Group By** dropdown list to confirm that the C_q (C_t or C_{rt}) values of all replicate samples meet the experimental criteria.

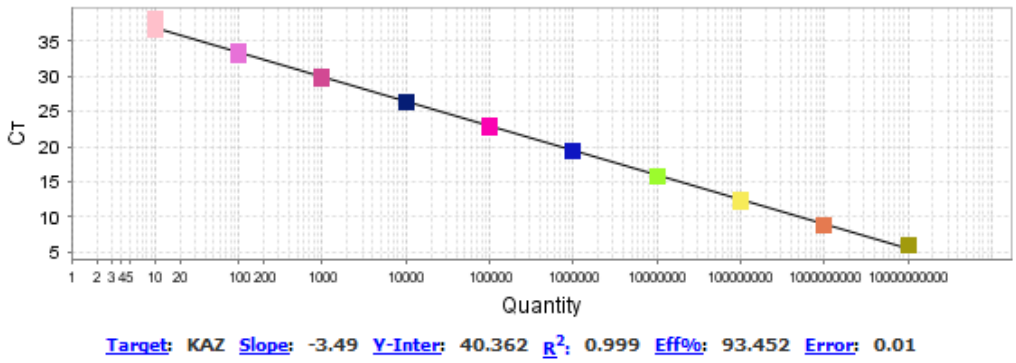


Figure 10 Example Standard Curve Plot

If the results do not meet the experimental criteria, troubleshoot using one of the following strategies:

- Omit wells, then reanalyze. For more information, see "Omit outliers from analysis" on page 33.
- Repeat the experiment, adjusting the template setup and analysis settings to improve results.

To learn more about the Standard Curve Plot, see "Standard Curve Plot overview" on page 45.

Standard curve settings overview

You can use the standard curve from another experiment and apply it to the current experiment. The two experiments must be from the same instrument type, block type, and run method.

To import an external standard curve, select ⚙ **Analysis Settings** ▸ **Standard Curve Settings**, then follow the instructions on the screen.

For step-by-step instructions for adjusting the standard curve settings, see the desktop software Help.

Gene Expression Plot overview

The Gene Expression Plot displays the results of relative quantification calculations for relative standard curve and comparative C_t experiments.

Review the Gene Expression Plot to evaluate the fold change in expression level of the targets of interest in the test samples relative to the reference sample.

There are two plots available, depending on the experimental focus. Each plot can be viewed on a linear, $\log_{10}$, Ln, and $\log_2$ scale.

Table 13 Gene Expression plots

Plot type	Description
RQ vs. Target	Groups the relative quantification (RQ) values by target. Each sample is plotted for each target.
RQ vs. Sample	Groups the relative quantification (RQ) values by sample. Each target is plotted for each sample.

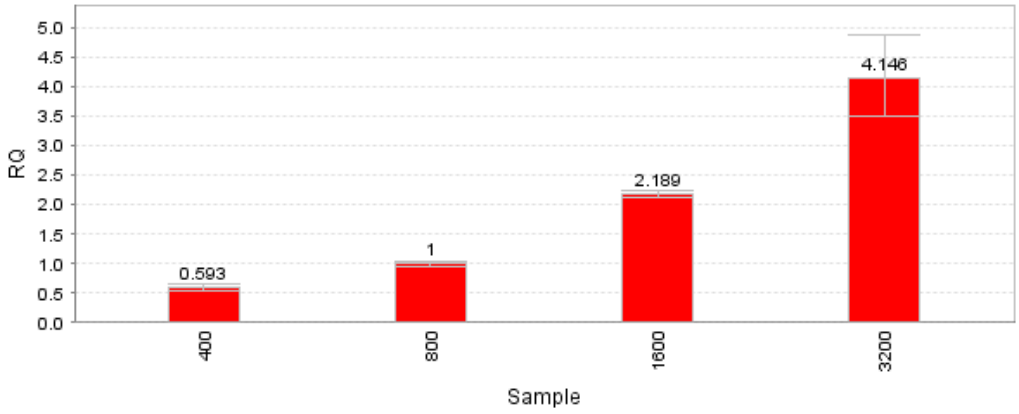


Figure 11 Example Gene Expression Plot

In this example, there is one target of interest, and the reference sample (calibrator) is sample 800.

QC Plot overview

The QC Plot is a visual display of the C_q (C_t or C_{rt}) levels of potential endogenous control targets across all samples (Endogenous Control Profile).

Use the QC Plot to help choose the best endogenous control for an experiment. Select the target with a quantity (indicated by C_q (C_t or C_{rt}) value) that does not change under experimental conditions.

All targets can be displayed in the QC Plot. You can view up to four potential endogenous controls at a time.

View and assess the QC Plot

You can view and assess the **QC Plot** in the **Results** tab.

If no data are displayed, click **Analyze**.

1. In the **Results** tab, select **QC Plot** from the dropdown list.
   The **Endogenous Control Profile** is displayed.
2. In the right pane, select the targets to display, then select the color and shape from the dropdown lists.
3. (*Optional*) In the **View Replicate Results Table** tab, select the samples to omit from analysis.
4. (*Optional*) To change the endogenous controls used for analysis, select **Analysis Settings ▸ Relative Quantification Settings** (see "Relative quantification settings overview" on page 59).
5. Click **Analyze** to see the result of the adjustments.

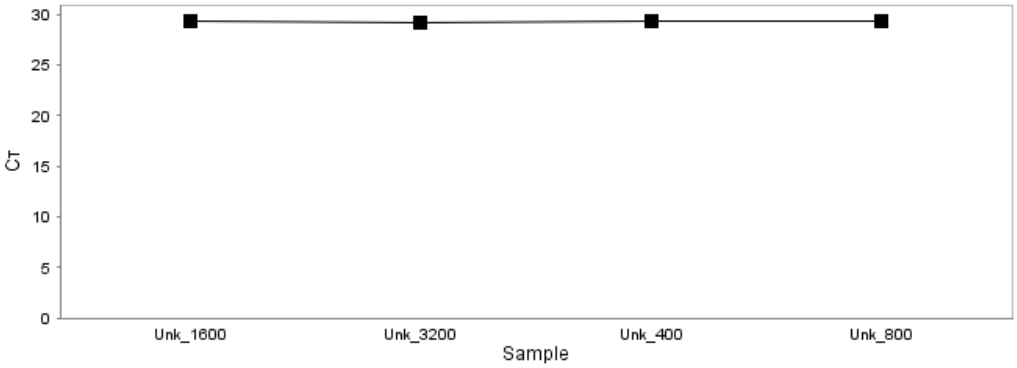


Figure 12 Example QC Plot

Relative quantification settings overview

In the **Results** tab, select ⚙ **Analysis Settings** ▸ **Relative Quantification Settings** to configure the following parameters:

Parameter	Description
Analysis Type	Select **Multiplex** or **Singleplex** analysis.
References	Set the reference sample, or set a biological replicate group as the reference sample.
Endogenous Controls	Change the endogenous control, or select multiple endogenous controls.
Efficiency *(Comparative C_t experiments only)*	Set the amplification efficiency for a target. The amplification efficiency for each target is calculated from the standard dilution series in relative standard curve experiments.
Outlier Rejection *(Multiplex reactions only)*	Outliers with ΔC_q (ΔC_t or ΔC_{rt}) values less than or equal to the entered value are rejected.
RQ Min/Max Calculations	Determines the algorithm used to calculate the relative quantification minimum and maximum values (error bars). • **Confidence Level**—Select to calculate the RQ minimum and maximum values based on the selected confidence level. • **Standard Deviations**—Select to calculate the RQ minimum and maximum values based on the selected number of standard deviations.

For step-by-step instructions for adjusting the relative quantification settings, see the desktop software Help.

6 Set up, run, and review genotyping experiments

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- Review results 63

Genotyping experiments

Overview

Use genotyping experiments to detect single nucleotide polymorphism (SNP) variants of a target nucleic acid sequence.

Genotyping experiments use preformulated TaqMan® SNP Genotyping Assays that include the following components:

- Two sequence-specific primers for amplification of sequences containing the SNP of interest
- Two allele-specific TaqMan® probes for Allele 1 and Allele 2

In a genotyping experiment, the software performs the following tasks.

1. The software normalizes the fluorescence of the reporter dyes to the fluorescence of the passive reference dye in each well.
2. The software plots the normalized reporter dye signal of each sample well on an Allelic Discrimination Plot, which contrasts the reporter dye intensities of the allele-specific probes.
3. The software algorithmically clusters the sample data, and assigns a genotype call to the samples of each cluster according to its position on the plot.

Reaction types

Table 14 Reaction types for genotyping experiments

Reaction type (task)	Sample description
Unknown	Test sample
No-template control	Water or buffer No amplification of the target should occur in NTC wells.
Allele control (1/1)	Control sample that is homozygous for allele 1
Allele control (1/2)	Control sample that is heterozygous allele 1/allele 2
Allele control (2/2)	Control sample that is homozygous for allele 2

Allele controls are optional but recommended. Including allele controls helps to improve the clustering algorithm, particularly in situations where a limited number of samples are run.

In genotyping experiments, the software makes calls for individual wells. Running 3 or more replicates of each reaction can help identify outlier wells that may be present.

Compatible PCR options

Table 15 PCR options for genotyping experiments

Single- or multiplex PCR	PCR or RT-PCR[1]	Detection chemistry
Multiplex[2]	PCR	TaqMan®

[1] RT-PCR: reverse transcription PCR

[2] Each SNP genotyping assay is a multiplex assay with a probe for each allele. Multiple SNP assays can be performed in a single well.

Genotyping calls are based either on end-point data (data collected outside of any PCR cycling stage) or on real-time data (data collected during a PCR cycling stage). For detailed information, see the analysis settings section of this guide.

We recommend collecting real-time amplification data during the PCR stage, for troubleshooting purposes.

Set up a genotyping experiment in the software

1. In the **Home** screen, create or open a template.
   - In the **New Experiment** pane, click **Create New Experiment** to create a new template.
   - In the **Open Existing Experiment** pane, click **Open** to select and open an existing template.
2. In the **Properties** tab, enter the template information.
3. In the **Method** tab, adjust the reaction volume.
   For most experiments, the default run method is appropriate.

4. In the **Plate** tab (**Quick Setup**), assign plate attributes.
   a. In the **Plate Attributes** pane, select the **Passive Reference** from the dropdown list.

5. In the **Plate** tab (**Quick Setup**), define and assign well attributes.
   a. Select wells in the **Plate Layout** or the **Well Table**.

   b. Assign samples and SNP assays to selected wells.
      - Enter new sample and SNP assay names in the text fields.
      - Select previously defined samples and SNP assays from the dropdown lists.

      Note: New sample or SNP assay names entered in the **Quick Setup** subtab are automatically populated with the following default values:

Allele 1	Reporter	Quencher	Allele 2	Reporter	Quencher	Task
Allele 1	VIC	NFQ-MGB	Allele 2	FAM	NFQ-MGB	U Unknown

      Edit these values in the **Advanced Setup** subtab.

6. (*Optional*) In the **Plate** tab (**Advanced Setup**), assign tasks.
   a. Select wells in the **Plate Layout** or the **Well Table**.

   b. In the **SNP Assays** table, select the checkbox of a SNP assay, then select a task from the dropdown list.

Reaction type	Task
Unknown (test sample)	U
No-template control	N
Allele control (1/1)[1]	1 1
Allele control (1/2)[1]	1 2
Allele control (2/2)[1]	2 2

[1] Optional but recommended

In the **Plate** tab (**Advanced Setup**), ensure the **Samples** table contains the following samples:

- Unknown samples
- (*Optional*) Allele control samples

Set up and run the PCR reactions

1. Assemble the PCR reactions. Follow the manufacturer's instructions for the reagents and follow the plate layout set up in the software (see "Prepare reactions" on page 21).
2. In the desktop software, open the appropriate template (EDT file).
3. Load the reaction plate into the instrument and start the run (see "Start and monitor a run" on page 22).

Review results

Workflow: Review genotyping experiments

Assess the Allelic Discrimination Plot (page 64)

▼

(Optional) View the Amplification Plot (page 30)

▼

Review data for outliers and *(optional)* omit wells (page 33)

▼

(Optional) View the Multicomponent Plot to review the dye signal profile (page 36)

▼

(Optional) View the Raw Data Plot to review the signal profile (page 37)

▼

(Optional) Review flags in the QC Summary (page 38)

▼

(Optional) Configure the analysis settings (page 66, page 100)

IMPORTANT! If you omit wells or configure the analysis settings, click **Analyze** to reanalyze the data.

Allelic Discrimination Plot overview

The Allelic Discrimination Plot contrasts the Rn or the ΔRn of the reporter dyes for the allele-specific probes of the SNP assay. It is an intermediary step in the software algorithm for genotyping calls.

Data points tend to cluster along the horizontal axis (Allele 1), vertical axis (Allele 2), or diagonal (Allele 1/Allele 2).

Table 16 Data clusters in the Allelic Discrimination Plot

A substantial increase in...	Clusters along...	Indicates...
Fluorescence of VIC™ dye-labeled probe only	Horizontal axis	Homozygosity for Allele 1
Fluorescence of FAM™ dye-labeled probe only	Vertical axis	Homozygosity for Allele 2
Fluorescence of both VIC™ and FAM™ dye-labeled probes	Diagonal	Heterozygosity for Allele 1 – Allele 2

Review the allelic discrimination plot to assess data clusters.

- Confirm that clustering of control samples is as expected.
- Visually assess clusters for the three possible genotypes.

Note: The desktop software clustering algorithm does not call genotypes if all the samples are one genotype (form one cluster).

View and assess the Allelic Discrimination Plot

You can view and assess the **Allelic Discrimination Plot** in the **Results** tab.

If no data are displayed in the **Results** tab, click **Analyze**.

1. In the **Results** tab, select **Allelic Discrimination Plot** from the dropdown list.
2. Click to configure the plot, then make the following selections:
   - **SNP Assay**: select the assay of interest
   - **Plot Type**: **Cartesian** or **Polar**

   The Allelic Discrimination Plot is displayed for the selected SNP assay.

   Note: Initially, all points in the plot are cyan because all of the wells in the **Plate Layout** are selected. Click anywhere in the plot or **Plate Layout** to deselect all wells. The data points in the plot change to the call colors.
3. Confirm that control data clusters as expected.
   a. In the **Well Table** or **Plate Layout**, select the wells containing a control to highlight the corresponding data points in the plot.
   b. Check that the data points for each genotype control cluster along the expected axis of the plot.

4. Select the cluster at the bottom-left corner of the plot, then confirm that only the negative control wells are selected in the **Plate Layout** or **Well Table**.

   Samples can unexpectedly cluster with the negative controls for one of the following reasons.

   - Samples contain no DNA.
   - Samples contain PCR inhibitors.
   - Samples are homozygous for a sequence deletion.

5. Review the other clusters in the plot.
   a. Click–drag a box around a cluster to select the associated wells.
   b. Confirm that the expected wells are selected in the **Plate Layout** or **Well Table**.

6. Look for outliers outside the three genotype clusters.

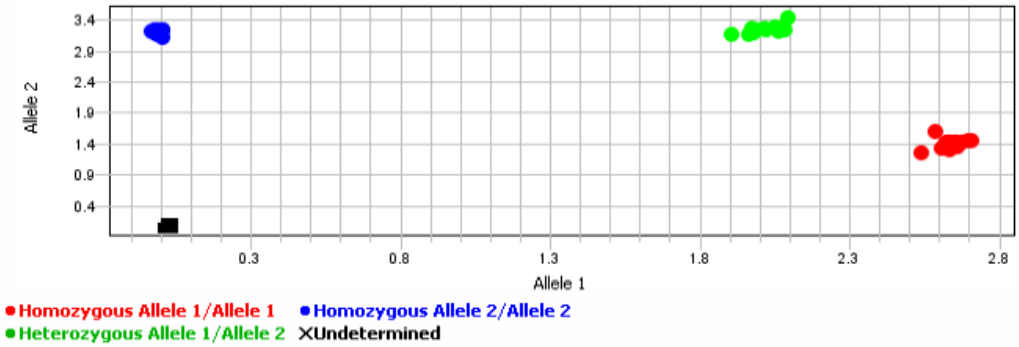

Figure 13 Example Allelic Discrimination Plot

To confirm results, retest outliers and samples with no amplification (cluster with negative controls).

Perform manual calls

You can perform manual calls in the **Results** tab.

1. In the **Results** tab, select **Allelic Discrimination Plot** from the dropdown list.
2. If the data are not analyzed, click **Analyze**.
3. (*For multiple assays only*) Click , then select a SNP assay from the dropdown list.
4. In the **Allelic Discrimination Plot**, use the lasso tool to select the samples to be manually called.
5. Click , then select the allele call from the **Apply Call** dropdown list.

6. Click **Analyze**.

IMPORTANT! To maintain manual calls after reanalysis, select ⚙ **Analysis Settings** ▸ **Call Settings**, then deselect **Default Settings** and select **Keep Manual Calls from Previous Analysis**.

Note: To remove manual calls, select ⚙ **Analysis Settings** ▸ **Call Settings**, deselect **Keep Manual Calls from Previous Analysis**, then reanalyze.

Call settings overview (genotyping)

In the **Results** tab, select ⚙ **Analysis Settings** ▸ **Call Settings** to edit the following settings:

- Data analysis settings
- Default call settings for SNP assays without custom call settings
- Custom call settings for individual SNP assays

Table 17 Options for data analysis settings (genotyping experiments)

Data analysis setting	Description
Analyze Data from Post-PCR Read Only	Only post-PCR read data is used to determine calls.
Analyze Data from Pre-PCR Read and Post-PCR Read[1]	The pre-PCR read is subtracted from the post-PCR read to determine calls.
Analyze Real-Time Rn Data[2,3]	The normalized reporter data (Rn) from the user-selected cycle of the cycling stage is used to determine calls.
Analyze Real-Time Rn - Median (Rna to Rnb)[2,4,3]	A *quick baseline-subtracted Rn* from the user-selected cycle of the cycling stage is used to determine calls. The quick baseline-subtracted Rn is the Rn minus the median value of the baseline region. The median subtraction provides improved data accuracy.
Analyze Real-Time dRn Data[2,3]	The regular ΔRn (dRn) from the user-selected cycle of the cycling stage is used to determine calls. The ΔRn is calculated by subtracting the best-fit line through the baseline region. This method is better if the baselines are not flat.

[1] The run method must include a pre-read stage.
[2] Data collection must be on during the PCR stage.
[3] Analysis is not restricted to the last cycle; adjust the analysis cycle using the **Reveal Traces** feature while viewing the Allelic Discrimination Plot.
[4] Rna to Rnb refers to all the cycles from the Start Cycle Number to the End Cycle Number of the baseline region.

Table 18 Options for call settings (genotyping experiments)

Call setting	Description
Autocaller Enabled	The autocaller algorithm is used to make genotype calls.
Keep Manual Calls from Previous Analysis	If autocaller is enabled, maintains manual calls after reanalysis.
Quality Value	The Quality Value is a proprietary estimation of the likelihood that a genotyping call is correct (associated with the correct cluster). If the Quality Value is less than the setting, the call is undetermined.

For step-by-step instructions for adjusting the call settings, see the desktop software Help.

7 Set up, run, and review presence/absence experiments

- Presence/absence experiments 68
- Set up the presence/absence experiment in the software 69
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- Review results 71

Presence/absence experiments

Overview

Use presence/absence experiments to determine the presence or absence of a target nucleic acid sequence in a sample.

The software calls the target present or absent based on an algorithmically determined call threshold. (The call threshold is different from the C_t threshold; the C_t threshold is not used to make calls.)

Reaction types

Presence/absence reaction types depend on whether the experiment is set up with or without an internal positive control (IPC).

- **Presence/absence experiments with IPC (recommended)** are multiplex assays for the target of interest and the IPC target. The IPC is used to confirm that a negative result for the target of interest is not caused by a failed PCR.

Table 19 Reaction types for presence/absence experiments with IPC

Reaction type (task)	Sample description
Unknown	Test sample *and* IPC template
Negative control	Water or buffer *and* IPC template
No amplification control (NAC; blocked IPC)[1]	Water or buffer plus a blocking agent *and* IPC template; amplification prevented by blocking agent

[1] Minimum of two replicates is required for this control.

- **Presence/absence experiments without IPC** are singleplex reactions.

  Table 20 Reaction types for presence/absence experiments without IPC

Reaction type (task)	Sample description
Unknown	Test sample
Negative control	Water or buffer

The software makes calls for individual wells. Running three or more replicates of each reaction can help identify outlier wells that may be present.

Compatible PCR options

Table 21 PCR options for presence/absence experiments

Single- or multiplex PCR	PCR or RT-PCR[1]	Detection chemistry
Singleplex (without IPC) Multiplex (with IPC)	PCR 1-step RT-PCR 2-step RT-PCR	TaqMan®

[1] RT-PCR: reverse transcription-PCR

Presence/absence calls are based on end-point data (data collected after the PCR stage).

- The data collected is the normalized intensity of the reporter dye, or Rn.
- If end-point experiments include pre-PCR data points, the software calculates the delta Rn (ΔRn) value according to the following formula:
  $\Delta Rn = Rn_{(post\text{-}PCR\ read)} - Rn_{(pre\text{-}PCR\ read)}$, where Rn = normalized readings.

We recommend collecting real-time amplification data during the PCR stage, for troubleshooting purposes.

Set up the presence/absence experiment in the software

1. In the **Home** screen, create or open a template.
   - In the **New Experiment** pane, click **Create New Experiment** to create a new template.
   - In the **Open Existing Experiment** pane, click **Open** to select and open an existing template.
2. In the **Properties** tab, enter the template information.
3. In the **Method** tab, adjust the reaction volume.
   For most experiments, the default run method is appropriate.
4. In the **Plate** tab (**Quick Setup**), assign plate attributes.
   a. In the **Plate Attributes** pane, select the **Passive Reference** from the dropdown list.
5. In the **Plate** tab (**Quick Setup**), define and assign well attributes.
   a. Select wells in the **Plate Layout** or the **Well Table**.

b. Assign samples and targets to selected wells.
   - Enter new sample and target names in the text fields.
   - Select previously defined samples and targets from the dropdown lists.

   Note: New sample or target names entered in the **Quick Setup** subtab are automatically populated with default values for **Reporter** (FAM) and **Quencher** (NFQ-MGB) and assigned a task (U **Unknown**). Edit these values in the **Advanced Setup** subtab.

6. (*Optional*) In the **Plate** tab (**Advanced Setup**), assign tasks.
   a. Select wells in the **Plate Layout** or the **Well Table**.
   b. In the **Targets** table, select the checkbox of a target, then select a task from the dropdown list.

Reaction type	Target	Task
Unknown (test sample)	Target of interest	U
	IPC	I
Negative control	Target of interest	N
	IPC	I
NAC (blocked IPC)	Target of interest	N
	IPC	I

Set up and run the PCR reactions

1. Assemble the PCR reactions. Follow the manufacturer's instructions for the reagents and follow the plate layout set up in the software (see “Prepare reactions“ on page 21).
2. In the desktop software, open the appropriate template (EDT file).
3. Load the reaction plate into the instrument and start the run (see “Start and monitor a run“ on page 22).

Review results

Workflow: Review presence/absence experiments

Assess the Presence/Absence Plot (page 72)

▼

(Optional) View the Amplification Plot (page 30)

▼

Review data for outliers and *(optional)* omit wells (page 33)

▼

(Optional) View the Multicomponent Plot to review the dye signal profile (page 36)

▼

(Optional) View the Raw Data Plot to review the signal profile (page 37)

▼

(Optional) Review flags in the QC Summary (page 38)

▼

(Optional) Configure the analysis settings (page 73, page 100)

IMPORTANT! If you omit wells or configure the analysis settings, click **Analyze** to reanalyze the data.

Presence/Absence Plot overview

The Presence/Absence Plot displays the intensity of the fluorescence for each well.

Review the Presence/Absence Plot to confirm that amplification in the control wells is as expected and to review the calls for the unknown samples.

Table 22 Expected results for control reactions

Reaction type	Target	Result	Call
Negative control	IPC	Amplification	IPC Succeeded
	Target of interest[1]	No amplification	Negative control
NAC (blocked IPC)	IPC[2]	No amplification	Blocked IPC Control
	Target of interest	No amplification	Negative control

[1] The target threshold is calculated from the negative control reactions.
[2] The IPC threshold is calculated from the NAC reactions.

Table 23 Criteria for calls in unknown reactions

Target signal	IPC Signal	Call
Above the target threshold	Above *or* below the IPC threshold	Presence
Below the target threshold	Above the IPC threshold	Absence
Below the target threshold	Below the IPC threshold	Unconfirmed

View and assess the Presence/Absence Plot

You can view and assess the **Presence/Absence Plot** in the **Results** tab.

If no data are displayed in the **Results** tab, click **Analyze**.

1. In the **Results** tab, select **Presence/Absence Plot** from the dropdown list.
2. Click to configure the plot, making the following selections:
   - **Target Reporter**: target defined for the target of interest
   - **Control Reporter**: target defined for the IPC
   - For the initial review of the **Presence/Absence Plot**, select the following options:
     - **Show Calls**: **All Calls**
     - **Show IPC**
     - **Show Controls**

   The **Presence/Absence Plot** is displayed for data points selected in the plot settings. The data points for selected wells in the **Plate Layout** or **Well Table** are highlighted in the plot (see Figure 14).
3. Confirm that amplification in the negative and blocked IPC control wells is as expected. Use one of the following options:
   - Select control wells in the **Plate Layout** or **Well Table**, then confirm the location of the data points in the **Presence/Absence Plot**.
   - In the **Well Table**, select **Group By** ▸ **Task**, then examine the wells with the Blocked IPC ( ) and NTC ( ) tasks. The C_q (C_t or C_{rt}) values should be Undetermined.
   - View the amplification plots for the negative controls (see Figure 15 and "Optimize display of negative controls in the Amplification Plot" on page 34).
4. In the **Presence/Absence Plot**, view the signal intensity and calls for the unknown samples.

   Use the plot settings (click) to filter out the IPC results and control wells, or to select only one type of call.

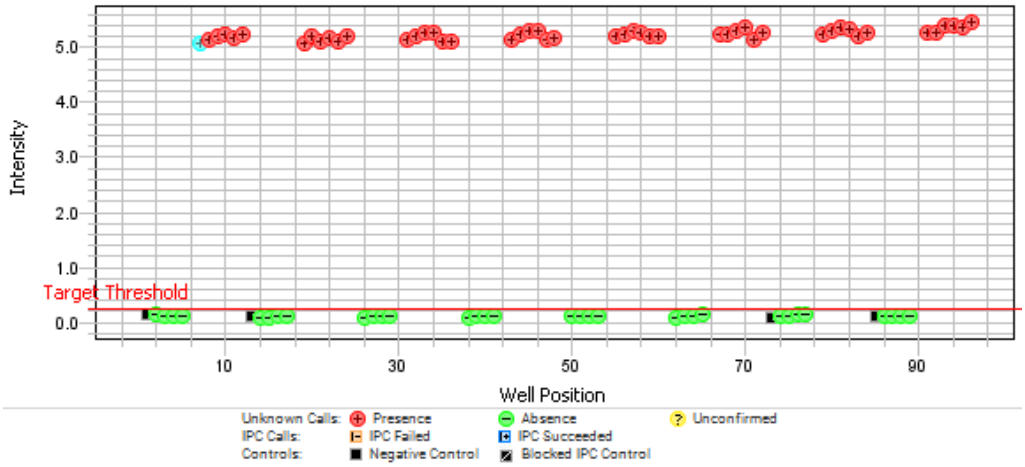

Figure 14 Example Presence/Absence Plot

The IPC results are not displayed in this example.

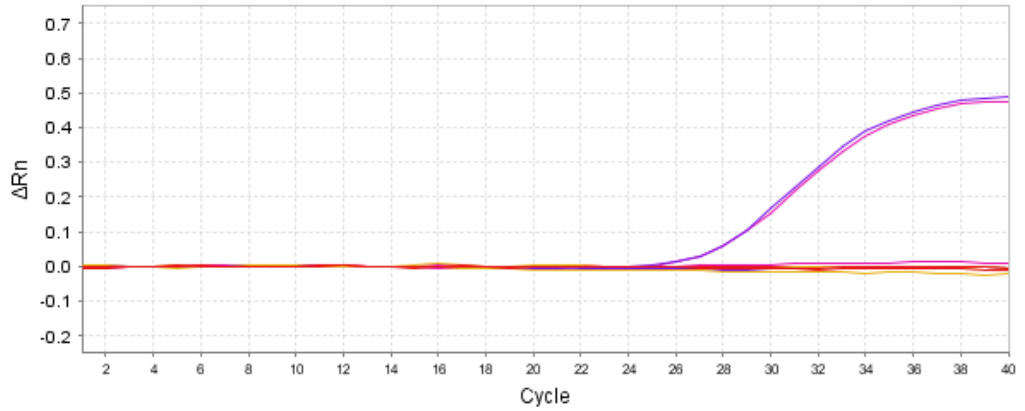

Figure 15 Example Amplification Plot for negative control and blocked IPC

Amplification of the IPC target (blue lines) is seen in the negative control wells but not the blocked IPC (NAC) wells. No amplification of the target of interest (red lines) is seen in either negative control or blocked IPC wells.

Call settings overview (presence / absence)

Use the **Call Settings** tab to edit the following settings:

- Data analysis settings
- Default call settings for assays without custom call settings
- Custom call settings for individual assays

Table 24 Options for data analysis settings (presence/absence experiments)

Data analysis setting	Description
Analyze Data from Post-PCR Read Only	Only post-PCR read data is used to determine calls.
Analyze Data from Pre-PCR Read and Post-PCR Read	The pre-PCR read is subtracted from the post-PCR read to determine calls.

Table 25 Options for call settings (presence/absence experiments)

Call setting	Description
Confidence Value	What confidence value is used to determine the target and IPC call thresholds. • A lower confidence value or more controls typically results in a lower calculated threshold. • A higher confidence value or fewer controls typically results in a higher calculated threshold.

For step-by-step instructions for adjusting the call settings, see the desktop software Help.

8 Set up, run, and review melt curve experiments

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- Review results 77

Melt curve experiments

Overview

Use melt curve experiments to determine the melting temperature (T_m) of the amplification products of a PCR that used intercalating dyes.

Melting temperature (T_m) is the temperature at which 50% of the DNA is double-stranded and 50% is dissociated into single-stranded DNA. The melt curve of a single amplification product displays a single peak at the product's T_m. Multiple peaks in a melt curve experiment indicate additional amplification products, usually from non-specific amplification or formation of primer-dimers.

In the software, melt curve analysis is included in the default run method for any experiment type that uses intercalating dyes.

1. The software plots a melt curve based on the fluorescence of the dye with respect to change in temperature.
2. Using the melt curve, the software calculates the melting temperature (T_m).

Reaction types

Table 26 Reaction types for melt curve experiments

Reaction type (task)	Sample description
Unknown	Previously run PCR reactions that used intercalating dyes
No-template control (NTC/ Negative Control)	Previously run PCR reactions that used water or buffer **Note:** No DNA should be present in NTC wells.

Set up a melt curve experiment in the software

1. In the **Home** screen, create or open a template.
   - In the **New Experiment** pane, click **Create New Experiment** to create a new template.
   - In the **Open Existing Experiment** pane, click **Open** to select and open an existing template.
2. In the **Properties** tab, enter the template information.
3. In the **Method** tab, adjust the reaction volume.
   (*Optional*) Edit the ramp increment in the melt curve (see "Edit the ramp increment for the melt curve dissociation step" on page 91).
4. In the **Plate** tab (**Quick Setup**), assign plate attributes.
   a. In the **Plate Attributes** pane, select the **Passive Reference** from the dropdown list.
5. In the **Plate** tab (**Quick Setup**), define and assign well attributes.
   a. Select wells in the **Plate Layout** or the **Well Table**.
   b. Assign samples and targets to selected wells.
      - Enter new sample and target names in the text fields.
      - Select previously defined samples and targets from the dropdown lists.

      Note: New sample or target names entered in the **Quick Setup** subtab are automatically populated with default values for **Reporter** (FAM) and **Quencher** (NFQ-MGB) and assigned a task (U **Unknown**). Edit these values in the **Advanced Setup** subtab.
6. (*Optional*) In the **Plate** tab (**Advanced Setup**), assign tasks.
   a. Select wells in the **Plate Layout** or the **Well Table**.
   b. In the **Targets** table, select the checkbox of a target, then select a task from the dropdown list.

Reaction type	Task
Unknown (test sample)	U
No-template control	N

Set up melt curve reactions

Melt curve experiments are performed using previously amplified PCR products, usually at the end of the PCR run method. You can also use a plate from an intercalating dyes-based PCR run on another instrument.

Review results

Workflow: Review melt curve experiments

Assess the Melt Curve Plot (page 78)

▼

(Optional) View the Multicomponent Plot to review the dye signal profile (page 36)

▼

(Optional) View the Raw Data Plot to review the signal profile (page 37)

▼

(Optional) Review flags in the QC Summary (page 38)

▼

(Optional) Configure the analysis settings (page 78, page 100)

IMPORTANT! If you omit wells or configure the analysis settings, click **Analyze** to reanalyze the data.

Melt Curve Plot overview

The Melt Curve Plot displays the melt curve of the amplification products in the selected wells.

Review the Melt Curve Plot to confirm that the amplification products in a well display a single melting temperature (T_m). Multiple peaks in a melt curve indicate non-specific amplification or primer-dimer formation.

Table 27 Melt Curve plots

Plot	Description
Derivative Reporter vs. Temperature	Displays the derivative reporter signal in the y-axis as a function of temperature. The peaks in the plot indicate significant decrease in SYBR™ Green signal, and therefore the T_m of the amplified products. Use this plot to confirm a single T_m of the amplification products.
Normalized Reporter vs. Temperature	Displays the fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference, as a function of temperature. You can use this plot to check the quality of the fluorescence data.

View and assess the Melt Curve Plot

You can view and assess the **Melt Curve Plot** in the **Results** tab.

If no data are displayed in the **Results** tab, click **Analyze**.

1. In the **Results** tab, select **Melt Curve Plot** from the dropdown list.
2. Click ◉ to configure the plot, making the following selections:
   - **Plot Type**: **Derivative Reporter**
   - **Color**: **Sample**, **Target**, or **Well**
   - **Target**: **All** or a target of interest
   - (*For custom experiments with more than one Melt Curve stage*) Select the Melt Curve stage to view.

   The **Melt Curve Plot** is displayed for the selected wells of the selected stage.
3. Review the plot for evidence of unexpected multiple peaks, which can indicate non-specific amplification or formation of primer-dimers.
4. Review the ≣ **Well Table** for the calculated T_m in each well.

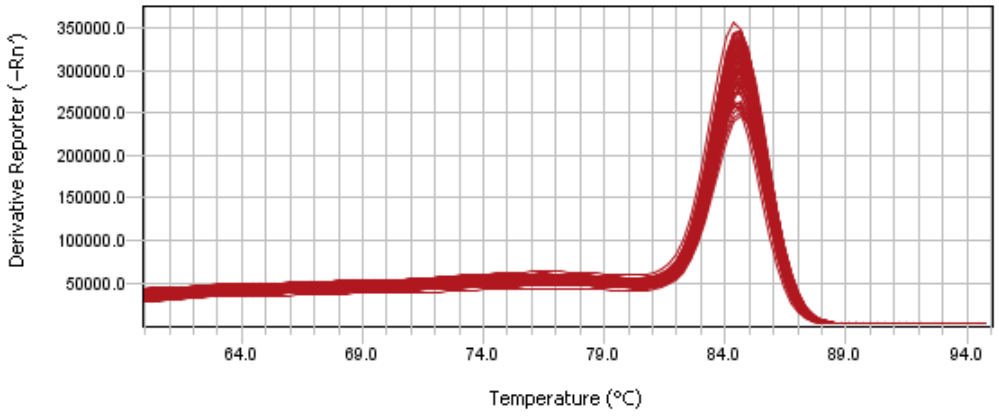

Figure 16 Example **Melt Curve Plot**

Melt curve settings overview

Use the **Melt Curve Settings** tab to enable or disable the Multi-Peak Calling function and adjust the detection levels for additional peaks, if needed.

- Enable or disable **Multi-Peak Calling**.

Multi-Peak Calling	Description
Enabled	– More than one PCR product is expected to amplify. – T_m will be determined for more than one peak.
Disabled	– A single PCR product is expected to amplify. – T_m will be determined for one peak.

- (*For multi-peak calling only*) Adjust the detection levels for additional peaks.

Option	Description
Peak level relative to the dominant peak (%)	Specify a fractional-level value as the additional peak detection threshold. The detected peaks are measured relative to the height of the tallest peak, which has a perfect fractional level of 100%. The default value is 10%. For example, set a fractional-level detection threshold value at 40, then peaks above 40% of the tallest peak are reported, and peaks below 40% are regarded as noise.
Peak Calling Threshold	Specify an absolute fluorescence-level value as the peak calling threshold. The absolute fluorescence is measured on the derivative reporter (-dRn') axis. Only peaks that appear above the peak calling threshold will be detected. For example, set a fluorescence-level value at 90,000, then peaks with fluorescence above 90,000 are reported, and peaks below 90,000 are regarded as noise.

For step-by-step instructions for adjusting the melt curve settings, see the desktop software Help.

A Instrument overview

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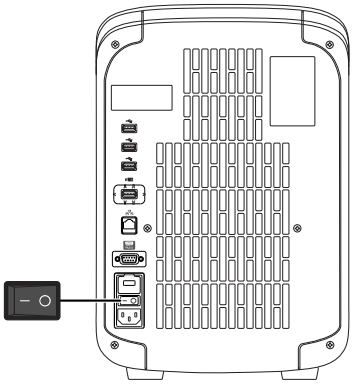
- View run history and delete or transfer files from the instrument 80
- Load and unload the plate in the instrument 81

Power on the instrument

1. Touch anywhere on the touchscreen to determine if the instrument is in sleep mode. If the home screen is displayed, the instrument is already powered on.
2. If the home screen does not display, power on the instrument by pressing the switch on the rear panel.



If left unattended (for about two hours), the instrument automatically enters sleep mode (enabled by default) to conserve power.

Note: To customize the sleep mode setting, touch ⚙ **Settings ▸ Instrument Settings ▸ Sleep Mode**.

View run history and delete or transfer files from the instrument

In the home screen, touch ⚙ **Settings ▸ Run History**.

- Touch an individual run record to view its details, then complete one of the following actions:
  - Touch **Delete** to delete the run record.
  - Touch **Transfer** to export the run data.
- Touch **Manage** to select multiple run records for simultaneous viewing, deletion, or transfer.

Note:
- Guests (users not signed-in) can only view guest run records.
- Users signed into their instrument profiles can also view their own run records.
- Administrators can view all run records.

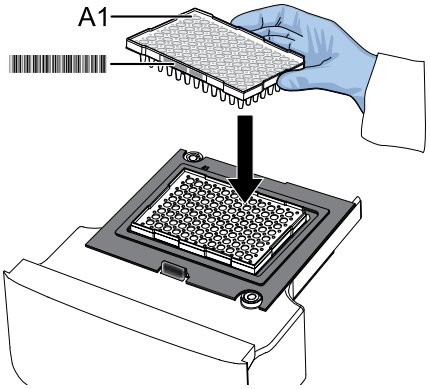
Note: If the connection between the instrument and the desktop software is interrupted during the run, the instrument still completes the run. However, the run data (EDS file) must be transferred from the instrument to the desktop software using a USB drive or a network drive.

Load and unload the plate in the instrument

Load and unload a plate in the QuantStudio™ 1 Real-Time PCR Instrument

CAUTION! Use flat caps for 0.2-mL tubes. Rounded caps can damage the heated cover.

1. Load the plate.
   a. Open the instrument drawer.
   b. Load the plate onto the plate adapter so that the following criteria are met.
      - Well A1 of the plate is in the top-left corner of the plate adapter.
      - The barcode faces the front of the instrument.



   Note: Do not remove the black plate adapter before loading a plate or tube strips. If used, tube strips can fit loosely in the adapter, but the heated cover will apply the appropriate pressure to seat the tube strips securely in the adapter.

   c. Close the instrument drawer.

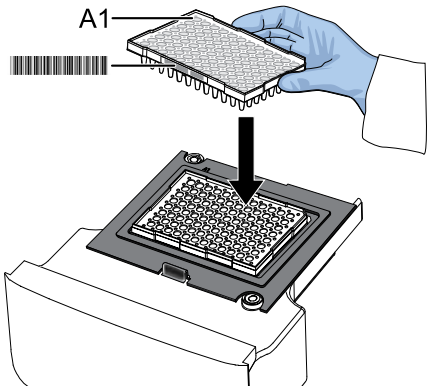
2. When the run ends, unload the plate.
   a. Open the instrument drawer.
   b. Remove the plate.
   c. Close the instrument drawer.

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

Load and unload a plate in the QuantStudio™ 3 Real-Time PCR Instrument or QuantStudio™ 5 Real-Time PCR Instrument

CAUTION! Use flat caps for 0.2-mL tubes and 0.1-mL tubes. Rounded caps can damage the heated cover.

1. Load the plate.
   a. Touch ⏏ to eject the instrument drawer.
   b. Load the plate onto the plate adaptor so that the following criteria are met.
      - Position well A1 of the plate in the top-left corner of the plate adapter.
      - Ensure that the barcode faces the front of the instrument.



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

Note: (*For 96-well 0.2-mL blocks only*) Do not remove the black plate adapter before loading a plate or tube strips. If used, tube strips can fit loosely in the adapter, but the heated cover will apply the appropriate pressure to seat the tube strips securely in the adapter.

Note: The 384-well and 96-well Fast (0.1-mL) block configurations do not require a plate adapter.

   c. Touch ⏏ to close the instrument drawer.

2. When the run ends, unload the plate.
   a. Touch ⏏ to eject the instrument drawer.
   b. Remove the plate.
   c. Touch ⏏ to close the instrument drawer.

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 plate, contact Support.

B Alternative procedures to set up a template

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Set up a custom experiment

Custom experiments overview

Custom experiment setup is required for assays that use multiple PCR stages, such as TaqMan® Mutation Detection Assays. A custom experiment also allows flexibility for secondary analysis.

The default settings for custom experiments are that of a standard curve experiment, but most settings are editable.

Table 28 Default settings in custom experiments

Setting	Default
Run method (thermal protocol)	Equivalent to standard curve experiment default
Tasks	Unknown U Negative control N Standard S
C_t settings	Baseline threshold equivalent to standard curve experiment default
Flag settings	QC flags on No automatic omissions
Auto Export	Off

Table 29 Editing options in custom experiments

Setting	Description
Run method (thermal protocol)	• Ramp rates can be edited within software limits. • Data collection can be enabled at any step and during any ramp within a melt stage. • Multiple instances of any type of stage can be added, with exceptions noted. • Any stage can be added at any point in a run method, with exceptions noted. Noted exceptions: • Only one infinite hold (must be added at the end). • Only one pre-PCR read and one post-PCR read stage. If both exist in a run method, the pre-read must be before the post-read. For example, the following order is valid: melt–PCR–Pre-Read–Melt–PCR.
Analysis settings	Editable
Flags	Editable
Optical filters	Editable

Set up a custom experiment in the software

1. In the **Home** screen, create or open a template.
   - In the **New Experiment** pane, click **Create New Experiment** to create a new template.
   - In the **Open Existing Experiment** pane, click **Open** to select and open an existing template.
2. In the **Properties** tab, enter the template information.
3. In the **Method** tab, edit the default run method according to the experiment requirements.
4. In the **Plate** tab (**Quick Setup**), assign plate attributes.
   a. In the **Plate Attributes** pane, select the **Passive Reference** from the dropdown list.
5. In the **Plate** tab (**Quick Setup**), define and assign well attributes.
   a. Select wells in the **Plate Layout** or the **Well Table**.
   b. Assign samples and targets to selected wells.
      - Enter new sample and target names in the text fields.
      - Select previously defined samples and targets from the dropdown lists.

      Note: New sample or target names entered in the **Quick Setup** subtab are automatically populated with default values for **Reporter** (FAM) and **Quencher** (NFQ-MGB) and assigned a task (**Unknown**). Edit these values in the **Advanced Setup** subtab.

6. (*Optional*) In the **Plate** tab (**Advanced Setup**), assign tasks.
   a. Select wells in the **Plate Layout** or the **Well Table**.
   b. In the **Targets** table, select the checkbox of a target, then select a task from the dropdown list.
7. (*Optional*) In the **Plate** tab (**Advanced Setup**), define and assign biological replicate groups (see "Define and assign biological replicate groups" on page 95).

Assign samples using a sample definition file

About sample definition files

Import sample information from a sample definition file to include in the plate setup. A sample definition file is a CSV file or a TXT file that contains the following setup information:

- Sample name
- *(Optional)* Custom sample properties

Create a sample definition file

1. In a spreadsheet program, create the following column headers:
   - Well
   - Sample Name
   - (*Optional*) Column header names for up to 32 user-defined custom fields (for example, **Custom 1**, **Custom 2**, etc.)
2. Enter the well number and sample name in the appropriate columns.
3. (*Optional*) Enter the custom properties for the sample.
4. Save the file as a tab-delimited text file (TXT) or a comma-separated values file (CSV).

Import sample information from a sample definition file

Example setup files are provided with the software in:

`<drive>:\Program Files (x86)\Applied Biosystems\QuantStudio Design and Analysis Software\examples\User Sample Files`,

where <*drive*> is the drive on which the software is installed.

1. In an open experiment, select **File** ▸ **Import Plate Setup**.
2. Click **Browse**, navigate to a sample definition text file, then click **Select**.
3. Click **Apply**.
4. If the experiment already contains plate setup information, the software prompts for the replacement of the plate setup with the data from the file. Click **Yes** to replace the plate setup information.

The samples appear in the **Samples** table for the experiment. All samples and well assignments in the experiment are replaced with those in the file. If defined, the custom sample properties also appear in the **Well Table** of the **Results** tab and in

the **Plate Layout** tooltips in both the **Plate** and **Results** tabs. The custom fields can be exported with the results data.

Note: To modify custom sample properties information, edit the custom fields in the sample definition file and import the file again. All of the sample information in the experiment is replaced with the information in the new file.

Assign samples and targets using plate setup files

About plate setup files

Plate setup files contain setup information such as the well number, sample name, sample color, target name, dyes, and other reaction plate contents.

Plate setup files can be exported from previously run experiments. For instructions on exporting an experiment, see "Export experiments or results" on page 40.

Import plate setup data

Import the plate setup for a new experiment from an exported file with one of the following formats:

- EDS – EDS file format
- EDT – user-created and system templates files format
- TXT – text format
- XML – XML format
- CSV – comma separated values format
- SDT – Sequence Detection System (SDS) template files format
- SDS – 7900 v2.4 format

Note: Import plate setup information from a 96-well plate into a 384-well plate, provided that the sample file is a TXT file.

IMPORTANT! The file must contain only plate setup data and it must match the experiment type.

---

1. In the **Plate** tab, select **File ▸ Import Plate Setup**.
2. Click **Browse**, navigate to and select the file to import, then click **Select**.
   Example setup files are provided with the software in:
   <*drive*>`:\Program Files (x86)\Applied Biosystems\QuantStudio Design and Analysis Software\examples\User Sample Files`,
   where <*drive*> is the drive on which the software is installed.
3. Click **Apply**.
   The setup data from the selected file is imported into the open experiment.

Assign targets, samples, and biological replicate groups from an XLS file

For wells with single targets, you can paste assignment information from an XLS file into the plate layout of the desktop software.

An example copy and paste file is provided with the software in:

<*drive*>:\Program Files (x86)\Applied Biosystems\QuantStudio Design and Analysis Software\examples\User Sample Files.

where <*drive*> is the drive on which the software is installed.

1. In the custom properties tab of the example Microsoft™ Excel™ file, ensure that the **Well** column is sorted in order 1 through 96, then select the **Well** column and the **Sample Name** column, *including* headers.
2. In the **Plate** tab of the software, click ≣ **Well Table**, then ensure that the well numbers are in order from 1 through 96.
3. In the ≣ **Well Table**, hover the mouse in the first cell underneath the **Sample** header (adjacent to A1), right-click, then select either **Paste** or **Paste only samples**.
   Any of the columns not copied are treated as NULL values for those columns.

Create new EDT files using existing EDT and EDS files

About experiment templates

Use templates to create experiments with the same parameters or with pre-existing settings. Experiments can be saved as unlocked or locked (password-protected) templates.

You can save the following information in an experiment template (EDT) file:

- Plate setup information (defined sample and targets or SNP assays, plate assignment of samples and targets or SNP assays)
- Reagent information
- Run method (thermal protocol)
- Analysis settings

Example templates are provided with the software in:

<*drive*>:\Program Files (x86)\Applied Biosystems\QuantStudio Design & Analysis Software\templates,

where <*drive*> is the drive on which the software is installed.

C

Detailed procedures to create or edit a method

- Adjust method parameters 88
- Set up advanced temperature zones (Auto Delta and VeriFlex™ Zones) 89
- Add or adjust a pause step 90
- Select optical filters 90
- Edit the ramp increment for the melt curve dissociation step 91

Adjust method parameters

For an overview of the method as it is graphically represented, see "Method elements" on page 18.

- In the **Method** tab, click a method parameter field to edit the following information:
  - Reaction volume
  - Temperature ramp rate
  - Step temperature
  - Step hold time
  - Number of cycles
- Click-drag ≡ to increase or decrease a step temperature.
- Click 📷 to switch data collection on or off at each step.

  Data Collection On enables analysis of data that is collected throughout the PCR, for real-time analysis and troubleshooting.
- *(QuantStudio™ 3 Real-Time PCR System and QuantStudio™ 5 Real-Time PCR System only)* Click ⚙ to configure settings for **Auto Delta** or VeriFlex™ Zones for individual steps (see "Set up advanced temperature zones (Auto Delta and VeriFlex™ Zones)" on page 89).

  Note: In melt curve stages, **Advanced Settings** are not applicable.

  An A or V is displayed in the PCR stage when **Auto Delta** or VeriFlex™, respectively, is enabled.
- Click ⏸ to configure pause settings.

- Adjust the heated cover temperature via the instrument settings (see *QuantStudio™ 1 Real-Time PCR System Installation, Use, and Maintenance Guide* (Pub. No. MAN0017853) or *QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide* (Pub. No. MAN0010407)).

- See the Help to learn more about adjusting the following parameters:
  - Adding or subtracting a stage
  - Adding or subtracting a step from a stage
  - Configuring optical filter settings

Set up advanced temperature zones (Auto Delta and VeriFlex™ Zones)

(QuantStudio™ 3 Real-Time PCR System and QuantStudio™ 5 Real-Time PCR System only)
Configure settings for **Auto Delta** and **VeriFlex™ Zones**.

- **Auto Delta**—Incremental increase or decrease of a cycle's temperature or hold time for a step in a cycling stage (not applicable for **Hold** or **Infinite Hold** stages).
- **VeriFlex™ Zones**—Independent temperature zones within 5°C of adjacent zones.
  - QuantStudio™ 3 Real-Time PCR Instrument: 3 zones
  - QuantStudio™ 5 Real-Time PCR Instrument: 6 zones

  Note: VeriFlex™ Zones temperature settings are not available for 384-well blocks.

1. In the **Method** tab, click **Advanced Settings** in a step.

   Note: Any changes apply only to the step in which you clicked.

2. Configure either the **VeriFlex™ Zones** or **Auto Delta** for the selected step.
   - Select **VeriFlex™**, then enter a temperature for each zone.

     Note: In the **Plate** tab, the VeriFlex™ Zones display on the plate layout.
     - To view setting details, hover over the **V** in each zone.
     - To hide the display of zones, click **Action** ▸ **Hide VeriFlex™ Zones**.
   - Select **Auto Delta**, then enter a starting cycle, temperature, and time.

3. Click **Save**.

Add or adjust a pause step

1. In the **Method** tab, click ⏸ in the step.
2. Select **Pause**.
3. Enter the cycle after which the pause should occur.
4. Enter a pause temperature between 4°C and 99.9°C.

   CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the plate temperature can reach 100°C. If you want to access the plate during a run pause, enter room temperature as the pause temperature and allow the plate to cool to room temperature before handling.

5. Click outside of the pause dialog box to return to the method.
6. (*Optional*) To remove a pause, click ⏸, then deselect **Pause**.

Select optical filters

The need to edit optical filter settings is rare, and it is intended for advanced or custom uses only.

Use the optical filters settings feature to select a filter set to match the profile of a custom dye.

1. In the **Method** tab, select **Action** ▸ **Optical filter settings**.
   The excitation (x) and emission (m) wavelengths that correspond to each filter are shown on the screen.
2. Select the check boxes to enable or disable filters.
   A **Melt Curve Filter** table is accessible if the method contains a melt curve stage. Otherwise, use the **PCR Filter** table to select optical filters.
3. (*Optional*) Click **Revert to Defaults** to reset filters.
4. Click **Close**.

For information on the dyes read by each filter, see *QuantStudio™ 1 Real-Time PCR System Installation, Use, and Maintenance Guide* (Pub. No. MAN0017853) or *QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide* (Pub. No. MAN0010407)

Edit the ramp increment for the melt curve dissociation step

In the **Method** tab, you can perform the following tasks to edit the ramp increment for the melt cure dissociation step.

- Select the ramp increment method for the dissociation step (located under the graphical representation of the thermal protocol).

Option	Description
Continuous (default)	Continuously increases the temperature by the ramp increment (°C/sec).
Step and Hold	Increases the temperature by the ramp increment (°C), then holds at that temperature for the specified time.
No. of Data Points per Degree	Increases the temperature by the ramp increment (°C) and collects the specified number of data points per degree increased.

- (*For all options*) Edit the temperature ramp increment.
  a. Click the ramp increment element in the **Dissociation** step.
  b. Enter a value or use the up/down arrows (default is 0.15°C/s).
- (*Step and Hold only*) Edit the hold time after each temperature increase.
  a. Click the time field next to **Step and Hold**.
  b. Enter a value or use the up/down arrows (default is 5 seconds).
- (*No. of Data Points per Degree only*) Edit the number of data points to be collected with each degree increase.
  a. Click the number of data points element in the **Dissociation** step.
  b. Enter a value or use the up/down arrows (default is 10 data points).

Detailed procedures to set up plate / well details and libraries

- Assign well attributes (Quick Setup subtab) 92
- Define and assign well attributes (Advanced Setup subtab) 93
- Assign a task to wells 93
- Define and set up standard dilutions 94
- Define and assign biological replicate groups 95
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Assign well attributes (Quick Setup subtab)

In the **Quick Setup** subtab, assign well attributes by direct entry into text fields or by selecting user-defined samples and targets or SNP assays from dropdown lists.

1. In the **Plate** tab, click **Quick Setup**.
2. Select plate wells in the **Plate Layout** or the **Well Table**.
3. Assign the well attributes for the selected wells.
   - Into the text fields, enter the names of the new sample and the new target or SNP assay.
   - From the dropdown lists, select a user-defined sample and target or SNP assay.

     For more information about defining or importing samples and targets or SNP assays, see "Define and assign well attributes (Advanced Setup subtab)".

   Note: In the **Advanced Setup** subtab, change the default selections for the reporter and quencher dyes and for tasks (see "Assign a task to wells" on page 93).
4. (*Optional*) Enter comments for the selected wells.

Define and assign well attributes (Advanced Setup subtab)

In the **Advanced Setup** subtab, define or import samples and targets or SNP assays, then assign well attributes.

1. In the **Plate** tab, click **Advanced Setup**.
2. In the **Samples** table, define samples (see "Define samples in the Samples table" on page 97).
3. In the **Targets** or **SNP Assays** table, define targets or SNP assays, then select detection tasks.
   a. Define targets or SNP assays (see "Define targets in the Targets table" on page 98 or "Define SNP assays in the SNP Assays table" on page 99, respectively).
   b. Select a detection task from the **Task** column dropdown list (see "Assign a task to wells" on page 93).
4. Assign well attributes.
   a. Select plate wells in the **Plate Layout** or the **Well Table** (see "Select plate wells" on page 19).
   b. Select the checkbox of a defined sample.
   c. Select the checkbox of a defined target or SNP assay.

Assign a task to wells

1. In the **Plate** tab, click **Advanced Setup**.
2. Select plate wells in the **Plate Layout** or the **Well Table** (see "Select plate wells" on page 19).
3. In the **Targets** or **SNPs** table, select the check box of a target or SNP assay.
4. Select a detection task from the **Task** column dropdown list.

Detection tasks for targets and SNP assays

Task	Description
U Unknown (default)	The well contains test samples with unknown genotype.
N Negative Control / No template control	The well contains water or buffer instead of sample.
S Standard [1]	The well contains samples with known standard quantities. **Note:** For a standard detection task, enter the standard quantity in the quantity column.

Task	Description
Positive Control Allele 1 / Allele 1 [2]	The well contains samples homozygous for allele 1.
Positive Control Allele 2 / Allele 2 [2]	The well contains samples homozygous for allele 2.
Positive Control Allele 1 / Allele 2 [2]	The well contains samples heterozygous for allele 1 and 2.
Internal positive control [3]	The PCR reaction contains a short synthetic DNA template to distinguish between true negative results (that is, the target is absent in the samples) and negative results caused by PCR inhibitors, incorrect assay setup, or reagent or instrument failure.
Blocked IPC [3]	The well contains an IPC blocking agent, which blocks amplification of the IPC.
NAC – No amplification control [3]	The PCR reaction contains an IPC blocking agent instead of sample. No amplification should occur in negative control-blocked IPC wells because the reaction contains no sample and amplification of the IPC is blocked.

[1] For standard curve and relative standard curve experiments only.
[2] For genotyping experiments only.
[3] For presence/absence experiments only.

Define and set up standard dilutions

Note: This information is applicable for standard curve and relative standard curve experiments only.

1. In the **Plate** tab, select **Action ▸ Define and Set Up Standards**.
2. Select **Singleplex** or **Multiplex** from the **Model** dropdown list.
3. (*Optional*) Select the target from the dropdown list.
4. Enter the parameters for the dilution series.
   - **Number of dilution points**—5 recommended
   - **Number of replicates**—3 recommended
   - **Starting Quantity**—The highest or lowest standard quantity, without units.

     Note: The quantity can be expressed as copies, copies/µL, ng/µL, or as relative dilutions.

     Note: Use **E** to indicate the exponent number using scientific notation. For example, to indicate 1.23×10^4, enter 1.23E4.
   - **Serial Factor**

     Note: The serial factor calculates quantities for all standard curve points.

     – Starting quantity is the highest value—Select 1:10 to 1:2.
     – Starting quantity is the lowest value—Select 2× to 10×.

   Note: The **Standard Curve Preview** y-axis values are calculated from the starting quantity and serial factor. Actual results may differ from the preview.

5. Select and arrange the wells to use for the standards.
   - Select **Automatically Select Wells for Me**.
   - Select **Let Me Select Wells**, then select wells using the displayed plate layout.
6. Select to arrange the standards in **Columns** or **Rows**.
7. (*Optional*) Click **Reset** to revert to default values.
8. Click **Apply**, then click **Close** to return to the **Plate** tab.

Assign the standard dilutions manually

Note: Applicable for standard curve and relative standard curve experiments only.

1. In the **Plate** tab, select wells in the **Plate Layout** or **Well Table**.
2. Select the check box for the target, select S from the **Task** dropdown list, then enter a quantity.
3. Repeat to complete the standard dilution series.

Define and assign biological replicate groups

Biological Replicate Groups can be used in standard curve, relative standard curve, comparative C_t, and custom experiments.

1. In the **Plate** tab, click **Advanced Setup**.
2. Define **Biological Replicate Groups**.
   a. In the **Biological Replicate Groups** table, click + **Add**.
   b. (*Optional*) Click a cell to edit color, name, or comments.
   c. (*Optional*) Click ✕ to delete a biological replicate group from the table.
3. Assign **Biological Replicate Groups**.
   a. Select plate wells in the **Plate Layout** or the **Well Table** (see "Select plate wells" on page 19).
   b. In the **Biological Replicate Groups** table, select the check box of a biological replicate group.

Sample, target, and SNP assay libraries

Libraries overview

Libraries contain saved information to reuse in future templates.

The following libraries are available in the software:

- Dye Library
- Sample Library
- Target Library
- SNP Assay Library
- Analysis Settings Library

To access the libraries, use the **Tools** menu or the **Plate** tab.

- In the menu bar, select **Tools** ▸ **(Library of choice)**.
- In the **Plate** tab of an open EDT or EDS file, click **Advanced Setup**, then select **Action** ▸ **Import from Library**.

Apply a filter to search a library

You can filter the Sample, SNP assay, Target, and Analysis Settings Libraries.

1. Access a library of interest.
   - In the menu bar, select **Tools** ▸ **(Library of interest)**.
   - In the **Plate** tab of an open EDT or EDS file, click **Advanced Setup**, then select **Action** ▸ **Import from Library**.
2. Select a feature from the first dropdown list. Each column of the table is an available feature.
3. Select a condition from the second dropdown list to define the feature. The conditions will vary by feature.
4. Enter a value or text by which to filter.
5. Click **Apply Filter**.

Define samples in the Samples table

In the **Plate** tab, click **Advanced Setup**, then perform one of the following actions in the **Samples** table.

Option	Action
Manually define a sample	1. Click ⊞ **Add**. 2. Click a cell to edit the attributes for the sample. 3. (*Optional*) Click ➕ in the table header to add a **Custom Attribute** column. a. Click the **Custom Attribute** column header, then edit the header with a new sample attribute. b. Click a **Custom Attribute** cell in the table, then enter the attribute information.
Import samples from a TXT or XLS file	1. Select **Action** ▸ **Import from File**. 2. Navigate to and select a file, then click **Open**.
Import samples from the **Sample Library**	1. Select **Action** ▸ **Import from Library**. 2. (*Optional*) Apply a filter to search for a specific sample (see page 96). 3. Select one or more samples, then click **Add Selected**. **Note:** Shift-click or Ctrl-click to select multiple samples.
Save a sample to the **Sample Library**	Select a sample row, then select **Action** ▸ **Save to Library**.
Delete a sample from the table	Select a sample row, then click ✕.

Define targets in the Targets table

In the **Plate** tab, click **Advanced Setup**, then perform one of the following actions in the **Targets** table.

Option	Action
Manually define a target	1. Click ⊞ **Add**. 2. Click a cell to edit the attributes for the target.
Import targets from a TXT or XML file	1. Select **Action** ▸ **Import from Library**. 2. Click **Import** or **Import AIF**. 3. Navigate to and select a file, then click **Import**. 4. Select one or more targets, then click **Add Selected**. **Note:** Shift-click or Ctrl-click to select multiple targets.
Import targets from the **Target Library**	1. Select **Action** ▸ **Import from Library**. 2. (*Optional*) Apply a filter to search for a specific target (see page 96). 3. Select one or more targets, then click **Add Selected**. **Note:** Shift-click or Ctrl-click to select multiple targets.
Save a target to the **Target Library**	Select a target row, then select **Action** ▸ **Save to Library**.
Delete a target from the table	Select a target row, then click ✕.

Define SNP assays in the SNP Assays table

In the **Plate** tab, click **Advanced Setup**, then perform one of the following actions in the **SNP Assays** table.

Option	Action
Manually define a SNP assay	1. Click ⊞ **Add**. 2. Click a cell to edit the attributes for the SNP assay.
Import SNP assays from a TXT or XML file	1. Select **Action** ▸ **Import from Library**. 2. Click **Import** or **Import AIF**. 3. Navigate to and select a file, then click **Import**. 4. Select one or more SNP assays, then click **Add Selected**. **Note:** Shift-click or Ctrl-click to select multiple SNP assays.
Import SNP assays from the **SNP Assay Library**	1. Select **Action** ▸ **Import from Library**. 2. (*Optional*) Apply a filter to search for a specific SNP assay (see page 96). 3. Select one or more SNP assays, then click **Add Selected**. **Note:** Shift-click or Ctrl-click to select multiple SNP assays.
Save a SNP assay to the **SNP Assay Library**	Select a SNP assay row, then **Action** ▸ **Save to Library**.
Delete a SNP assay from the table	Select a SNP assay row, then click ✕.

Configure analysis settings

- Guidelines for the analysis settings 100
- View and configure the analysis settings 101
- Ct settings overview 101
- Flag settings overview 102
- Advanced settings overview 102

This section describes the analysis settings that apply to all experiment types, unless otherwise noted.

Guidelines for the analysis settings

- We recommend analyzing the experiment with the default analysis settings.
- If the default analysis settings are not suitable for the experiment, modify the settings in the **Analysis Settings** dialog box, then reanalyze the experiment.
- Save modified analysis settings to the **Analysis Settings Library**.

The default analysis settings are different for each experiment type. The analysis settings determine for following parameters.

- How the baseline, threshold, and threshold cycle (C_t) are calculated
- Which flags are enabled
- Other analysis options that are specific to an experiment type

For detailed information about different types of analysis settings, see the following sections.

- "Ct settings overview" on page 101
- "Flag settings overview" on page 102
- "Advanced settings overview" on page 102
- "Standard curve settings overview" on page 47
- "Relative quantification settings overview" on page 59
- "Call settings overview (genotyping)" on page 66
- "Call settings overview (presence / absence)" on page 73
- "Melt curve settings overview" on page 78

View and configure the analysis settings

1. In the **Results** tab, click ⚙ (in top-right corner).
2. View and (*optional*) configure the analysis settings.
   For step-by-step instructions for adjusting analysis settings, see the desktop software Help.
3. Click **Apply**.
4. Click **Analyze** to reanalyze to experiment with the new settings.
5. (*Optional*) To save the settings in the **Analysis Settings Library**, click **Save**.
6. (*Optional*) To return to the default settings, click **Revert**.

C_t settings overview

The default C_t settings are appropriate for most applications. Configuration of the settings is an option for analysis of atypical or unexpected run data.

For step-by-step instructions for adjusting the C_t settings, see the desktop software Help.

Note: The **C_t Settings** feature is not available for experiments without a PCR stage, such as melt curve experiments.

Table 30 C_t Settings

Setting	Description
Data Step Selection	Determines the stage/step combination for C_t analysis (when there is more than one data collection point in the run method).
Algorithm Settings – **Baseline Threshold**	The **Baseline Threshold** Algorithm is used to calculate the C_t values. This algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescence threshold in the exponential region.
Algorithm Settings – **Relative Threshold**	The **Relative Threshold** (C_{rt}) Algorithm is used to calculate the C_{rt} values.
Default C_t Settings	Determines how the **Baseline Threshold** Algorithm is set. The **Default C_t Settings** are used for targets unless they have custom settings. For recommendations on adjusting baseline and threshold settings, see Table 31.
C_t Settings for Target	• **Default Settings** selected—The **Default C_t Settings** are used to calculate the C_t values for the target. • **Default Settings** deselected—The software allows manual setting of the baseline or the threshold. For recommendations for adjusting baseline and threshold settings, see Table 31.

Table 31 Recommendations for manual threshold and baseline settings

Setting	Recommendation
Threshold	Enter a value for the threshold so that the threshold is: • Above the background. • Below the plateau and linear phases of the amplification curve. • Within the exponential phase of the amplification curve.
Baseline	Select the **Start Cycle** and **End Cycle** values so that the baseline ends before significant fluorescence signal is detected.

Flag settings overview

Use the **Flag Settings** to configure the following parameters.

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the software for each experiment type.

For step-by-step instructions for configuring the flag settings, see the desktop software Help.

Advanced settings overview

Use the **Advanced Settings** tab to change baseline settings for individual wells.

For step-by-step instructions for adjusting the advanced settings, see the desktop software Help.

Note: The **Advanced Settings** feature is not available for experiments without a PCR stage, such as melt curve experiments.

Documentation and support

- Related documentation for the QuantStudio™ 1 Real-Time PCR System 103
- Related documentation for the QuantStudio™ 3 Real-Time PCR System and QuantStudio™ 5 Real-Time PCR System 104
- Customer and technical support 104
- Limited product warranty 104

Related documentation for the QuantStudio™ 1 Real-Time PCR System

Document	Publication number
QuantStudio™ 1 Real-Time PCR System Installation, Use, and Maintenance Guide	MAN0017853
QuantStudio™ Design and Analysis Desktop Software Command-Line Application Guide	MAN0010409
QuantStudio™ Design and Analysis Desktop Software User Guide	MAN0010408
QuantStudio™ 1 Real-Time PCR System Site Preparation Guide	MAN0017854

Related documentation for the QuantStudio™ 3 Real-Time PCR System and QuantStudio™ 5 Real-Time PCR System

Document	Publication number
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407
QuantStudio™ Design and Analysis Desktop Software Command-Line Application Guide	MAN0010409
QuantStudio™ Design and Analysis Desktop Software User Guide	MAN0010408
QuantStudio™ 5 Real-Time PCR System SAE Admin Console User Guide	MAN0010410
QuantStudio™ 3 and 5 Real-Time PCR Systems Site Preparation Guide	MAN0010405

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

Limited product warranty

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Glossary

biological replicates	Reactions that contain identical components and volumes, but evaluate separate samples of the same biological source (for example, samples from three different mice of the same strain, or separate extractions of the same cell line or tissue sample). For runs that use biological replicate groups in a gene expression project, the values displayed in the biological replicates lists are calculated by combining the results of the separate biological samples and treating this collection as a single population (that is, as one sample). For ΔC_t computations (normalizing by the endogenous control) in a singleplex experiment, the software averages technical replicates. The averages from the technical replicates are then averaged together to determine the value for that biological replicate.
endogenous control	A gene that is used to normalize template differences and sample-to-sample or run-to-run variation.
endpoint read	See post-PCR read.
post-PCR read	In genotyping and presence/absence experiments, the part of the instrument run that occurs after amplification. In genotyping experiments, fluorescence data collected during the post-PCR read are displayed in the allelic discrimination plot and used to make allele calls. In presence/absence experiments, fluorescence data collected during the post-PCR read are displayed in the presence/absence plot and used to make detection calls. Also called endpoint read.
pre-PCR read	In genotyping and presence/absence experiments, the part of the instrument run that occurs before amplification. The pre-PCR read is optional but recommended. Fluorescence data collected during the pre-PCR read can be used to normalize fluorescence data collected during the post-PCR read.
reference sample	In relative standard curve and comparative C_t ($\Delta\Delta C_t$) experiments, the sample used as the basis for relative quantification results. Also called the calibrator.
target	The nucleic acid sequence that is amplified and detected during PCR.
task	In the software, the type of reaction performed in the well for the target.
technical replicates	Reactions that contain identical components and volumes, and that evaluate the same sample; important for evaluating precision.

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