appliedbiosystems

QuantStudio™ Design and Analysis Software

USER GUIDE

Getting started with design and analysis of experiments in the desktop software v1.2.x

for use with: QuantStudio™ 3 and 5 Real-Time PCR Systems

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About this guide

Revision history

Revision	Date	Description
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.

Scope of the guide

This guide is intended to help users get started with design and analysis of experiments in the QuantStudio™ Design and Analysis desktop Software.

The software includes multiple options for many steps. This guide presents a single, straightforward procedure for setup and analysis of each experiment type. Alternative options and shortcuts are included in Appendix A, "Alternative procedures and shortcuts for setting up an experiment".

Also, the guide directs the user to the desktop software Help system for step-by-step instructions for procedures that are not routinely performed by the user.

1 General procedures to set up and run experiments

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Set up an experiment

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

- "Set up a Standard Curve experiment in the software" on page 40
- "Set up a Relative Standard Curve experiment in the software" on page 48
- "Set up a Comparative CT experiment in the software" on page 50
- "Set up a Genotyping experiment in the software" on page 59
- "Set up the Presence/Absence experiment in the software" on page 68
- "Set up a Melt Curve experiment in the software" on page 74
- "Set up a custom experiment in the software" on page 80

Create or open an experiment

In the Home screen:

- Create a new experiment.

To	Action
Create an experiment without preexisting settings	Click **Create New Experiment**.
Create an experiment from a system template	1. Select **Create New Experiment** ▸ **Template**. 2. Navigate to and select the desired file, then click **Open**. 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.

- Open an existing experiment.
  a. Click **Open**.

b. Navigate to and select the desired file, then click **Open**.

Continue to "Enter experiment properties" on page 10.

Workflow bar

The workflow bar is used for navigation through experiment design and analysis.

Enter experiment properties

In the Properties tab:

1. (*Optional*) Modify the default experiment Name.

   The experiment Name determines the file name of the experiment. However, when you save your experiment, changes to the experiment name from the Properties tab do not update the file name. To change the file name *after* an initial save, select **Save ▸ Save as...**.

2. (*Optional*) Enter or scan a Barcode.

   Note: Click in the Barcode field before using a barcode scanner.

3. (*Optional*) Enter a User name and Comments.

4. Select an Instrument type, Block type, Experiment type, Chemistry (reagents), and Run Mode (Fast or Standard) from the drop-down lists.

   Note: Selecting the experiment type defines the available options for the experimental setup. See the "Experiment definitions" on page 11 for the parameters defined in each experiment type.

5. (*Optional*) Click **Manage chemistry details** (see "Enter reagent information" on page 10).

6. (*Optional*) Click **Save** or select **Save ▸ Save as...** to save the experiment (.edt) file before continuing.

Click **Next** to advance to "Adjust method parameters" on page 81.

Enter reagent information

Refer to the software Help system for step-by-step instructions for entering reagent information.

Confirm or edit the experiment method

In the Method tab:

- (*Optional*) Adjust the reaction volume.
- (*Optional*) Edit the default run method (thermal protocol) (see "Adjust method parameters" on page 81).

  The default run method has been optimized for TaqMan™ assays and a broad range of other reagents.

Define samples and targets or SNP assays

These are general instructions. For specific instructions for each experiment type, see the appropriate chapter in this guide.

Refer to the software Help system for step-by-step instructions for defining samples and targets or SNP assays.

(*Optional*) Select **Action** ▸ **Save to Library** to save a sample, target, or SNP assay to its respective library.

Experiment definitions

The parameters 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	✓		✓	✓	✓	

Manually define a sample

In the Plate tab:

1. Click **Advanced Setup**.
2. In the Samples table, click **Add**.
3. (*Optional*) Click a cell to edit Color or Sample Name.
4. (*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 a sample definition.
5. (*Optional*) Click to delete a sample from the table.

Manually define a target

In the Plate tab:

1. Click **Advanced Setup**.
2. In the Targets table, click **Add**.
3. (*Optional*) Click a cell to edit Color, Name, Reporter dye, Quencher, or to add Comments.
4. (*Optional*) Click to delete a target from the table.

Manually define a SNP assay

Only Genotyping experiments contain SNP assay options.

In the Plate tab:

1. Click **Advanced Setup**.
2. In the SNPs table, click **Add**.
3. In the New SNP Assay dialog box, enter:
   - SNP Assay Name
   - Color
   - Assay ID
   - Gene Symbol
   - Gene Name
   - NCBI SNP Reference
   - Allele 1 Name or Bases(s), Color, Reporter, and Quencher
   - Allele 2 Name or Bases(s), Color, Reporter, and Quencher
   - Context Sequence
   - Comments
4. Click **OK**.
5. (*Optional*) Click to delete a SNP assay from the table.

Define biological replicate groups

Biological Replicate Groups can be defined in Standard Curve, Relative Standard Curve, Comparative C_T, and custom experiments.

In the Plate tab:

1. Click **Advanced Setup**.
2. In the Biological Replicate Groups table, click **Add**.
3. (*Optional*) Click a cell to edit color, name, or comments.
4. (*Optional*) Click to delete a biological replicate group from the table.

Assign samples, targets or SNP assays, and tasks to wells

These are general instructions. For specific instructions for each experiment type, see the appropriate chapter in this guide.

Refer to the software Help system for step-by-step instructions for defining samples and targets or SNP assays.

Note: Only Genotyping experiments contain SNP assay options.

Note: You can start a run without well assignments, but the amplification plots are displayed only after wells are assigned in the software.

Select plate wells

Click the appropriate icon to access the Plate Layout or the Well Table.

- 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 on a well to define a corner, then Shift-click another well on the opposite corner
Deselect a single well	Ctrl-click in the selected well

- 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 in the selected row

Assign samples, targets or SNP assays, and tasks to wells (Quick Setup)

Use the Quick Setup pane to assign well attributes by direct entry into the text fields or by selecting user-defined samples and targets or SNP assays from the drop-down lists (see "Manually define a sample" and "Manually define a target" or "Manually define a SNP assay").

Note: Only Genotyping experiments contain SNP assay options.

In the Plate tab:

1. Click **Quick Setup**.
2. Select plate wells in the Plate Layout or the Well Table.

3. Assign the well attributes for the selected well(s).
   - Enter the names of the new sample and the new target or SNP assay into their respective text fields.
   - Select a user-defined sample and target or SNP assay from their respective drop-down lists.

   Note: Assign a task to the well(s) in the Advanced Setup pane (see "Assign a task to wells" on page 14).

4. (*Optional*) Enter comments for the selected well(s).

Assign samples, targets or SNP assays, and tasks to wells (Advanced Setup)

Use the Advanced Setup pane to define and assign samples and targets or SNP assays concurrently. See "Manually define a sample" and "Manually define a target" or "Manually define a SNP assay" for detailed instructions on defining samples and targets or SNP assays.

Note: Only Genotyping experiments contain SNP assay options.

In the Plate tab:

1. Click **Advanced Setup**.
2. Select plate wells in the Plate Layout or the Well Table.
3. In the Samples table:
   a. Select the check box of a sample to assign it to the selected well(s).
   b. Define the sample (see "Manually define a sample").
4. In the Targets or SNP Assays table:
   a. Select the check box of a target or SNP assay to assign it to the selected well(s).
   b. Define the target or SNP assay (see "Manually define a target" or "Manually define a SNP assay").
   c. Select a detection task from the Task column drop-down list (see "Assign a task to wells" on page 14).

Assign a task to wells

In the Plate tab:

1. Click **Advanced Setup**.
2. Select plate wells in the Plate Layout or the Well Table.
3. In the Targets or SNP Assays table, select the check box of a target or SNP assay.

   Note: Only Genotyping experiments contain SNP assay options.

4. Select a detection task from the Task column drop-down list.

Option	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 (*Standard Curve and Relative Standard Curve experiments only*)	The well contains samples with known standard quantities. **Note:** If you select Standard as the detection task, enter the standard quantity in the Quantity column.
1 1 Positive Control Allele 1/Allele 1	The well contains samples homozygous for allele 1.
2 2 Positive Control Allele 2/Allele 2	The well contains samples homozygous for allele 2.
1 2 Positive Control Allele 1/Allele 2	The well contains samples heterozygous for allele 1 and 2.
I Internal positive control (*Presence/Absence experiments only*)	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 (*Presence/Absence experiments only*)	The well contains an IPC blocking agent, which blocks amplification of the IPC.
NAC – No amplification control (*Presence/Absence experiments only*)	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.
Custom task (if defined)	The well contains reaction components that do not match the default task selections.

Assign a biological replicate group to wells

Biological Replicate Groups can be assigned in Standard Curve, Relative Standard Curve, Comparative C_T, and custom experiments.

In the Plate tab:

1. Click **Advanced Setup**.
2. Select plate wells using the Plate Layout or the Well Table.
3. In the Biological Replicate Groups table, select the check box of a biological replicate group to assign it to the selected well(s).

Save the experiment

Experiment files saved before a run have an .edt extension. After the run is complete, the experiment file containing the run data has an .eds extension.

1. In any tab, click **Save**.

   Note: The file name is created from the experiment Name entered in the Properties tab. Changes to the experiment name in the Properties tab *after* the first save do not update the file name.

2. (*Optional*) To change the file name:

   - In the menu bar, select **File** ▸ **Save As**.
   - In any tab, select **Save** ▸ **Save as...**.

See also "Set up an experiment using templates" on page 89.

Shortcuts for experiment setup

- Use sample and target or SNP assay definitions saved in a library (see "Sample, target, and SNP assay libraries" on page 84).
- Import some or all of an experiment setup:

To	Action	Setup information
Import sample and well assignments	Import a sample definition file (page 86)	– Well number – Sample name – *(Optional)* Custom sample properties
Import samples, targets, and well assignments	Import a plate setup file (page 87)	Plate setup information: – Well number – Sample name – Sample color – Target name – Dyes – *(Optional)* Other well 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	Paste from an *.xls file (page 88)	Plate setup information, as above
Import complete experimental setup	Create an experiment from a template (page 89)	– Plate setup information, as above – Reagent information – Thermal protocol – Analysis settings

Prepare reactions

See Appendix C, "Parts and materials" for information about compatible reagents and required materials for PCR reactions.

Follow the manufacturer's instructions for preparing reactions.

Follow the other guidelines described in this section.

Good laboratory practices for PCR and RT-PCR

When preparing samples for PCR or RT-PCR amplification:

- Wear clean gloves and a clean lab coat (not previously worn while handling amplified products or during sample preparation).
- Change gloves whenever 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*Zap*™ Solutions (Cat. no. AM9890).

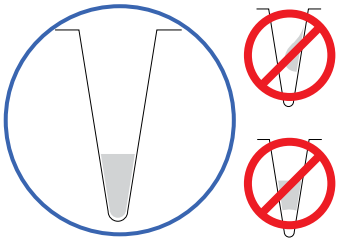
Guidelines for handling samples and reagents

- Use calibrated pipettors and aerosol-resistant tips.
- Follow the manufacturer's recommendations for the master mixes and assay mixes to prepare reaction mixes.
- Include excess volume in calculations to provide excess volume for the loss that occurs during reagent transfers.
- Use TE buffer or water to dilute samples and any standards.
- Use care when diluting samples and standards. Mistakes or inaccuracies in making the dilutions directly affect data accuracy.
- Keep the dilutions and assay mix protected from light, in the freezer, until you are ready to use it. Excessive exposure to light may affect the fluorescent probes or dyes.
- Prior to 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

- Follow the guidelines in "Good laboratory practices for PCR and RT-PCR" on page 17.
- Ensure that the arrangement of the PCR reactions matches the plate layout displayed in the QuantStudio™ desktop 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.

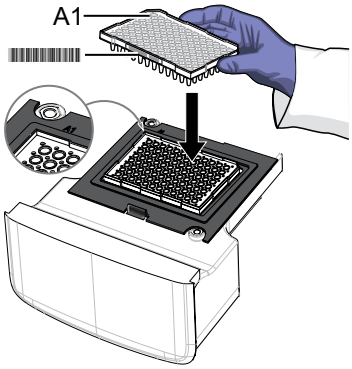


- Ensure that plates or tubes are properly sealed.
- Keep the reaction plate or tubes at 4°C, in the dark, until you are ready to load it into the instrument.
- Keep the bottom of the plate clean. Fluids and other contaminants on the bottom of the plate can contaminate the sample block and cause an abnormally 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.

Run an experiment from the desktop software

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

1. Open an experiment (.edt) file in the desktop software.
2. Load the plate.
   a. Touch ⏏ to eject the instrument drawer.
   b. Load the plate onto the plate adaptor so that:
      - Well A1 of the plate is in the top-left corner of the plate adapter.
      - The barcode faces the front of the instrument.



IMPORTANT! Plates should be loaded and unloaded 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 strip tubes. Strip tubes may fit loosely in the adapter, but when the drawer closes, 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.
3. In the Run tab of the desktop software, click **START RUN**.

   Note: If multiple instruments are connected on a local area network, first select the appropriate instrument from the drop-down list.
4. When the run is complete, touch ⏏ on the instrument home screen, unload the plate from the instrument, and close the drawer.

The experiment (.eds) file in the desktop software is automatically updated with experiment data when the run is complete.

If the connection between the instrument and the desktop software is interrupted during the run, the instrument completes the run. However, the experiment data must be transferred via a USB drive (see "Transfer files from the instrument" on page 93).

2 General procedures to review results

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This section includes information about viewing results and configuring analysis settings for all experiment types. For analysis information for a specific experiment type see the corresponding chapter.

Refer to the software Help system for step-by-step instructions for procedures that are not described in this guide.

Overview of the Results tab

View and analyze experiment data in the Results tab. In the Results tab, two additional tools are displayed at the right-end of the workflow bar (Figure 1).

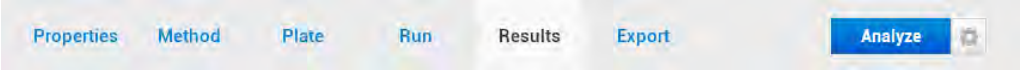


Figure 1 Workflow bar in the Results tab

- Click **Analyze** to analyze data after omission of wells or changes to the analysis settings
- Click ⚙ to access analysis settings (varies by experiment)

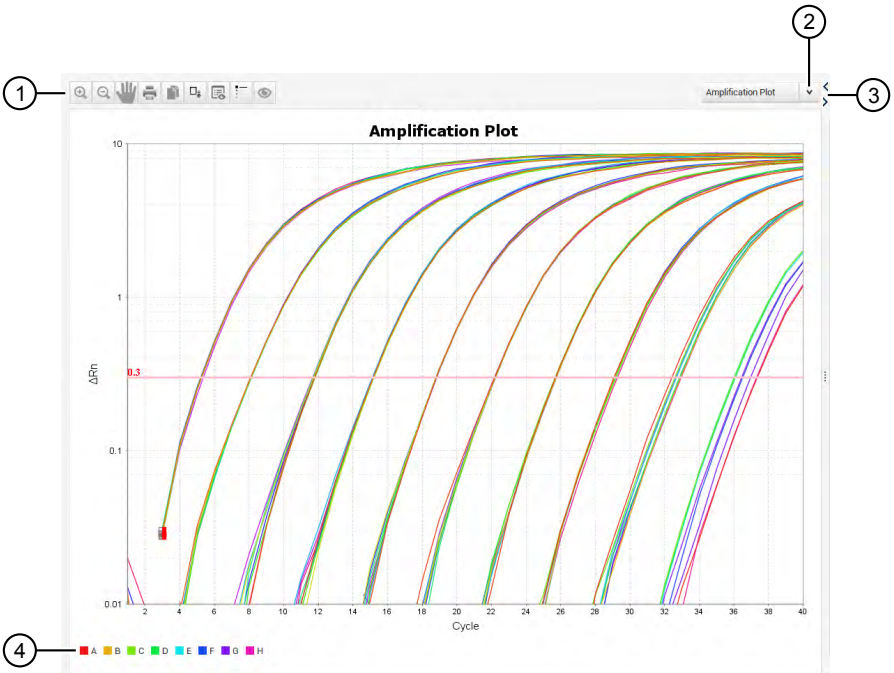


Figure 2 Plot pane

① Plot toolbar (left to right):
- Zoom in
- Zoom out
- Print plot image
- Copy plot image
- Save plot as image file
- Configure plot properties
- Show/hide plot legend
- Configure plot settings

② Plot selection list (varies by experiment)

③ Expand/contract the plot pane display

④ Plot legend

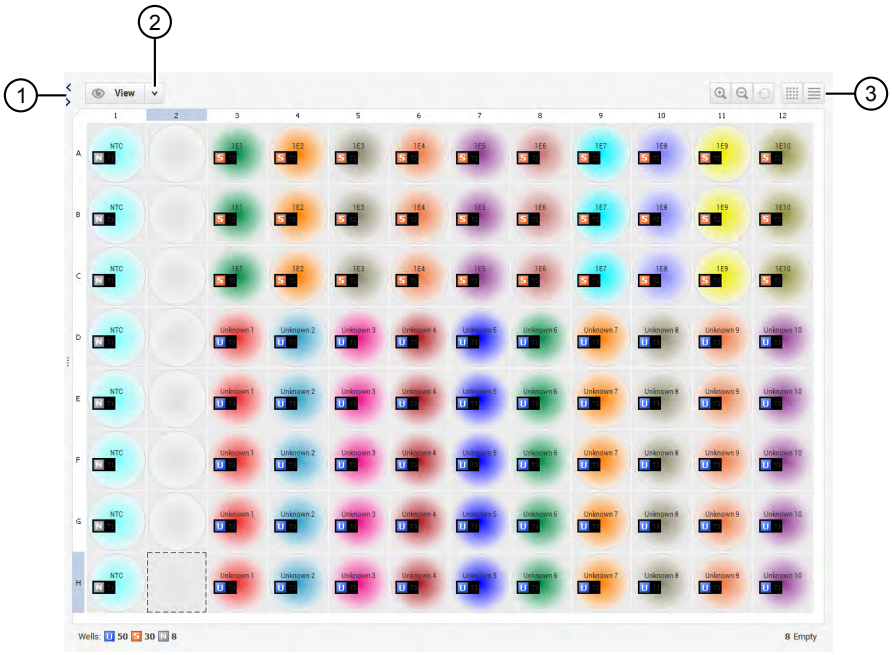


Figure 3 Plate Layout

1. Expand/contract the Plate Layout display (layout is expanded in this figure)
2. View: Select well properties to display
3. Plate Layout toolbar (left to right):
   - Zoom in
   - Zoom out
   - Fit plate to window
   - Display Plate Layout
   - Display Well Table

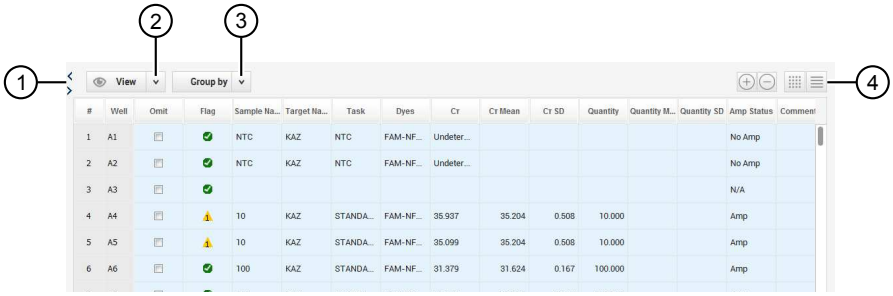


Figure 4 Well Table

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 (left to right):
   - Expand grouped rows
   - Collapse grouped rows
   - Display Plate Layout
   - Display Well Table

General workflow for analysis of experiments

When the run is complete, the QuantStudio™ desktop Software automatically analyzes the data using the default analysis settings, then displays the Amplification Plot in the Results tab.

View the Amplification Plot to confirm or correct threshold and baseline settings (page 25)

▼

Assess the experiment plot for the experiment

(refer to the appropriate chapter)

▼

Review data for outliers and omit wells, if necessary (page 29)

▼

(Optional) View the Multicomponent Plot (page 31)

▼

(Optional) View the Raw Data Plot (page 33)

▼

(Optional) Review flags in the QC Summary (page 34)

▼

(Optional) Configure the analysis settings (page 34)

IMPORTANT! After omission of wells or configuration of analysis settings, click **Analyze** to reanalyze the experiment.

Guidelines for viewing and analyzing results

- Refer to the software Help system for information about adjusting the views in the Results tab.
- 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

About the Amplification Plot

The Amplification Plot displays sample amplification as a function of cycle number or well. View the amplification plot to:

- Confirm or correct baseline and threshold values.
- Identify outliers.
- Identify and examine abnormal amplification, including:
  - 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, refer to the instrument user guide for troubleshooting information.

There are three plots available. Each plot can be viewed as a linear or $\log_{10}$ graph.

Table 1 Amplification plots

Plot type	Description	Use to
ΔRn vs Cycle	ΔRn is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification.	Identify and examine irregular amplification and to view threshold and baseline 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.
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

In the Results tab:

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

1. Select **Amplification Plot** (default) from the drop-down list.
2. Click to configure the plot:
   - Plot Type: **ΔRn vs Cycle** (default)
   - Graph Type: **Log** (default)
   - Plot Color: **WELL** (default)

   The Amplification Plot is displayed for the selected well(s) in the Plate Layout.

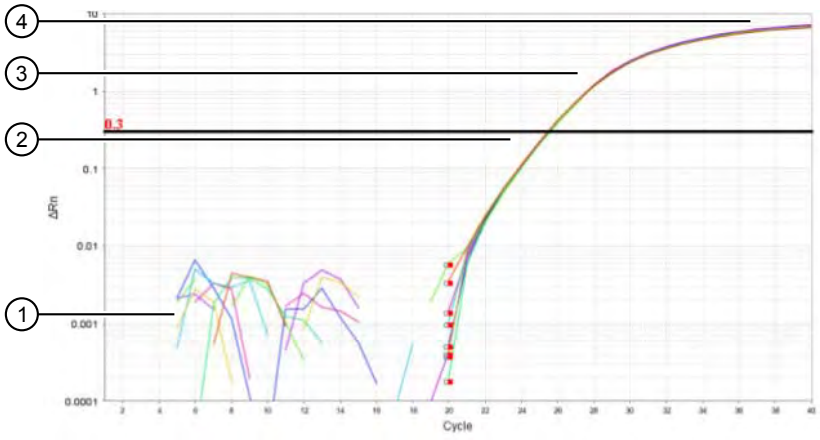


Figure 5 Typical amplification plot

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

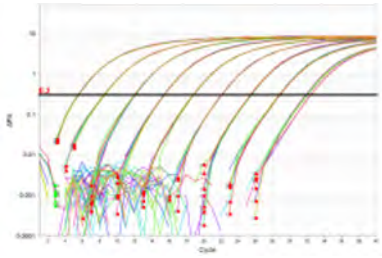
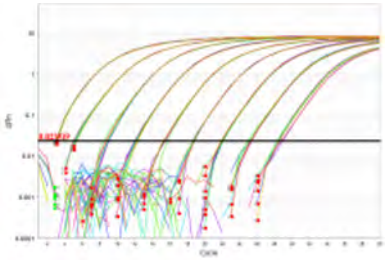
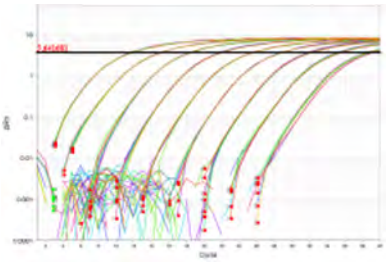
In the Results tab:

1. Select **Amplification Plot** (default) from the drop-down list.
2. Click to configure the plot:
   - Plot Type: **ΔRn vs Cycle** (default)
   - Graph Type: **Log** (default)
   - Plot Color: **WELL** (default)

   The Amplification Plot is displayed for the selected well(s) 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 page 35).

Examples of threshold settings

The threshold should be set 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 set too low.	
Threshold set too high.	

Confirm or correct baseline settings

In the Results tab:

1. Select **Amplification Plot** (default) from the drop-down list.
2. Click to configure the plot:
   - Plot Type: **ΔRn vs Cycle** (default)
   - Graph Type: **Linear** (default)
   - Plot Color: **WELL** (default)

   The Amplification Plot is displayed for the selected well(s) in the Plate Layout.
   The start and end cycles (green and red squares, respectively) that are used to calculate the baseline are displayed on the Amplification Plot for that well.
3. (*Optional*) Adjust the start and end cycle values for the baseline (see "About the CT settings" on page 35).

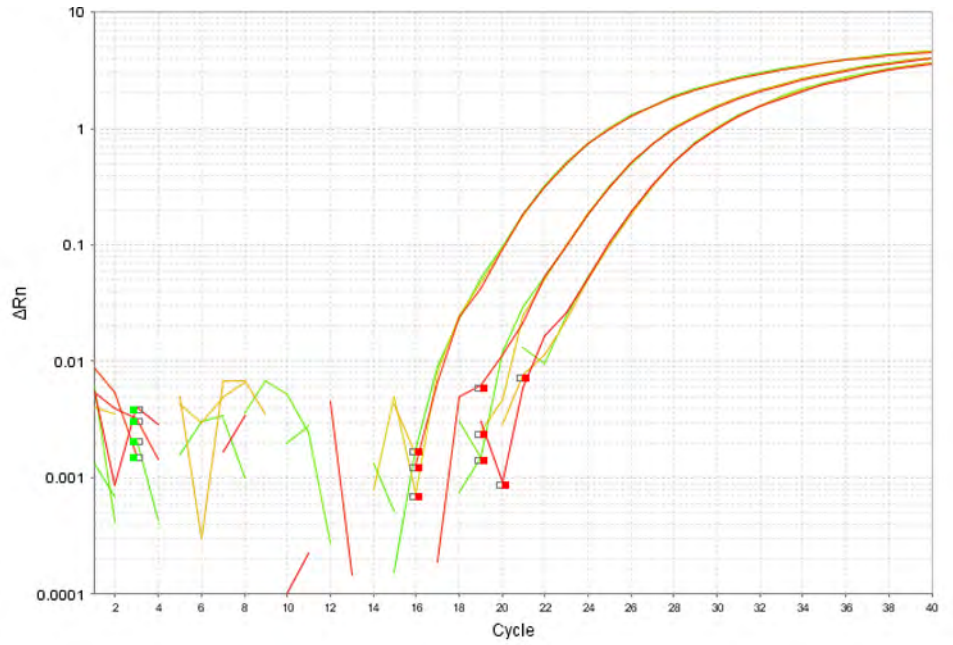


Figure 6 Example of correct baseline

The end cycle (indicated by the red squares) should be set a few cycles before the cycle number where significant fluorescent signal is detected.

Omit outliers from analysis

Outlier wells have C_T values that differ significantly from the average for the associated replicate wells. To ensure C_T precision, omit the outliers from the analysis.

In the Results tab:

1. Select **Amplification Plot** (default) from the drop-down list.
2. Click to configure the plot:
   - Plot Type: **C_T vs Well**
   - Graph Type: **Linear** (default)
   - Plot Color: **WELL** (default)

   The C_T values are displayed for the selected well(s) 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 the Well Table, select **Omit** in outlier rows of the table.

   or
   - In the Plate Layout, right-click a well, then select **Omit**.
5. Click **Analyze** to reanalyze the experiment data with any outliers removed.

Optimize display of negative controls in the Amplification Plot

In the Results tab:

1. Select **Amplification Plot** (default) from the drop-down list.
2. Click to configure the plot:
   - Plot Type: **ΔRn vs Cycle**
   - Graph Type: **Linear**
   - Plot Color: **TARGET**
   - Deselect **Threshold** and **Show: Baseline Start/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. Configure the plot.
   a. Deselect **Auto-adjust range**.
   b. Enter Minimum value of –1.
   c. Enter Maximum value of 2.

d. Click **Save**.

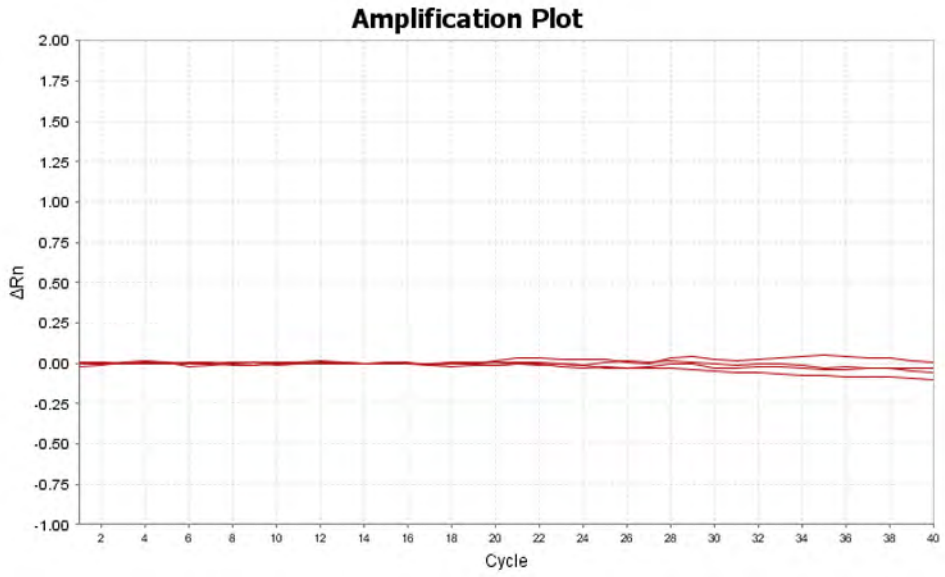


Figure 7 Example Amplification Plot display of negative controls

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

Assess results in the Well Table view

About the Well Table

The Well Table displays data for each well in the reaction plate, including:

- The sample name, target name, task, and dyes
- The calculated threshold cycle (C_T), C_T standard deviation, and normalized fluorescence (Rn)
- Specific experimental values (for example, quantities or calls)
- Comments
- Flags

Group or sort the Well Table as described in the following table.

Note: You can select only one category at a time.

Group category	Description	Notes
Replicate	Grouped by replicate For example, negative controls, standards, and samples	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 has found a potential error in the flagged well. Refer to the software Help system for more information about flags.

Group category	Description	Notes
C_T	Grouped by C_T value: • Low (C_T <24) • Medium (C_T from 24 to 30) • High (C_T >30) • No C_T	• A C_T value <8 indicates that there may be too much template in the reaction. • A C_T value >35 indicates a low amount of target in the reaction; for C_T values >35, expect a higher standard deviation.

Confirm accurate dye signal using the Multicomponent Plot

About the Multicomponent Plot

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

Use the Multicomponent Plot to:

- 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

In the Results tab:

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

1. Select **Multicomponent Plot** from the drop-down list.
2. Click to configure the plot:
   - Plot Color: **DYE**

   The Multicomponent Plot is displayed for the selected well(s) in the Plate Layout.

3. Select wells one at a time in the Plate Layout, and examine the Multicomponent Plot for the following:

Plot characteristic	Description
Passive reference dye	The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
Reporter dye	The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
Irregularities in the signal	Spikes, dips, and/or sudden changes in the fluorescent signal may have an impact on the data.
Negative control wells	There should not be any amplification in the negative control wells.

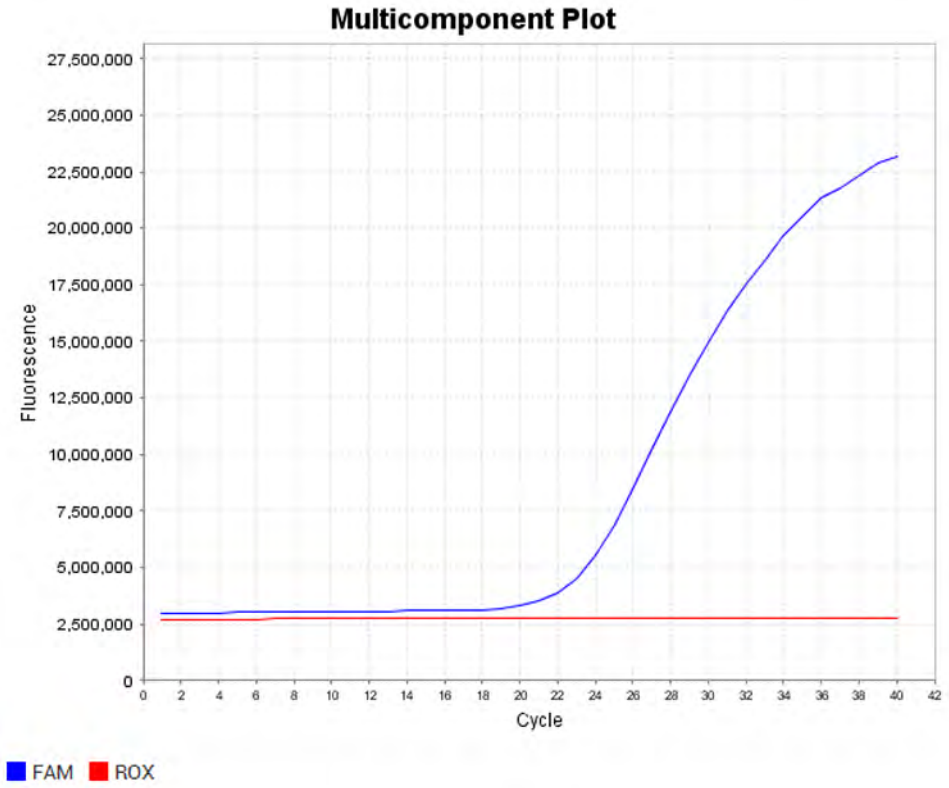


Figure 8 Example Multicomponent Plot (single well)

Determine signal accuracy using the Raw Data Plot

About the Raw Data Plot

The Raw Data Plot screen 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

In the Results tab:

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

1. Select **Raw Data Plot** from the drop-down list.
   The Raw Data Plot is displayed for the selected well(s) 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 characteristic signal growth is seen for each filter.
   The filter set for each system dye is provided in the *QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide* (Pub. no. MAN0010407).

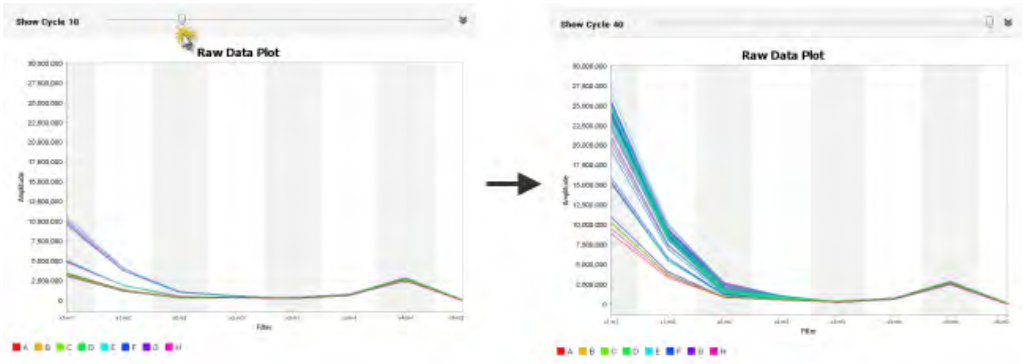


Figure 9 Example Raw Data Plot

Review the flags in the QC Summary

The QC Summary displays a list of the software flags, including the flag frequency and location.

In the Results tab:

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

1. Select **QC Summary** from the drop-down list.
2. Review the Flag Details table and the summary.
   The Flag Details table identifies any wells that triggered a flag in the Well column.
3. (*Optional*) In the Flag Details table, click each flag to display information about the flag.
   Refer to the software Help system for more information about the flag.

Configure analysis settings

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

About the analysis settings

Analysis settings determine how the baseline, threshold, and threshold cycle (C_T) are calculated, what flags are enabled, and other analysis options that are specific to an experiment type. The default analysis settings are different for each experiment type.

We recommend analyzing the experiment with default analysis settings. If the default analysis settings are not suitable for the experiment, change the settings in the Analysis Settings dialog box, then reanalyze the experiment.

Save changed analysis settings to the Analysis Settings Library.

See also:

- "About the CT settings" on page 35
- "About the flag settings" on page 36
- "About Advanced Settings" on page 36
- "About the Standard Curve settings" on page 44
- "About relative quantification settings" on page 57
- "About call settings for Genotyping experiments" on page 64
- "About call settings for Presence/Absence experiments" on page 72
- "About Melt Curve settings" on page 77

View and configure the analysis settings

In the Results tab:

1. Click ⚙ (in top-right corner).
2. View and, if necessary, configure the analysis settings.
   Refer to the software Help system for step-by-step instructions for adjusting analysis settings.

3. Click **Apply**.
4. Click **Analyze** to reanalyze to experiment with the new settings.
5. (*Optional*) To save the analysis settings in the Analysis Settings Library, click **Save...**.
6. (*Optional*) To return to the default settings, click **Revert**.

About the C_T settings

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

Refer to the software Help system for step-by-step instructions for adjusting the C_T settings.

The C_T Settings feature is not available for Melt Curve experiments without a PCR stage.

Table 2 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 (Crt) algorithm is used to calculate the C_T values.
Default C_T Settings	Determines how the Baseline Threshold algorithm is set. The Default C_T Setting is used for targets unless they have custom settings. See Table 3 for recommendations on adjusting baseline and threshold.
C_T Settings for Target	• Selected Default Settings uses the Default C_T Settings to calculate the C_T values for the target. • Deselected Default Settings allows manual setting of the baseline or the threshold. See Table 3 for recommendations for adjusting baseline and threshold.

Table 3 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.

About the flag settings

Use the Flag Settings tab to:

- 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.

Refer to the software Help system for step-by-step instructions for configuring the flag settings.

About Advanced Settings

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

Refer to the software Help system for step-by-step instructions for adjusting the advanced settings.

The Advanced Settings feature is not available for Melt Curve experiments.

About overriding the calibration data

Each experiment (.eds) file stores the calibration data from the QuantStudio™ 3 or 5 Instrument on which it was run.

You can use calibration data from another QuantStudio™ 3 or 5 Instrument for analysis of your experiment.

The calibration data used must be from the same block type and instrument type (QuantStudio™ 3 Instrument or QuantStudio™ 5 Instrument). Calibration data from a QuantStudio™ 5 Instrument experiment can be used to override calibration data for a QuantStudio™ 3 Instrument experiment, but not vice-versa.

Refer to the software Help system for step-by-step instructions for overriding calibration data.

Export experiments or results

Refer to the software Help system for step-by-step instructions for exporting experiments or results.

Options for publishing the analyzed data

To	Action
Save a plot as an image file	Click
Print a plot	Click
Copy a plot to the clipboard	Click
Export data	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 or application
Plate setup files for future experiments	Plate setup information (such as the well number, sample name, sample color, target name, dyes, and other reaction plate contents)	• .txt (for future import) • .xls • .xlsx
Analyzed data for further analysis	QuantStudio™ format	• .txt • .xls • .xlsx
	RDML format (for Standard Curve, Relative Standard Curve, Comparative C_T, and Melt Curve experiments)	.rdml

3 Getting started with Standard Curve experiments

- About Standard Curve experiments 38
- Set up a Standard Curve experiment in the software 40
- Set up and run the PCR reactions 41
- Review results 42

About Standard Curve experiments

Overview

A Standard Curve experiment is used to determine absolute target quantity in samples.

In a Standard Curve experiment:

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. Using the standard curve, the software interpolates 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 quantities of the target **Note:** The target should be previously quantified in the standard sample using an independent method.
Unknown	Test sample
No-template control (NTC)	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.

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

Compatible PCR options

Table 5 PCR options for Standard Curve experiments

Single- or multiplex PCR	PCR or RT-PCR[1]	Reagent system
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 an experiment.
2. In the Properties tab, enter the information for the experiment.
3. In the Method tab, enter the reaction volume for the experiment.
   For most experiments, the default run method is appropriate.
4. Set up standard dilutions in the Plate tab.
   a. Select **Action ▸ Define and Set Up Standards**.
   b. Select **Singleplex** or **Multiplex** from the Model drop-down list.
   c. (*Optional*) Select the target.
   d. Enter the parameters for the dilution series.
      - **Number of dilution points**: 5 recommended
      - **Number of replicates**: 3 recommended
      - **Starting quantity**: enter the highest or lowest standard quantity, without units (the quantity can be expressed as copies, copies/µL, or ng/µL)
      - **Serial dilution factor**:
        – If the starting quantity is the highest value, select a serial factor from 1:10 to 1:2.
        – If the starting quantity is the lowest value, select a serial factor from 2× to 10×.
   e. Select and arrange the wells for the standards.
      - Select to arrange standards in columns or rows.
      - Select **Automatically Select Wells for Me** or select **Let Me Select Wells**, then drag the cursor to select the appropriate block of wells.
   f. Click **Apply**.

   Alternatively, see "Assign the standard dilutions manually" on page 83.
5. Define and assign well attributes in the Quick Setup pane of the Plate tab.
   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 their respective fields.
      - Select previously defined samples and targets from their respective drop-down lists.

      Note: When you enter a new sample or target name in the Quick Setup pane, the software automatically populates default values for Reporter (FAM) and Quencher (NFQ-MGB) and assigns the task (U Unknown).

To change these default values, click **Advanced Setup** (see "Define samples and targets or SNP assays" on page 11 or "Sample, target, and SNP assay libraries" on page 84).

6. Assign plate attributes in the Quick Setup pane of the Plate tab.
   a. In the Plate Attributes group, select the Passive Reference from the drop-down list.

7. (*Optional*) Assign tasks in the Advanced Setup pane of the Plate tab.
   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 drop-down list.

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

   Note: You can define and setup standards in the Targets table: Select the task of S Standard, then enter the quantity for that target.

8. (*Optional*) Define and assign biological replicate groups in the Advanced Setup pane of the Plate tab.
   a. In the Biological Replicates Groups table, enter a name and select a color for each biological replicate group.

   b. Select wells, then select the checkbox of a biological replicate group to assign it to the selected well(s).

In the Advanced Setup pane of Plate tab, ensure the Samples table contains:

- One sample for each dilution of an unknown sample
- (*Optional*) A standard sample for each target

Set up and run the PCR reactions

1. Assemble the PCR reactions, following the manufacturer's instructions for the reagents, and following the plate layout set up in the software.

2. In the desktop software, open the appropriate experiment (.edt) file.

3. Load the reaction plate into the instrument and start the run.

See also:

- "Prepare reactions" on page 17
- "Run an experiment from the desktop software" on page 19

Review results

General workflow for analysis of Standard Curve experiments

View the Amplification Plot to confirm or correct threshold and baseline settings (page 25)

▼

Assess the Standard Curve Plot (page 43)

▼

Review data for outliers and omit wells, if necessary (page 29)

▼

(Optional) View the Multicomponent Plot (page 31)

▼

(Optional) View the Raw Data Plot (page 33)

▼

(Optional) Review flags in the QC Summary (page 34)

▼

(Optional) Configure the analysis settings (page 34)

IMPORTANT! After omission of wells or configuration of analysis settings, click **Analyze** to reanalyze the experiment.

About the Standard Curve Plot

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 to evaluate in the Standard Curve Plot

Result	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: • **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 in the reaction can reduce amplification efficiency.
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_T 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.

Result	Description	Criteria for evaluation
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. • A C_T value <8 indicates that there may be too much template in the reaction. • A C_T value >35 indicates 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

In the Results tab:

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

1. Select **Standard Curve** from the drop-down list.
2. Click to configure the plot:
   - Target: select the target of interest
   - Plot Color: **FLAG-STATUS**, **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_T values fall within the standard curve range.

5. In the Well Table, use the Group By drop-down list to confirm that the C_T values of all replicate samples meet the experimental criteria.

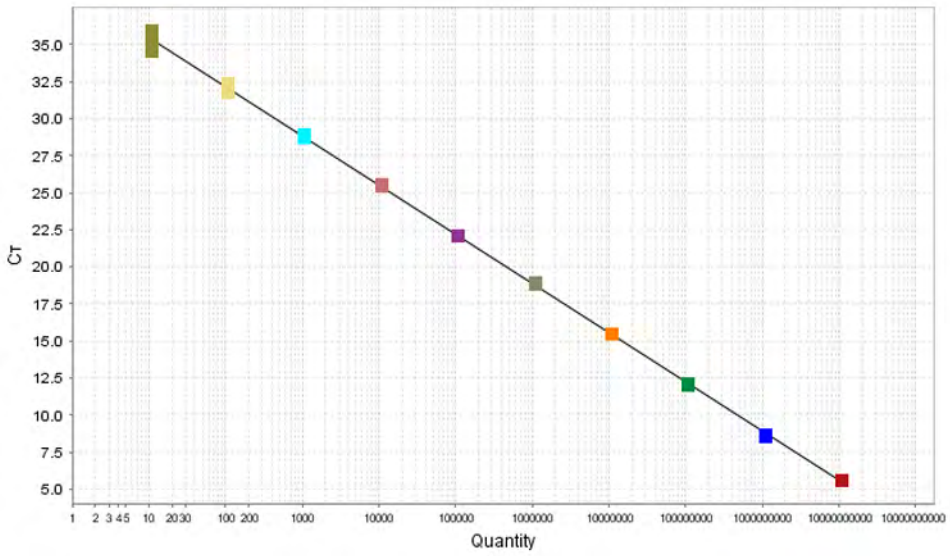


Figure 10 Example Standard Curve Plot

If the experiment does not meet the guidelines above, troubleshoot as follows:

- Omit wells (see "Omit outliers from analysis" on page 29).

  or

- Repeat the experiment, adjusting the experimental setup to improve results.

See also "About the Standard Curve Plot" on page 42.

About the Standard Curve settings

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. Use the Standard Curve Settings tab in the Analysis Settings to import an external standard curve.

Refer to the software Help system for step-by-step instructions for adjusting the Standard Curve settings.

4 Getting started with Relative Standard Curve and Comparative C_T experiments

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- Relative quantitation: Relative Standard Curve vs Comparative CT 48
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About Relative Standard Curve experiments

Overview

The Relative Standard Curve experiment is used to determine relative target quantity in samples.

In a Relative Standard Curve experiment:

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. Using the standard curves, the software interpolates 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.

See “Relative quantitation: Relative Standard Curve vs Comparative CT“ on page 48 for a comparison of this method to the Comparative C_T ($\Delta\Delta C_T$) method.

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 quantities of the target **Note:** The target should be previously quantified in the standard sample using an independent method.
Reference sample	The sample that is used as the basis for relative quantification results
Unknown	Test sample
No-template control (NTC)	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.
  If the amount of standard is limited, the target is in low abundance, or the target is known to fall within a given range, a narrow range of standard quantities may be appropriate.

Compatible PCR options

Table 8 PCR options for Relative Standard Curve experiments

Single- or multiplex PCR	PCR or RT-PCR[1]	Reagent system
Singleplex Multiplex	PCR 1-step RT-PCR 2-step RT-PCR	TaqMan™ SYBR™ Green

[1] RT-PCR: reverse transcription PCR

About Comparative C_T experiments

Overview

The Comparative C_T ($\Delta\Delta C_T$) experiment is used to determine relative target quantity in samples.

In a Comparative C_T experiment:

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 C_T (ΔC_T) for the sample to the normalized C_T (ΔC_T) for the reference sample.

See "Relative quantitation: Relative Standard Curve vs Comparative CT" on page 48 for a comparison of this method to the Relative Standard Curve method.

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	The sample that is used as the basis for relative quantification results
Unknown	Test sample
No-template control (NTC)	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.

Compatible PCR options

Table 10 PCR options for Comparative C_T experiments

Single- or multiplex PCR	PCR or RT-PCR[1]	Reagent system
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

Relative Standard Curve experiments and Comparative C_T ($\Delta\Delta C_T$) experiments have the same purpose: 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 to:

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

Table 11 Comparison of Relative Standard Curve 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 an experiment.
2. In the Properties tab, enter the information for the experiment.
3. In the Method tab, enter the reaction volume for the experiment.
   For most experiments, the default run method is appropriate.
4. Set up standard dilutions in the Plate tab.
   a. Select **Action ▸ Define and Set Up Standards**.

b. Select **Singleplex** or **Multiplex** from the Model drop-down list.

c. (*Optional*) Select the target.

d. Enter the parameters for the dilution series.
   - **Number of dilution points**: 5 recommended
   - **Number of replicates**: 3 recommended
   - **Starting quantity**: enter the highest or lowest standard quantity, without units (the quantity can be expressed as copies, copies/µL, or ng/µL)
   - **Serial dilution factor**:
     – If the starting quantity is the highest value, select a serial factor from 1:10 to 1:2.
     – If the starting quantity is the lowest value, select a serial factor from 2× to 10×.

e. Select and arrange the wells for the standards.
   - Select to arrange standards in columns or rows.
   - Select **Automatically Select Wells for Me** or select **Let Me Select Wells**, then drag the cursor to select the appropriate block of wells.

f. Click **Apply**.

Alternatively, see "Assign the standard dilutions manually" on page 83.

5. Define and assign well attributes in the Quick Setup pane of the Plate tab.
   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 their respective fields.
      - Select previously defined samples and targets from their respective drop-down lists.

      Note: When you enter a new sample or target name in the Quick Setup pane, the software automatically populates default values for Reporter (FAM) and Quencher (NFQ-MGB) and assigns the task (U Unknown). To change these default values, click **Advanced Setup** (see "Define samples and targets or SNP assays" on page 11 or "Sample, target, and SNP assay libraries" on page 84).

6. Assign plate attributes in the Quick Setup pane of the Plate tab.
   a. In the Plate Attributes group, select a Passive Reference, Reference Sample, and Endogenous Control from their respective drop-down lists.

7. (*Optional*) Assign tasks in the Advanced Setup pane of the Plate tab.
   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 drop-down list.

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

Note: You can define and setup standards in the Targets table: Select the task of S Standard, then enter the quantity for that target.

8. (*Optional*) Define and assign biological replicate groups in the Advanced Setup pane of the Plate tab.
   a. In the Biological Replicates Groups table, enter a name and select a color for each biological replicate group.
   b. Select wells, then select the checkbox of a biological replicate group to assign it to the selected well(s).

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

In the Advanced Setup pane of Plate tab, ensure the Samples table contains:

- One sample for each dilution of an unknown sample
- Reference sample
- (*Optional*) A standard sample for each endogenous control target and target of interest

Set up a Comparative C_T experiment in the software

1. In the Home screen, create or open an experiment.
2. In the Properties tab, enter the information for the experiment.
3. In the Method tab, enter the reaction volume for the experiment.
   For most experiments, the default run method is appropriate.
4. Define and assign well attributes in the Quick Setup pane of the Plate tab.
   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 their respective fields.
      - Select previously defined samples and targets from their respective drop-down lists.

      Note: When you enter a new sample or target name in the Quick Setup pane, the software automatically populates default values for Reporter (FAM) and Quencher (NFQ-MGB) and assigns the task (U Unknown).

To change these default values, click **Advanced Setup** (see "Define samples and targets or SNP assays" on page 11 or "Sample, target, and SNP assay libraries" on page 84).

5. Assign plate attributes in the Quick Setup pane of the Plate tab.
   a. In the Plate Attributes group, select a Passive Reference, Reference Sample, and Endogenous Control from their respective drop-down lists.

6. (*Optional*) Assign tasks in the Advanced Setup pane of the Plate tab.
   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 drop-down list.

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

7. (*Optional*) Define and assign biological replicate groups in the Advanced Setup pane of the Plate tab.
   a. In the Biological Replicates Groups table, enter a name and select a color for each biological replicate group.

   b. Select wells, then select the checkbox of a biological replicate group to assign it to the selected well(s).

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

In the Advanced Setup pane of Plate tab, ensure the Samples table contains:

- Unknown samples
- Reference sample

Set up and run the PCR reactions

1. Assemble the PCR reactions, following the manufacturer's instructions for the reagents, and following the plate layout set up in the software.

2. In the desktop software, open the appropriate experiment (.edt) file.

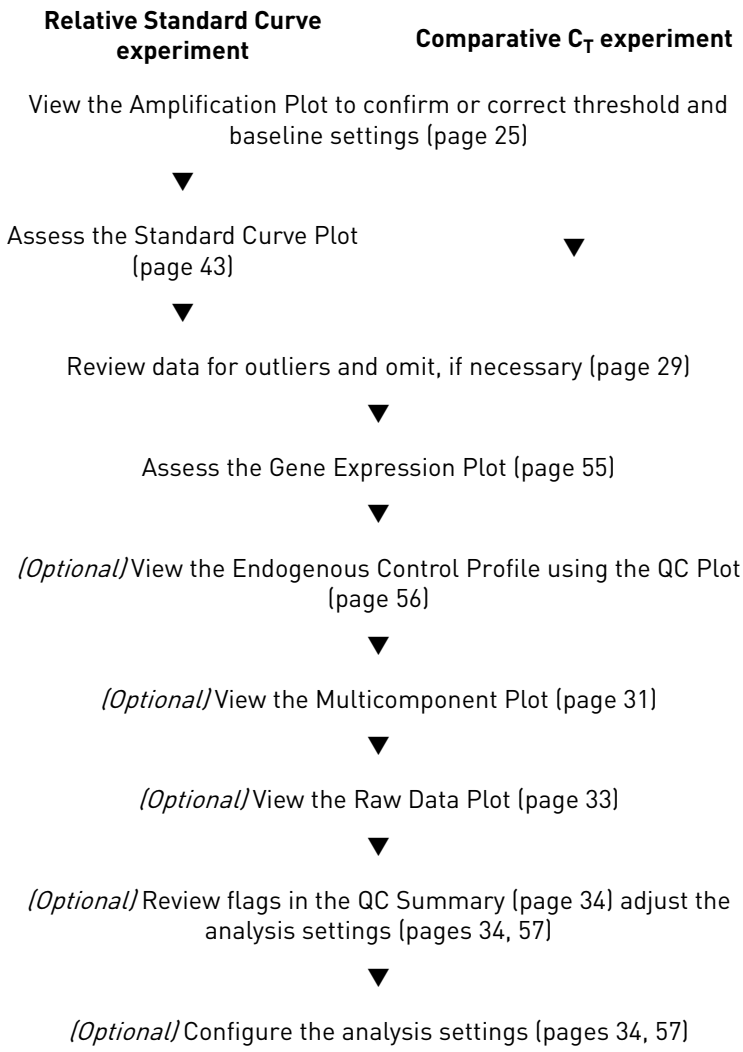
3. Load the reaction plate into the instrument and start the run.

See also:

- "Prepare reactions" on page 17
- "Run an experiment from the desktop software" on page 19

Review results

General workflows for analysis of Relative Standard Curve and Comparative C_T experiments



IMPORTANT! After omission of outliers or configuration of analysis settings, click **Analyze** to reanalyze the experiment.

View and assess the Standard Curve Plot

This section only applies to Relative Standard Curve experiments.

About the Standard Curve Plot

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 to evaluate in the Standard Curve Plot

Result	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: • **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 in the reaction can reduce amplification efficiency.
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_T 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. • A C_T value <8 indicates that there may be too much template in the reaction. • A C_T value >35 indicates 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

In the Results tab:

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

1. Select **Standard Curve** from the drop-down list.
2. Click to configure the plot:
   - Target: select the target of interest
   - Plot Color: **FLAG-STATUS**, **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_T values fall within the standard curve range.
5. In the Well Table, use the Group By drop-down list to confirm that the C_T values of all replicate samples meet the experimental criteria.

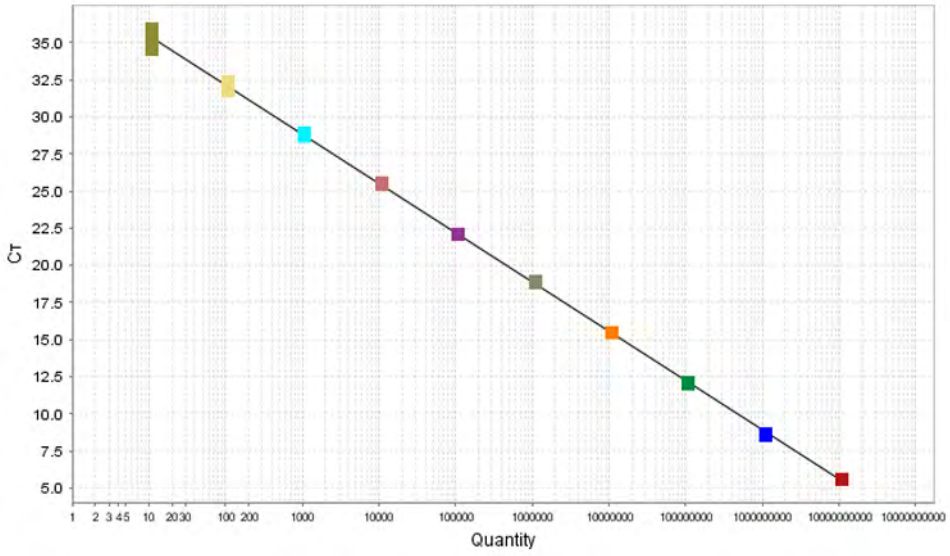


Figure 11 Example Standard Curve Plot

If the experiment does not meet the guidelines above, troubleshoot as follows:

- Omit wells (see "Omit outliers from analysis" on page 29).

  or
- Repeat the experiment, adjusting the experimental setup to improve results.

See also "About the Standard Curve Plot" on page 42.

About the Standard Curve settings

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. Use the Standard Curve Settings tab in the Analysis Settings to import an external standard curve.

Refer to the software Help system for step-by-step instructions for adjusting the Standard Curve settings.

About the Gene Expression Plot

The Gene Expression Plot displays the results of relative quantification calculations for Relative Standard Curve and Comparative C_T experiments.

The purpose of reviewing the Gene Expression Plot is to evaluate the fold change in expression level of the target(s) 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.

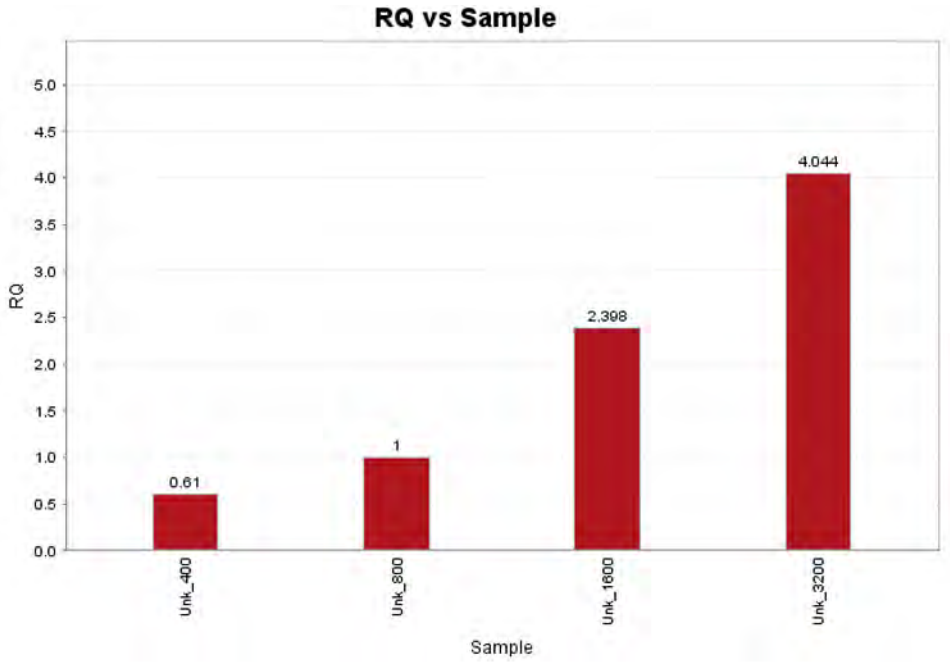


Figure 12 Example Gene Expression Plot

In this example, there is one target of interest, and the sample Unk_800 is the reference sample (calibrator).

About the QC Plot

The QC Plot is a visual display of the C_T levels of potential endogenous control targets across all samples (Endogenous Control Profile). The QC Plot serves as a tool to help users choose the best endogenous control(s) for an experiment by choosing the target whose quantity (indicated by C_T value) 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

In the Results tab:

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

1. Select **QC Plot** from the drop-down list.
2. In the right-side pane, select the target(s) to display, then select the color and shape from the drop-down lists.
3. (*Optional*) In the View Replicate Results Table tab, select the samples to omit from analysis.
4. (*Optional*) To change the endogenous control(s) used for analysis, select ⚙ ▸ **Relative Quantification Settings** (see "About relative quantification settings" on page 57).

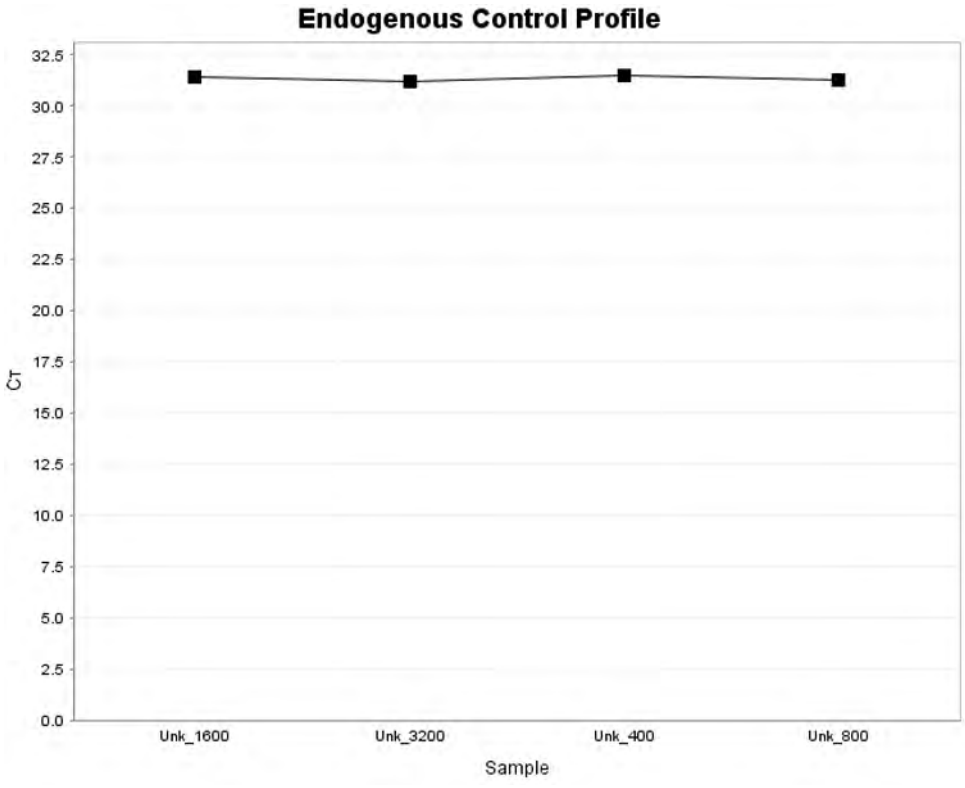


Figure 13 Example QC Plot

Click **Analyze** to see the result of the adjustments.

About relative quantification settings

Select **Results** ▸ ⚙ ▸ **Relative Quantification Settings** to change these parameters:

Parameter	Description
Analysis Type	Select **Multiplex** or **Singleplex** analysis.
Reference(s)	Set the reference sample, or set a biological replicate group as the reference sample.
Endogenous Control(s)	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_T 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.

Refer to the software Help system for step-by-step instructions for adjusting the relative quantification settings.

5 Getting started with Genotyping experiments

- About Genotyping experiments 58
- Set up a Genotyping experiment in the software 59
- Set up and run the PCR reactions 61
- Review results 61

About Genotyping experiments

Overview

Genotyping experiments are used to detect single nucleotide polymorphism (SNP) variants of a target nucleic acid sequence.

Genotyping experiments use preformulated TaqMan™ SNP Genotyping Assays that include:

- 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:

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 intensities (Rn) of the reporter dyes in 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]	Reagent system
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 on end-point data, that is, data collected after the PCR is completed.

- 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:
  ΔRn = Rn (post-PCR read) – Rn (pre-PCR read), where Rn = normalized readings.

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

Set up a Genotyping experiment in the software

Only Genotyping experiments contain SNP assay options.
Note:

1. In the Home screen, create or open an experiment.
2. In the Properties tab, enter the information for the experiment.
3. In the Method tab, enter the reaction volume for the experiment.
   For most experiments, the default run method is appropriate.

4. Define and assign well attributes in the Quick Setup pane of the Plate tab.
   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 their respective fields.
      - Select previously defined samples and SNP assays from their respective drop-down lists.

      Note: When you enter a new sample or SNP assay name in the Quick Setup pane, the software automatically populates 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

      To change these default values, click **Advanced Setup** (see "Define samples and targets or SNP assays" on page 11 or "Sample, target, and SNP assay libraries" on page 84).

5. Assign plate attributes in the Quick Setup pane of the Plate tab.
   a. In the Plate Attributes group, select the Passive Reference from the drop-down list.

6. (*Optional*) Assign tasks in the Advanced Setup pane of the Plate tab.
   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 drop-down 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 Advanced Setup pane of Plate tab, ensure the Samples table contains:

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

Set up and run the PCR reactions

1. Assemble the PCR reactions, following the manufacturer's instructions for the reagents, and following the plate layout set up in the software.
2. In the desktop software, open the appropriate experiment (.edt) file.
3. Load the reaction plate into the instrument and start the run.

See also:

- "Prepare reactions" on page 17
- "Run an experiment from the desktop software" on page 19

Review results

General workflow for analysis of Genotyping experiments

Assess the Allelic Discrimination Plot (page 62)

▼

(Optional) View the Amplification Plot (page 25)

▼

Review data for outliers and omit, if necessary (page 29)

▼

(Optional) View the Multicomponent Plot (page 31)

▼

(Optional) View the Raw Data Plot (page 33)

▼

(Optional) Review flags in the QC Summary (page 34)

▼

(Optional) Configure the analysis settings (pages 34, 64)

IMPORTANT! After omission of outliers or configuration of analysis settings, click **Analyze** to reanalyze the experiment.

About the Allelic Discrimination Plot

The Allelic Discrimination Plot contrasts the normalized intensities (Rn) 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...
VIC™ dye-labeled probe fluorescence only	Horizontal axis	Homozygosity for Allele 1
FAM™ dye-labeled probe fluorescence only	Vertical axis	Homozygosity for Allele 2
Both VIC™ and FAM™ dye-labeled probes fluorescence	Diagonal	Allele 1-Allele 2 heterozygosity

View the allelic discrimination plot to:

- 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). In this case, use the Applied Biosystems™ Genotyping Analysis Module GT in the Thermo Fisher Cloud.

View and assess the Allelic Discrimination Plot

In the Results tab:

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

1. Select **Allelic Discrimination Plot** from the drop-down list.
2. Click to configure the plot:
   - 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 Allelic Discrimination 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, and confirm that only the negative control wells are selected in the Plate Layout or Well Table.
   Samples that unexpectedly cluster with the negative controls may:
   - Contain no DNA.
   - Contain PCR inhibitors.
   - Be homozygous for a sequence deletion.
5. Review the other clusters in the plot.
   a. Click and 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.

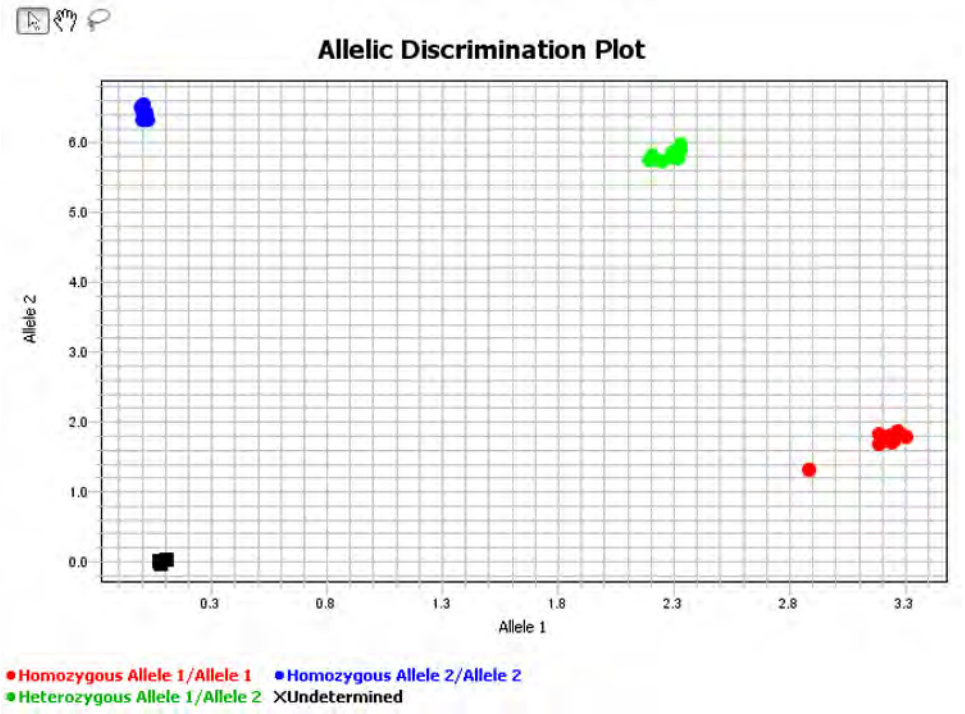


Figure 14 Example Allelic Discrimination Plot

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

Perform manual calls

1. Open a Genotyping experiment that contains results data.
2. In the Results tab, select **Allelic Discrimination Plot** from the drop-down list.
3. If the data are not analyzed, click **Analyze**.
4. Click to display the plot menu bar.
5. (*For multiple assays only*) Select a SNP assay from the drop-down list.

6. In the Allelic Discrimination Plot, click-drag to select the sample(s) to be manually called.
7. Click , then select the allele call from the Apply Call drop-down list.
8. Click **Analyze**.

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

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

About call settings for Genotyping experiments

Use the Call Settings tab in the Advanced Settings to:

- Change the default data analysis settings.
- Edit the default call settings (for SNP assays without custom call settings) or custom call settings for individual SNP assays.

Table 17 Data analysis settings options for 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]	The normalized reporter data (Rn) from the last cycle of the cycling stage is used to determine calls.
Analyze Real-Time Rn - Median (Rna to Rnb)[2,3]	A *quick baseline-subtracted Rn* from the last 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]	The regular ΔRn (dRn) from the last 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] Rna to Rnb refers to all the cycles from the Start Cycle Number to the End Cycle Number of the baseline region.

Table 18 Call settings for 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.

Refer to the software Help system for step-by-step instructions for adjusting the call settings.

6 Getting started with Presence/Absence experiments

- About Presence/Absence experiments 66
- Set up the Presence/Absence experiment in the software 68
- Set up and run the PCR reactions 69
- Review results 69

About Presence/Absence experiments

Overview

Presence/Absence experiments are used 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]	Reagent system
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, that is, data collected after the PCR is completed.

- 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:
  ΔRn = Rn (post-PCR read) – Rn (pre-PCR read), where Rn = normalized readings.

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

Set up the Presence/Absence experiment in the software

1. In the Home screen, create or open an experiment.
2. In the Properties tab, enter the information for the experiment.
3. In the Method tab, enter the reaction volume for the experiment.
   For most experiments, the default run method is appropriate.
4. Define and assign well attributes in the Quick Setup pane of the Plate tab.
   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 their respective fields.
      - Select previously defined samples and targets from their respective drop-down lists.

      Note: When you enter a new sample or target name in the Quick Setup pane, the software automatically populates default values for Reporter (FAM) and Quencher (NFQ-MGB) and assigns the task (U Unknown). To change these default values, click **Advanced Setup** (see "Define samples and targets or SNP assays" on page 11 or "Sample, target, and SNP assay libraries" on page 84).
5. Assign plate attributes in the Quick Setup pane of the Plate tab.
   a. In the Plate Attributes group, select the Passive Reference from the drop-down list.
6. (*Optional*) Assign tasks in the Advanced Setup pane of the Plate tab.
   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 drop-down 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	

Set up and run the PCR reactions

1. Assemble the PCR reactions, following the manufacturer's instructions for the reagents, and following the plate layout set up in the software.
2. In the desktop software, open the appropriate experiment (.edt) file.
3. Load the reaction plate into the instrument and start the run.

See also:

- "Prepare reactions" on page 17
- "Run an experiment from the desktop software" on page 19

Review results

General workflow for analysis of Presence/Absence experiments

Assess the Presence/Absence Plot (page 70)

▼

(Optional) View the Amplification Plot (page 25)

▼

Review data for outliers and omit, if necessary (page 29)

▼

(Optional) View the Multicomponent Plot (page 31)

▼

(Optional) View the Raw Data Plot (page 33)

▼

(Optional) Review flags in the QC Summary (page 34)

▼

(Optional) Configure the analysis settings (pages 34, 72)

IMPORTANT! After omission of outliers or configuration of analysis settings, click **Analyze** to reanalyze the experiment.

About the Presence/Absence Plot

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

The purpose of viewing the Presence/Absence Plot is to confirm that amplification in the control wells is as expected and to view 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

In the Results tab:

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

1. Select **Presence/Absence Plot** from the drop-down list.
2. Click to configure the plot:
   - Target Reporter: target defined for the target of interest
   - Control Reporter: target defined for the IPC
   - For initial view of the Presence/Absence Plot, select:
     - 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 15).

3. Confirm that amplification in the negative and blocked IPC control wells is as expected.
   - 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_T values should be Undetermined.
   - View the amplification plots for the negative controls (see Figure 16 and "Optimize display of negative controls in the Amplification Plot" on page 29).

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.

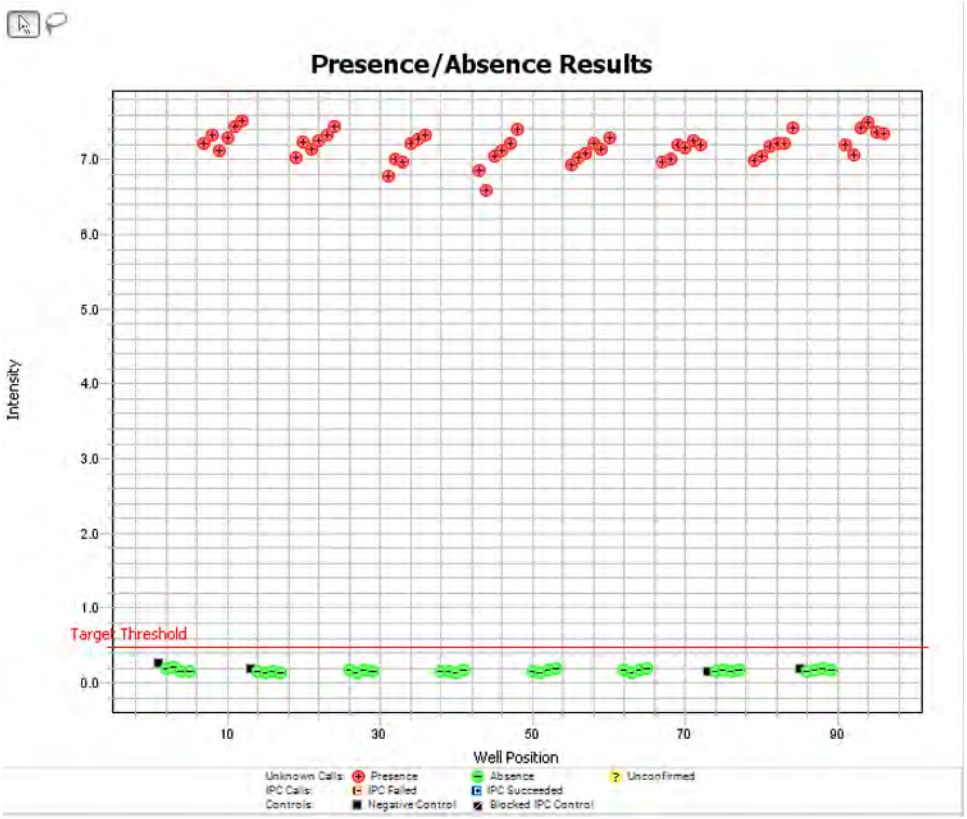


Figure 15 Example Presence/Absence Plot

The IPC results have been filtered out in this example.

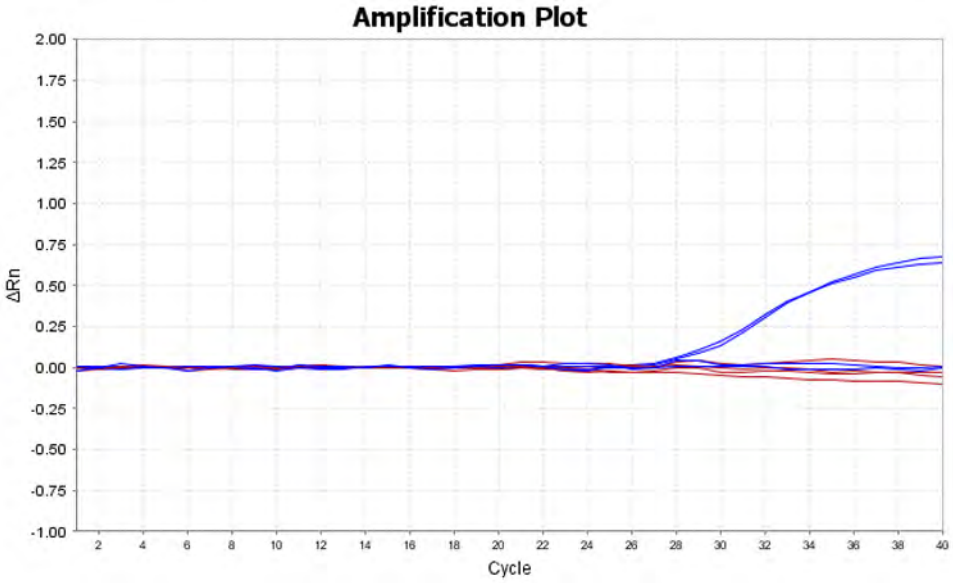


Figure 16 Example Amplification Plot for negative control and blocked IPC

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.

About call settings for Presence/Absence experiments

Use the Call Settings tab to:

- Change the default data analysis settings.

Data Analysis Settings option	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.

- Edit the default call settings (for assays without custom call settings) or custom call settings for individual assays.

Call setting	Description
Confidence Value	Select the confidence value 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.

Refer to the software Help system for step-by-step instructions for adjusting the call settings.

7 Getting started with Melt Curve experiments

- About Melt Curve experiments 73
- Set up a Melt Curve experiment in the software 74
- Set up Melt Curve reactions 75
- Review results 75

About Melt Curve experiments

Overview

The Melt Curve experiment is used to determine the melting temperature (Tm) of the amplification products of a PCR reaction that used SYBR™ Green dye.

Melting temperature (Tm) 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 Tm. 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 SYBR™ Green reagents.

Reaction types

Table 24 Reaction types for Melt Curve experiments

Reaction type (task)	Sample description
Unknown	Previously run PCR reactions that used SYBR™ Green reagents
No-template control (NTC)	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 an experiment.
2. In the Properties tab, enter the information for the experiment.
3. In the Method tab, enter the reaction volume for the experiment.
   (*Optional*) Edit the ramp increment in the melt curve (see "Edit the ramp increment for the Melt Curve dissociation step" on page 82).
4. Define and assign well attributes in the Quick Setup pane of the Plate tab.
   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 their respective fields.
      - Select previously defined samples and targets from their respective drop-down lists.

      Note: When you enter a new sample or target name in the Quick Setup pane, the software automatically populates default values for Reporter (FAM) and Quencher (NFQ-MGB) and assigns the task (U Unknown). To change these default values, click **Advanced Setup** (see "Define samples and targets or SNP assays" on page 11 or "Sample, target, and SNP assay libraries" on page 84).
5. Assign plate attributes in the Quick Setup pane of the Plate tab.
   a. In the Plate Attributes group, select the Passive Reference from the drop-down list.
6. (*Optional*) Assign tasks in the Advanced Setup pane of the Plate tab.
   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 drop-down 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 plate from a SYBR™ Green-based PCR run on another instrument.

Review results

General workflow for analysis of Melt Curve experiments

Assess the Melt Curve Plot (page 76)

▼

(Optional) View the Multicomponent Plot (page 31)

▼

(Optional) View the Raw Data Plot (page 33)

▼

(Optional) Review flags in the QC Summary (page 34)

▼

(Optional) Configure the analysis settings (pages 34, 77)

IMPORTANT! After omission of outliers or configuration of analysis settings, click **Analyze** to reanalyze the experiment.

About the Melt Curve Plot

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

The purpose of viewing the Melt Curve Plot is to confirm that the amplification products in a well display a single melting temperature (Tm). Multiple peaks in a melt curve indicate non-specific amplification or primer-dimer formation.

Table 25 Melt Curve plots

Plot	Description
Derivative Reporter (-Rn') vs. Temperature	Displays the derivative reporter signal in the y-axis. The peaks in the plot indicate significant decrease in SYBR™ Green signal, and therefore the Tm of the amplified products. Use this plot to confirm a single Tm of the amplification products.
Normalized Reporter (Rn) 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

In the Results tab:

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

1. Select **Melt Curve Plot** from the drop-down list.
2. Click to configure the plot:
   - 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, indicating non-specific amplification or formation of primer-dimers.
4. Review the Well Table for the calculated Tm in each well.

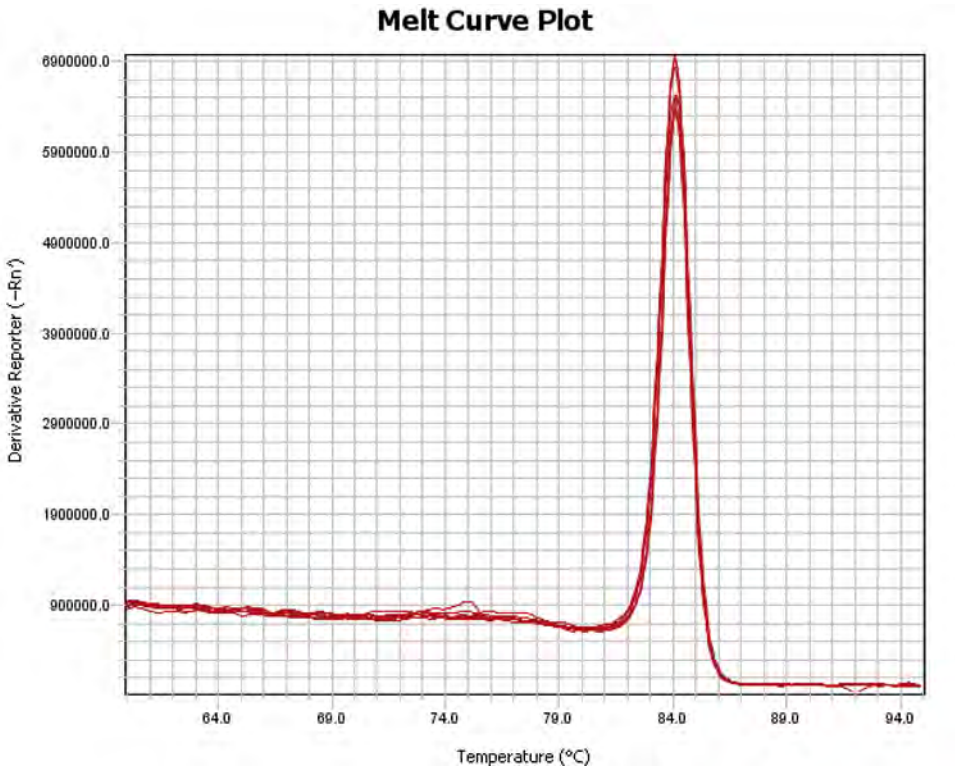


Figure 17 Example Melt Curve Plot

About Melt Curve settings

Use the Melt Curve Settings tab to:

- Enable or disable Multi-Peak Calling.

Multi-Peak Calling	Description
Enabled	More than one PCR product is expected, to determine the Tm for multiple peaks.
Disabled	Only one PCR product is expected.

- (*For multi-peak calling only*) Adjust the threshold 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.

Refer to the software Help system for step-by-step instructions for adjusting the Melt Curve settings.

Alternative procedures and shortcuts for setting up an experiment

- Set up a custom experiment 79
- Adjust method parameters 81
- Edit the ramp increment for the Melt Curve dissociation step 82
- Assign the standard dilutions manually 83
- Shortcuts for experiment setup 83
- Sample, target, and SNP assay libraries 84
- Assign samples using a sample definition file 86
- Assign samples and targets using plate setup files 87
- Assign targets, samples, and biological replicate groups from an *.xls file 88
- Set up an experiment using templates 89

Set up a custom experiment

About custom experiments

Custom experiment setup is required for assays that use multiple PCR stages, such as TaqMan™ Mutation Detection Assays. The 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 26 Default for key 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 27 Key editing options in custom experiments

Item	Description
Run method (thermal protocol)	• Multiple instances of any type of stage can be added, with exceptions noted. • Any stage can be added anywhere, with exceptions noted. Exceptions: – Only one infinite hold, which must be only 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, this order is valid: melt–PCR–Pre-Read–Melt–PCR. • Ramp rates can be edited within software limits. • Data collection can be enabled at any step and during any ramp within a melt stage.
Analysis settings	Editable
Flags	Editable
Filter channel	Editable

Set up a custom experiment in the software

1. In the Home screen, create or open an experiment.
2. In the Properties tab, enter the information for the experiment.
3. In the Method tab, edit the default run method according to the experiment requirements.
4. Define and assign well attributes in the Quick Setup pane of the Plate tab.
   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 their respective fields.
      - Select previously defined samples and targets from their respective drop-down lists.

      Note: When you enter a new sample or target name in the Quick Setup pane, the software automatically populates default values for Reporter (FAM) and Quencher (NFQ-MGB) and assigns the task (U Unknown). To change these default values, click **Advanced Setup** (see "Define samples and targets or SNP assays" on page 11 or "Sample, target, and SNP assay libraries" on page 84).
5. Assign plate attributes in the Quick Setup pane of the Plate tab.
   a. In the Plate Attributes group, select the Passive Reference from the drop-down list.
6. (*Optional*) Assign tasks in the Advanced Setup pane of the Plate tab.
   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 drop-down list.
7. (*Optional*) Define and assign biological replicate groups in the Advanced Setup pane of the Plate tab.
   a. In the Biological Replicates Groups table, enter a name and select a color for each biological replicate group.
   b. Select wells, then select the checkbox of a biological replicate group to assign it to the selected well(s).

Adjust method parameters

See "Method elements" on page 81 for an overview of the method as it is graphically represented.

In the Method tab:

- Click a method parameter field to edit:
  - Sample volume
  - Ramp rate
  - Step temperature
  - Step hold time
  - Number of cycles
- Click-drag to quickly increase or decrease a step temperature.
- Click to switch data collection on or off at each step.
  Data Collection On enables analysis of data collected throughout the PCR, for real-time analysis and troubleshooting.
- Click to configure settings for Auto Delta or VeriFlex™ Zones for individual steps (see "Set up specialized temperature zones" on page 81).

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

  An A or V displays in the PCR stage when Auto Delta or VeriFlex™, respectively, is enabled.
- Click to configure pause settings.
- Adjust the cover temperature via the instrument settings (refer to the touchscreen Help system).
- Refer to the software Help system to:
  - Add or subtract a stage
  - Add or subtract a step from a stage
  - Configure optical filter settings

Method elements

Set up specialized temperature zones

Configure settings for Auto Delta and VeriFlex™ Zones using (Advanced Settings).

The Auto Delta feature allows incremental increase or decrease of a cycle's temperature at a step within a cycling stage (not applicable for Hold and Infinite Hold stages).

VeriFlex™ Zones allow for independent temperature zones within 5°C of adjacent zones: 3 zones for QuantStudio™ 3 Systems and 6 zones for QuantStudio™ 5 Systems. VeriFlex™ Zones temperature settings are not available for 384-well blocks.

In the Method tab:

1. Click within 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 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 of the zones.
     - To hide the display of zones, select **Action ▸ Hide VeriFlex™ Zones**.

   - Select **Auto Delta**, then enter a starting cycle, temperature, and time.

3. Click **Save**.

Edit the ramp increment for the Melt Curve dissociation step

In the Method tab:

- Under the graphical representation of the thermal protocol, select the ramp increment method for the dissociation step.

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 (default is 0.15 °C/s) or use the up/down arrows to set the desired value.

- (*Step and Hold only*) Edit the hold time after each temperature increase.
  a. Click in the time field next to Step and Hold.
  b. Enter a value (default is 5 seconds) or use the up/down arrows to set the desired value.

- (*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 (default is 10) or use the up/down arrows to set the desired value.

Assign the standard dilutions manually

This procedure applies to Standard Curve and Relative Standard Curve experiments only.

In the Plate tab:

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

Shortcuts for experiment setup

- Use sample and target or SNP assay definitions saved in a library (see "Sample, target, and SNP assay libraries" on page 84).
- Import some or all of an experiment setup:

To	Action	Setup information
Import sample and well assignments	Import a sample definition file (page 86)	– Well number – Sample name – *(Optional)* Custom sample properties
Import samples, targets, and well assignments	Import a plate setup file (page 87)	Plate setup information: – Well number – Sample name – Sample color – Target name – Dyes – *(Optional)* Other well 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	Paste from an *.xls file (page 88)	Plate setup information, as above
Import complete experimental setup	Create an experiment from a template (page 89)	– Plate setup information, as above – Reagent information – Thermal protocol – Analysis settings

Sample, target, and SNP assay libraries

About libraries

Libraries contain saved information to reuse in future experiments.

The available libraries are:

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

To access the libraries:

- In the menu bar, select **Tools** ▸ **(Library of choice)...**

  or

- In the Plate tab of an open experiment or template, 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)**.

     or

   - In the Plate tab of an open experiment or template, click **Advanced Setup**, then select **Action** ▸ **Import from Library**.
2. Select a feature from the first drop-down list. Each column of the table is an available feature.
3. Select a condition from the second drop-down 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**.

The list narrows based on the filter applied.

Import to the Sample Library

In the Plate tab:

1. Click **Advanced Setup**.
2. In the Samples table, select **Action** ▸ **Import from Library**.
3. Select **Import...**.
4. Navigate to and select a file, then click **Import**.

Import from the Sample Library to the experiment

In the Plate tab:

1. Click **Advanced Setup**.
2. In the Samples table, select **Action ▸ Import from Library**.
3. (*Optional*) Import sample information from a file (see "Import to the Sample Library" on page 84).
4. (*Optional*) Apply a filter to search for a specific sample (see "Apply a filter to search a library" on page 84).
5. Select one or more samples, then click **Add Selected**.

   Note: **Shift-click** or **Ctl-click** to select multiple samples.

Import to the Target Library

Import target information from an assay information file (AIF) or library export file to the Target Library.

In the Plate tab:

1. Click **Advanced Setup**.
2. In the Targets table, select **Action ▸ Import from Library**.
3. Click **Import...** or **Import AIF...**
4. Navigate to and select a target .txt or .xml file, then click **Import**.

Import from the Target Library to the experiment

In the Plate tab:

1. Click **Advanced Setup**.
2. In the Targets table, select **Action ▸ Import from Library**.
3. (*Optional*) Import targets from an assay information file (AIF) or library export file (see "Import to the Target Library" on page 85).
4. (*Optional*) Apply a filter to search for a specific target (see "Apply a filter to search a library" on page 84).
5. Select one or more targets, then click **Add Selected**.

   Note: **Shift-click** or **Ctl-click** to select multiple targets.

Import to the SNP Assay Library

Only Genotyping experiments contain SNP assay options.

Import SNP assay information from an assay information file (AIF) or exported library file to the SNP Assay Library.

In the Plate tab:

1. Click **Advanced Setup**.
2. In the SNP Assays table, select **Action** ▸ **Import from Library**.
3. Click **Import...** or **Import AIF...**.
4. Navigate to and select a SNP assay file, then click **Import**.

Import from the SNP Assay Library to the experiment

Only Genotyping experiments contain SNP assay options.

In the Plate tab:

1. Click **Advanced Setup**.
2. In the SNP Assays table, select **Action** ▸ **Import from Library**.
3. (*Optional*) Import SNP assay information from an assay information file (AIF) or library export file (see "Import to the SNP Assay Library" on page 86).
4. (*Optional*) Apply a filter to search for a specific SNP assay (see "Apply a filter to search a library" on page 84).
5. Select a SNP assay in the table, then click **Add Selected**.

   Note: **Shift-click** or **Ctl-click** to select multiple SNP assays.

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 comma-delimited file (*.csv) or a tab-delimited text file (*.txt) that contains the following setup information:

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

Create a sample definition file

1. In a spreadsheet program such as Microsoft™ Excel™, 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 & 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 37.

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 & 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 QuantStudio™ desktop Software.

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

`<drive>:\Program Files (x86)\Applied BioSystems\QuantStudio Design & 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.

Set up an experiment using templates

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.

The information saved in an experiment template (.edt) file includes:

- 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.

Options for working with unlocked templates

To	Action
Create a new template (.edt file) from a locked template (.edt file)	1. In the Home screen, select **Create New Experiment ▸ Template**. 2. Navigate to and select the template, then click **Open**. In the new .edt file, all experiment parameters can be modified.
Save the experiment parameters of an experiment (.eds) file as an unlocked template (.edt file) for future runs	1. Open an .eds file in the desktop software. 2. Select **File ▸ Convert Experiment to Template...**. 3. Navigate to the desired location, enter a file name, then click **Save**.
Modify an existing template (.edt file)	1. In the menu bar, select **File ▸ Open...**. 2. Navigate to and select the template (.edt file), then click **Open**. 3. Modify the experiment parameters as needed; all experiment parameters can be modified. 4. Save changes to the new template. • In the menu bar, select **File ▸ Save As ...**. • In any tab, select **Save ▸ Save as...**.

Options for working with locked templates

To	Action
Create a new template (.edt file) from a locked template (.edt file)	1. In the menu bar, select **File ▸ Open...** 2. Navigate to and select the locked template. 3. Click **Open ▸ Enter run experiment information ▸ OK** (no password required). In the new .edt file, parameters that can be modified: • Experiment properties: name, barcode, username, chemistry details, comments • Sample names and assignments • Export configuration
Save the experiment parameters of an experiment (.eds) file as a locked template (.edt file) for future runs	1. In the menu bar, select **File ▸ Save As Locked Template...**. 2. Enter and confirm a password, then click **OK**. 3. Navigate to the desired location, enter a file name, then click **Save**.

To	Action
Modify an existing locked template (.edt file)	1. In the menu bar, select **File ▸ Open...** 2. Navigate to and select the locked template. 3. Click **Open ▸ Edit**, enter the password, then click **OK**. 4. Modify the experiment parameters as needed; all experiment parameters can be modified. 5. In any tab, click **Save** to save changes to the existing locked template. 6. (*Optional*) Save the changes as a new locked template. A locked template cannot be saved as an unlocked template. • In any tab, select **Save ▸ Save as...**, then enter the password. • In the menu bar, select **File ▸ Save As Locked Template...**.

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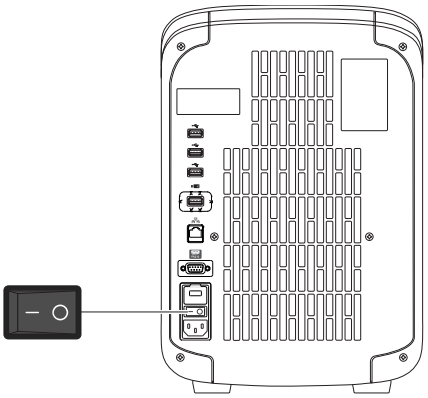
Power on the instrument

To power on the instrument from a powered-off state:

1. Touch anywhere on the touchscreen to determine if the instrument is in sleep mode. If the home screen displays, 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.



The instrument is ready to use when the home screen is displayed.

If left unattended (for about two hours), the instrument automatically enters sleep mode (enabled by default) to conserve power. Refer to the touchscreen Help system for step-by-step instructions for changing the sleep mode setting.

Transfer files from the instrument

If the instrument completes a run without a connection to the desktop software, the experiment data must be manually transferred.

You can use a USB drive plugged into the front-panel USB port to transfer files.

In the instrument home screen:

Touch **Settings** ▸ **Run History**.

- Touch an individual run to view details, then touch **Delete** to delete the record, or **Transfer** to export the experiment .eds file.
- Touch **Manage** to select multiple items for simultaneous viewing, deletion, or transfer.

Guests (users not signed-in) can only view guest records. Users with instrument profiles can also view their own records. Administrators can view all experiment records.

C

Parts and materials

Kits, consumables, and accessories

The following kits, consumables, and reagents are used with the QuantStudio™ 3 and 5 Systems.

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Note: Store ROI/Uniformity, Background, Dye, and RNase P plates at –20°C and use them by the expiration date on the packaging. All other consumables can be stored at room temperature.

Compatible reagents

To perform	Recommended reagent kits	Cat. no.
Reverse transcription	SuperScript™ VILO™ cDNA Synthesis Kit	4453650
TaqMan™ PCR	TaqMan™ Fast Advanced Master Mix	4444557
	TaqMan™ GTXpress™ Master Mix	4401892
	TaqMan™ Fast Virus 1-Step Master Mix	4444432
	TaqMan™ Gene Expression Master Mix	4369016
	TaqMan™ Genotyping Master Mix	4371355
	TaqMan™ Universal Master Mix II, with UNG	4440038
	TaqMan™ RNA-to-CT™ 1-Step Kit	4392938
SYBR™ Green PCR	Fast SYBR™ Green Master Mix	4385612
	Power SYBR™ Green PCR Master Mix	4367659
	Power SYBR™ Green RNA-to-CT™ 1-Step Kit	4389986

96-Well (0.2-mL) consumables

Consumable	Contents	Cat. no.
MicroAmp™ Optical 8-Cap Strip	300 strips	4323032
MicroAmp™ Optical 8-Tube Strip (0.2 mL)	125 strips	4316567
MicroAmp™ Optical Tube without Cap (0.2 mL)	2000 tubes	N8010933
MicroAmp™ 96-Well Tray/Retainer Set (blue) (for 0.2 mL)	10 sets	4381850
MicroAmp™ Optical 96-Well Reaction Plate (0.2 mL)	10 plates	N8010560
	500 plates	4316813
MicroAmp™ EnduraPlate™ Optical 96-Well Reaction Plate with Barcode (blue) (0.2 mL)	20 plates	4483343
MicroAmp™ Optical Adhesive Film Kit	1 kit	4313663

Calibration or instrument verification plate	Cat. no.
96-Well Region of Interest (ROI) and Background Plates (2 plates)	4432364
QuantStudio™ 3 or 5 10-Dye Spectral Calibration Kit, 96-Well 0.2-mL	A26343
96-Well 0.2-mL Spectral Calibration Plate 1 (containing FAM™, VIC™, ROX™, and SYBR™ dyes)	A26331
96-Well 0.2-mL Spectral Calibration Plate 2 (containing ABY™, JUN™, and MUSTANG PURPLE™ dyes)	A26332
96-Well 0.2-mL Spectral Calibration Plate 3 (containing TAMRA™, NED™, and Cy^{fi} 5 dyes)	A26333
96-Well 0.2-mL TaqMan™ RNase P Instrument Verification Plate	4432382

384-well consumables

Consumable	Contents	Cat. no.
MicroAmp™ Optical 384-Well Reaction Plate with Barcode	50 plates	4309849
	500 plates	4326270
	1000 plates	4343814
MicroAmp™ EnduraPlate™ Optical 384-Well Reaction Plate with Barcode (clear)	20 plates	4483285
	500 plates	4483273
MicroAmp™ Optical Adhesive Film Kit	1 kit	4313663

Calibration or instrument verification plate	Cat. no.
384-Well Region of Interest (ROI) and Background Plates (2 plates)	4432320
QuantStudio™ 3 or 5 10-Dye Spectral Calibration Kit, 384-Well	A26341
384-Well Spectral Calibration Plate 1 (containing FAM™, VIC™, ROX™, TAMRA™, and SYBR™ dyes)	A26334
384-Well Spectral Calibration Plate 2 (containing ABY™, JUN™, MUSTANG PURPLE™, NED™, and Cy^{fi} 5 dyes)	A26335
384-Well TaqMan™ RNase P Instrument Verification Plate	4455280

96-Well Fast (0.1-mL) consumables

Consumable	Contents	Cat. no.
MicroAmp™ Optical 8-Cap Strip	300 strips	4323032
MicroAmp™ Optical Fast 8-Tube Strip (0.1 mL)	125 strips	4358293
MicroAmp™ Optical Fast Tube with Cap (0.1 mL)	1000 tubes	4358297
MicroAmp™ 96-Well Tray (blue) (for 0.1 mL)	10 trays	4379983
MicroAmp™ Optical 96-Well Fast Reaction Plate (0.1 mL)	10 plates	4346907
MicroAmp™ EnduraPlate™ Optical 96-Well Fast Reaction Plate with Barcode (clear) (0.1 mL)	20 plates	4481194
	500 plates	4483494
MicroAmp™ Optical Adhesive Film Kit	1 kit	4313663

Calibration or instrument verification plate	Cat. no.
96-Well Fast Region of Interest (ROI) and Background Plates (2 plates)	4432426
QuantStudio™ 3 or 5 10-Dye Spectral Calibration Kit, 96-Well 0.1-mL	A26342
96-Well 0.1-mL Spectral Calibration Plate 1 (containing FAM™, VIC™, ROX™, and SYBR™ dyes	A26336
96-Well 0.1-mL Spectral Calibration Plate 2 (containing ABY™, JUN™, and MUSTANG PURPLE™ dyes)	A26337
96-Well 0.1-mL Spectral Calibration Plate 3 (containing TAMRA™, NED™, and Cy^{fi} 5 dyes)	A26340
96Well Fast TaqMan™ RNase P Instrument Verification Plate	4351979

QuantStudio™ 3 and 5 Systems accessories

Accessory	Contents	Cat. no.
MicroAmp™ Multi-Removal Tool	1 tool	4313950
MicroAmp™ Cap Installing Tool (handle style)	1 tool	4330015
MicroAmp™ Optical Adhesive Film Kit	1 kit	4313663
MicroAmp™ Optical Adhesive Film	25 films	4360954
	100 films	4311971
MicroAmp™ Adhesive Film Applicator	5 applicators	4333183
Real Time PCR Grade Water	10 × 1.5 mL tubes	AM9935
Handheld Barcode Scanner	1 scanner	448842
High Power USB WiFi Module	1 module	A26774

General-use materials and consumables

The following general-use materials and consumables are required to calibrate, maintain, and operate the instrument. Unless indicated otherwise, all materials shown below are available from major laboratory suppliers (MLS).

Material/consumable	Source
Bleach, 10% solution	MLS
Centrifuge with 96–well plate buckets	MLS
Cotton or nylon swabs and lint–free cloths	MLS
Ethanol, 95% solution	MLS
Optical clear adhesive film for PCR	MLS
Pipettors, 100–µL and 200–µL (with pipette tips)	MLS
Powder–free gloves	MLS
Safety glasses	MLS
Screwdriver, flathead	MLS
Tissue, lint–free	MLS
Deionized water	MLS

Documentation and support

Related documentation

Document	Publication number	Description
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407	Describes the QuantStudio™ 3 and 5 Real-Time PCR Systems hardware and software and provides information on preparing, using, maintaining, and troubleshooting the system.
QuantStudio™ Real-Time PCR System Help	MAN0010422	Describes the QuantStudio™ 3 and 5 Real-Time PCR Systems touchscreen and provides procedures for configuration, calibration, and performing a run.
QuantStudio™ Design and Analysis desktop Software Command-Line Application Guide	MAN0010409	Describes how to use the command-line interface of the QuantStudio™ Design and Analysis desktop Software and provides the procedure to automate the creation of new experiment files and export data from existing files.
QuantStudio™ Design and Analysis desktop Software User Guide	MAN0010408	Describes how to perform the six different experiments on the QuantStudio™ Design and Analysis desktop Software
QuantStudio™ Design and Analysis desktop Software Help	MAN0010415	Describes the QuantStudio™ Design and Analysis desktop Software and provides procedures for common tasks.
SAE Admin Console Help	MAN0010417	Describes the Security, Audit, and e-Signature (SAE) Administrator Console and provides procedures for common tasks.
SAE Admin Console User Guide	MAN0010410	Describes how to use the Security, Audit, and e-Signature (SAE) Administrator Console.

Document	Publication number	Description
QuantStudio™ Design and Analysis cloud Software Help	MAN0010414	Describes the QuantStudio™ Design and Analysis cloud Software and provides procedures for common tasks.
QuantStudio™ 3 and 5 Real-Time PCR Systems Site Preparation Guide	MAN0010405	Explains how to prepare your site to receive and install the QuantStudio™ 3 and 5 Real-Time PCR Systems. Intended for personnel who schedule, manage, and perform the tasks required to prepare the site for installation of the QuantStudio™ 3 and 5 Real-Time PCR Systems.

Note: For additional documentation, see "Customer and technical support" on page 99.

Obtain information from the Help system

The software has a Help system that describes how to use each feature of the software. Click ? to access the Help system.

Customer and technical support

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

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

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

Limited product warranty

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

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). When an experiment uses biological replicate groups in a gene expression project, the values displayed in the Biological Replicates tab 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 C_q 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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