Relative Quantification Analysis Module

USER GUIDE

for use with:

QuantStudio™ Design and Analysis Software v2

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Revision	Date	Description
D.0	15 April 2020	Changes for version 1.3: Remove send to the instrument run queue; add custom attribute; update analysis setting.
C.0	12 December 2019	Changes for version 1.2: Clarify EqC_q; minor UI changes.
B.0	14 October 2019	Changes for version 1.1: Add biogroup information; update overview of relative standard curve analysis; add 7900 file type; minor UI changes.
A.0	26 August 2019	New document for version 1.0.

Contents

1 About the Relative Quantification Analysis Module

The Relative Quantification Analysis Module for QuantStudio™ Design and Analysis Software v2 is used determine the relative quantity of a target of interest in a test sample relative to a reference sample. The analysis module supports relative quantification using either comparative C_t ($\Delta\Delta C_t$) analysis or relative standard curve analysis.

For more information about relative quantification analysis, see Chapter 5, “About relative quantification analysis”.

Compatible data files

The software is compatible with data files for the following real-time PCR instruments:

- QuantStudio™ 7 Pro Real-Time PCR System
- QuantStudio™ 6 Pro Real-Time PCR System
- QuantStudio™ 5 Real-Time PCR System (if the plate file for the instrument run is created using QuantStudio™ Design and Analysis Software v2)
- QuantStudio™ 3 Real-Time PCR System (if the plate file for the instrument run is created using QuantStudio™ Design and Analysis Software v2)
- QuantStudio™ 1 Real-Time PCR System (if the plate file for the instrument run is created using QuantStudio™ Design and Analysis Software v2)

Data files for the following instruments are a legacy file format that can be opened in the software, but can only be saved as the updated file format:

- QuantStudio™ 5 Real-Time PCR System (if the plate file for the instrument run was not created using QuantStudio™ Design and Analysis Software v2)
- QuantStudio™ 3 Real-Time PCR System (if the plate file for the instrument run was not created using QuantStudio™ Design and Analysis Software v2)
- QuantStudio™ 1 Real-Time PCR System (if the plate file for the instrument run was not created using QuantStudio™ Design and Analysis Software v2)
- QuantStudio™ 6 Flex Real-Time PCR System
- QuantStudio™ 7 Flex Real-Time PCR System
- QuantStudio™ 12K Flex Real-Time PCR System (except OpenArray™ format)
- StepOnePlus™ Real-Time PCR System
- ViiA™ 7 Real-Time PCR System

- 7500/7500 Fast Real-Time PCR System
- 7900HT Real-Time PCR System

Note: To convert a legacy data file into the updated file format, open the data file, then click **Actions ▸ Save As**.

2 Workflows for relative quantification analysis

Workflow: Relative standard curve analysis

Set up a plate file for relative quantification analysis (page 10)

Select a system template or existing plate file to set up a new plate file (page 10)

▼

Confirm or edit the run method for relative quantification analysis (page 11)

▼

Confirm or edit the plate setup for relative quantification analysis (page 11)

▼

Review and save the plate file (page 16)

▼

Perform relative quantification analysis (page 17)

Review results in the Amplification Plot (page 17)

▼

Select the Relative Quantification Analysis Module (page 17)

▼

Review results in the Standard Curve Plot (page 17)

▼

Review results in the Gene Expression Plot (page 18)

▼

(Optional) Review results in the Endogenous Plot (page 19)

▼

(Optional) Omit outliers from relative quantification analysis (page 20)

▼

(Optional) Review dye signal profile in the MultiComponent Plot (page 21)

▼

(Optional) Review signal profile in the Raw Data Plot (page 21)

▼

(Optional) Edit relative quantification analysis settings (page 21)

Workflow: Comparative C_t analysis

Set up a plate file for relative quantification analysis (page 10)

Select a system template or existing plate file to set up a new plate file (page 10)

▼

Confirm or edit the run method for relative quantification analysis (page 11)

▼

Confirm or edit the plate setup for relative quantification analysis (page 11)

▼

Review and save the plate file (page 16)

▼

Perform relative quantification analysis (page 17)

Review results in the Amplification Plot (page 17)

▼

Select the Relative Quantification Analysis Module (page 17)

▼

Review results in the Gene Expression Plot (page 18)

▼

(Optional) Review results in the Endogenous Plot (page 19)

▼

(Optional) Omit outliers from relative quantification analysis (page 20)

▼

(Optional) Review dye signal profile in the MultiComponent Plot (page 21)

▼

(Optional) Review signal profile in the Raw Data Plot (page 21)

▼

(Optional) Edit relative quantification analysis settings (page 21)

3 Set up a plate file for relative quantification analysis

For detailed instructions about setting up a plate file, see **Help ▸ Help Contents**.

Select a system template or existing plate file to set up a new plate file

A plate file contains the information that is necessary to perform an instrument run, including instrument setup, run method, plate setup, and analysis setting.

A system template is a non-editable plate file that is included with the software.

A new plate file must be created from a system template or a previously created plate file.

For detailed information about system templates and plate files, see **Help ▸ Help Contents**.

1. In the home screen, click **Set Up Plate**.
   The **Plate Gallery** opens to the **System Templates** tab.

2. **IMPORTANT!** Select a system template or a plate file that corresponds to your instrument, block, and run mode. These properties are not editable after the plate file has been created.

   In the left pane, select the appropriate options to filter the system template and plate file lists.

   - **Instrument**
   - **Block**
   - **Run Mode**
   - **Analysis**

     Note: Thermal protocol, plate setup, and post-run analysis options are independent of analysis module selection. Analysis module selection can be changed at any point during plate file set or post-run analysis (see “Select the Relative Quantification Analysis Module” on page 17).

3. Navigate to, then select a system template or plate file.

Tab	Description
System Templates	Contains system templates, non-editable plate files that are included with the software. Select a system template to automatically generate a new plate file that can be edited, then saved.
My Plate Files	Contains plate files that were previously saved to **My Plate Files**. plate files that are included with the software. Select an existing plate file to edit, then save, or to save as a new plate file.
Recents	Contains plate files that were recently opened. Recently opened plate files from **System Templates** and **My Plate Files** do not populate this tab. Select an existing plate file to edit, then save, or to save as a new plate file.

Note: To view all options for opening the plate file, mouse over the plate file, then click ··· **(Actions)**.

The plate file opens in the **Run Method** tab.

Confirm or edit the run method for relative quantification analysis

For most analysis, the default run method is appropriate. The following options are compatible for relative quantification analysis.

- PCR
- 1-step RT-PCR
- 2-step RT-PCR

- In a plate file, in the **Run Method** tab, adjust the run method elements as needed.

  For detailed instructions about editing the run method, see ⓘ **Help ▸ Help Contents**.

- Click ··· **(Actions) ▸ Filter Settings** to confirm or edit filter settings.

Confirm or edit the plate setup for relative quantification analysis

For detailed instructions about plate setup, or to download example plate setup files, see ⓘ **Help ▸ Help Contents**.

Add samples and assign to wells: relative standard curve analysis

For detailed instructions about plate setup, see ⓘ **Help ▸ Help Contents**.

Note: Multiple targets can be assayed using relative standard curve analysis, but each target requires its own standard curve. To set up the standard curve using the **Standard Curve Wizard** see "Set up the standard curve" on page 13.

1. In the **Plate Setup** tab, add samples and assign to wells using the following options.
   - Import a plate setup file
   - Manually add samples to the **Samples** table
   - Manually add samples to wells in the plate layout
2. Confirm or edit sample information in the **Samples** table.

Column	Description
Name	Sample name
Color	Sample color
Type[1]	Relative standard curve analysis uses the following sample types. • Standard[2] **Note:** You must enter the quantity for each standard sample in the **Quantity** column. • Unknown—test and reference samples **Note:** To identify a sample as the reference sample, see "(Optional) Edit relative quantification analysis settings" on page 21 • Negative Control
Quantity (standard samples only)	**Note:** The quantity entered for a standard sample in the **Samples** table is used to populate the **Quantity** column for standard tasks in the **Targets** table (see "Add targets and assign to wells" on page 14).

[1] For more information, see "Sample types for relative standard curve analysis" on page 26.
[2] Each target requires its own standard curve.

3. Confirm or edit sample well assignments in the plate layout.

Add samples and assign to wells: comparative C_t analysis

For detailed instructions about plate setup, see ⓘ **Help ▸ Help Contents**.

1. In the **Plate Setup** tab, add samples and assign to wells using the following options.
   - Import a plate setup file
   - Manually add samples to the **Samples** table
   - Manually add samples to wells in the plate layout

2. Confirm or edit sample information in the **Samples** table.

Column	Description
Name	Sample name
Color	Sample color
Type[1]	Comparative C_t analysis uses the following sample types. • Unknown—test and reference samples **Note:** To identify a sample as the reference sample, see "(Optional) Edit relative quantification analysis settings" on page 21 • Negative Control

[1] For more information, see "Sample types for comparative Ct analysis" on page 27.

3. Confirm or edit sample well assignments in the plate layout.

Set up the standard curve

Note:

- A standard curve is only required for relative standard curve analysis.
- Multiple targets can be assayed using relative standard curve analysis, but each target requires its own standard curve.
- You can also set up the standard curve during sample setup (see "Add samples and assign to wells: comparative Ct analysis" on page 12).

1. In the **Plate Setup** tab, in the plate setup pane, click ··· **(Actions)** ▸ **Standard Curve Setup**.
   The **Standard Curve Wizard** opens.

2. In the **Standard Curve Wizard** pane, enter the sample name prefix.

3. Select the target for the standard curve.

Option	Instructions
Target previously defined	Select the target from the dropdown list.
Target not previously defined	1. Type the target name, the press **Enter**. 2. Select a reporter from the dropdown list. 3. Select a quencher from the dropdown list.

4. Adjust the parameters for the dilution series.
   - **Number of points**—5 recommended
   - **Number of replicates**—3 recommended
   - **Starting Quantity**—The highest or lowest standard quantity, without units.
     Note: The quantity must be ≥1.
   - **Serial Factor**

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

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

5. Select an option to select the wells for the standard
   - Select **Automatically**.
   - Select **Manually**, then select wells using the displayed plate layout.
6. Select to arrange the standards in **Rows** or **Columns**.
7. Click **Apply Standard Curve**, then click **Close** to return to the **Plate Setup** tab.

Add targets and assign to wells

For detailed instructions about plate setup, see **Help ▸ Help Contents**.

Note: Multiple targets can be assayed using standard curve analysis, but each target requires its own standard curve. To set up the standard curve using the **Standard Curve Wizard** see "Set up the standard curve" on page 13.

1. In the **Plate Setup** tab, add targets and assign to wells using the following options.
   - Import an AIF file
   - Import a plate setup file
   - Import TaqMan™ assay plate and card files
   - Manually add targets to the **Targets** table
   - Manually add targets to wells in the plate layout
2. Confirm or edit target information in the **Target** table.

Column	Description
Name	Target name
Color	Target color
Task[1]	The software automatically assigns a task to the target in a well based on the sample type in that well. The following tasks are used for relative quantification analysis. • Standard (relative standard curve analysis only) • Unknown • Negative Control
Quantity (standard tasks only)	The quantity entered for a standard sample in the **Samples** table is used to populate the **Quantity** column for standard tasks in the **Targets** table.

[1] For more information, see "Sample types for relative quantification analysis" on page 26.

3. Confirm or edit target well assignments in the plate layout.

Add biogroups and assign samples

Biogroups, or Biological Replicate Groups, are reactions that contain identical components and volumes, but evaluate separate samples of the same biological source. Biogroups can be used in relative quantification analysis.

1. In the **Plate Setup** tab, in the upper right pane, select an option to add biogroups:

Option	Description
Add biogroups in the **Samples** table	In the **Samples** table, enter the new biogroup name in the **Biogroup** field. The biogroup is added to the following locations: • **Samples** table **Biogroup** dropdown list • **Biogroup** table
Add biogroups in the **Biogroup** table	1. Click **Biogroup**. 2. In the **Biogroup** table, click + **(Add)**.

2. In the **Samples** table, for each sample in a biogroup, select a biogroup from the dropdown list.

3. (*Optional*) Edit the biogroup name or color.
   a. Click **Biogroup**.
   b. In the **Biogroup** table, click a field to edit.

Add a custom attribute to samples

1. In the **Plate Setup** tab, in the **Samples** table, click ··· **(Actions)** ▸ **Add Custom Attribute**.

2. In the **Add Custom Attribute** window, enter the custom attribute name, then click **Done**.
   A column for the custom attribute is added to **Samples** table, and a new tab for the custom attribute is created.

3. Select an option to define the choices for the custom attribute:

Option	Description
Define in the **Samples** table	In the **Samples** table, define the custom attribute in the appropriate field in the custom attribute column.
Define in the custom attribute tab	1. In the custom attribute tab, click + **(Add)**. 2. In the table, click in the field to edit the custom attribute name.

   The custom attribute options are added to the following locations:
   - **Samples** table, in the dropdown list in the custom attribute column
   - Table in the custom attribute tab

4. In the **Samples** table, for each sample, select the custom attribute from the dropdown list.
5. (*Optional*) Edit the custom attribute name or color.
   a. Select the custom attribute tab.
   b. In the table, click a field to edit.

Edit reagent information

1. In the **Plate Setup** tab, in the **Targets/SNP Assays** table pane, click **Reagents**.
2. In the **Reagents** table, click + **(Add)**.
3. Enter the reagent type, name, part number, lot number, and expiration date.

   Note: If the master mix that you enter is not compatible with the current run method, you have the option to apply the recommended run method for your master mix, instrument, block, and run mode.
4. (*Optional*) Click ⊗ **(Remove)** in the row of a reagent to delete it from the table.

Select a passive reference

1. In the upper-left corner of the **Plate Setup** tab, select a passive reference from the dropdown list.
2. (*Optional*) Save the plate file or data file.

Review and save the plate file

1. In the **Run Summary** tab, review the run method selections, then edit if needed.
2. Review the plate setup, then edit if needed.
3. *(Optional)* Click the barcode field, then scan the plate barcode.
4. *(Optional)* Select **Add to My Plates**.
5. Select an instrument from the list.

   If the instrument does not appear on the list, click ⚙ **System ▸ Instruments** to add a new instrument.
6. Save the plate file.

Start the run on an instrument. For specifics on starting an instrument run, see the instrument documentation.

4 Perform relative quantification analysis

Review results in the Amplification Plot

For detailed instructions about reviewing results in the **Amplification Plot**, see **Help ▸ Help Contents**.

If no data are displayed in the **Quality Check** tab, or if reanalysis is required, click **Analyze**.

1. In the **Quality Check** tab, in the plot pane, select **Amplification Plot** from the dropdown list.
2. Review the amplification status for each well.
3. Review or edit threshold settings.
4. Review or edit baseline settings.

Select the Relative Quantification Analysis Module

1. In an open data file, click **Actions ▸ Analysis Modules**.
2. In the **Analysis Modules** window, select **Relative Quantification**, then click **Ok**.
   The Relative Quantification Analysis Module opens.

Click **Analyze**, then review the results in the **Relative Quantification** tab.

Review results in the Standard Curve Plot

1. In the **Relative Quantification** tab, in the plot pane, select **Standard Curve** dropdown list.
2. In the plot pane, click ⚙, then select an option from the **Color By** dropdown list: **Target**, **Sample**, or **Task**.
   The **Standard Curve Plot** is displayed. The target, slope, R^2 value, Y-intercept, amplification efficiency, and error are displayed below the plot.

3. Confirm that the slope, R^2 value, amplification efficiency, and error meet the analysis criteria.

   For more information, see “Standard Curve Plot overview” on page 28.

4. Visually check that all unknown sample C_q values fall in the standard curve range.

5. In the **Well Table**, confirm that the C_q values of all replicate samples meet the analysis criteria (see “Review the results in the Well Table” on page 19).

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

- Omit wells, then reanalyze (see “(Optional) Omit outliers from relative quantification analysis” on page 20).
- Repeat the plate run, adjusting plate file setup and analysis settings to improve results.

Review results in the Gene Expression Plot

For more information about the **Gene Expression Plot**, see “Gene Expression Plot overview” on page 30.

1. In the **Relative Quantification** tab, in the **Result Type** pane, select an option for data analysis:
   - **Sample**
   - **Biogroup**

2. In the plot pane, select **Gene Expression Plot** from the dropdown list.

3. Click **(Settings)** to select an option from the **Plot Type** dropdown list.
   - RQ vs Sample
   - RQ vs Target
   - RQ vs Biogroup

   Note: This option is only available for **Biogroup** analysis (see step 1).

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

(*Optional*) Review results in the Endogenous Plot

For more information about the **Endogenous Plot**, see "Endogenous Plot overview" on page 31.

Select the endogenous controls for analysis (see "(Optional) Edit relative quantification analysis settings" on page 21).

1. In the **Relative Quantification** tab, in the plot pane, select **Endogenous Plot** from the dropdown list.
2. Review the **Endogenous Control Profile**.
3. (*Optional*) To change the endogenous controls used for analysis, select ⚙ **Analysis Settings ▸ Relative Quantification Analysis Setting** (see "(Optional) Edit relative quantification analysis settings" on page 21).
4. Click **Analyze** to see the result using the updated analysis settings.

Review the results in the Well Table

- In the **Relative Quantification** tab, review the results in the Well Table.

Column	Description
Target	Target name
Sample	Sample name
EqC_q Mean	The Equivalent C_q Mean is the arithmetic average of the Equivalent C_q values for technical replicates. Equivalent C_q values are calculated to adjust for differences in PCR efficiency among individual targets within the relative quantification analysis. If the target efficiency set to less than 100% in the **Relative Quantification Analysis Settings**, then the Equivalent C_q for a target is calculated as if the PCR efficiency was 100%[1]. **Note:** The **C_q Mean** values in the **Quality Check** tab are not affected by the target efficiencies entered in the **Relative Quantification Analysis Settings**.
Adjusted EqC_q Mean	The Adjusted EqC_q Mean is the Equivalent C_q Mean adjusted based on the **Maximum Allowed C_q Mean** limit defined in the **Relative Quantification Analysis Settings**[2]. Wells with C_q scores greater than the maximum allowed value are adjusted to the specified C_q limit.
ΔEqC_q Mean	ΔEqC_q Mean is the arithmetic average of the technical replicate Equivalent C_q values for the sample replicate group. **Note:** The ΔEqC_q mean is calculated at the reaction plate level and represents the mean difference between the target C_q values and the endogenous control C_q values for all the technical replicates for that sample that are present on the plate.

Column	Description
ΔEqC_q SE	ΔEqC_q SE is the standard error of the sample-replicate-group-level Equivalent C_q values. **Note:** The ΔEqC_q SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level Equivalent C_q value variation between the target and the endogenous control.
$\Delta\Delta EqC_q$	$\Delta\Delta EqC_q$ is the fold change between the EqC_q values for a replicate group sample and the reference sample.
RQ	The calculated relative level of gene expression for the replicate group that is associated with the test sample.
RQ Min	View the minimum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The minimum includes the variability associated with the endogenous control and targets in only the test samples.
RQ Max	View the maximum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The maximum includes the variability associated with the endogenous control and targets in only the test samples.

[1] Adjust a target efficiency in the **Endo Controls** tab of the **Relative Quantification Analysis Settings** (see "(Optional) Edit relative quantification analysis settings" on page 21).

[2] Adjust the **Maximum Allowed C_q Mean** in the **General** tab of the **Relative Quantification Analysis Settings** (see "(Optional) Edit relative quantification analysis settings" on page 21).

(*Optional*) Omit outliers from relative quantification analysis

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

1. In the **Quality Check** tab, in the **Well Table**, select **Omit** in the row of the outlier well.
2. Click **Analyze** to reanalyze the data with any outliers removed.

(*Optional*) Review dye signal profile in the MultiComponent Plot

For more information about the **MultiComponent Plot**, see **Help ▸ Help Contents**.

If no data are displayed in the **Quality Check** tab, or if reanalysis is required, click **Analyze**.

1. In the **Quality Check** tab, in the plot pane, select **MultiComponent Plot** from the dropdown list.
2. Review the signal profiles for the passive reference dye, reporter dye, and negative control wells.
3. Review the plot to ensure that there are no irregularities in the dye signals.

(*Optional*) Review signal profile in the Raw Data Plot

For detailed instructions about reviewing results in the **Raw Data Plot**, see **Help ▸ Help Contents**.

If no data are displayed in the **Quality Check** tab, or if reanalysis is required, click **Analyze**.

1. In the **Quality Check** tab, in the plot pane, select **Raw Data Plot** from the dropdown list.
2. Click-drag the **Cycle Number** slider through all of the cycles, then confirm that each filter displays the characteristic signal increase.

(*Optional*) Edit relative quantification analysis settings

- We recommend that you analyze data with the default analysis settings. Edit the default analysis settings for analysis of unexpected run data, then reanalyze the data.
- For more information about analysis settings, see **Help ▸ Help Contents**.

Open the Relative Quantification Analysis Module.

1. Click **Actions** ▸ **Relative Quantification Analysis Setting**.

2. In the **General** tab, edit the analysis settings if needed.

Parameter	Description
RQ Min/Max Calculations	Select 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 Deviation**—Select to calculate the RQ minimum and maximum values based on the selected number of standard deviations.
Maximum Allowed C_q Mean	Turn on to automatically use the target that exceeds the designated C_q value as the default for analysis. **Maximum Allowed C_q Mean** is used to calculate the Adjusted EqC $_q$ Mean (see "Review the results in the Well Table" on page 19).
Analysis Type	Select **Singleplex** or **Multiplex** analysis.

3. In the **Endo Controls** tab, select the **Normalization Type**.

Option	Description
Specific endogenous controls	Select to specify one or more specific targets for use as endogenous controls in your analysis
Global normalization	Select to have the software algorithmically normalize the C_q scores for the relative quantification calculation. The global normalization option can be useful when analyzing data in which the amount of input RNA varies significantly from sample to sample. When configured for global normalization, the software first finds assays common to every sample in the analysis and then uses the median C_q of those assays as a normalization factor, on a per sample basis **Note:** Global normalization is not supported for multiplex analysis.
Skip normalization	Select to calculate the direct ΔC_q of the test and calibrator samples by omitting the intermediate target normalization step from the relative quantification calculation. By skipping normalization, the normalized quantities in the analyzed data reflect the fold change indicated between two samples directly. **Note:** We recommend target normalization for most analysis.

4. (*Optional*) If using specific endogenous controls, select the normalization preference for the targets.

5. (*Optional*) To adjust the target efficiency, enter a new value in the **Efficiency** field.

   Note: Target efficiency is used to calculate Equivalent C_q values (see "Review the results in the Well Table" on page 19). If the target efficiency set to less than 100, then the Equivalent C_q for a target is calculated as if the PCR efficiency was 100.

6. In the **References** tab, edit the analysis settings if needed.

Parameter	Description
Group Category	(*Optional*) Select the biogroup[1] or custom attribute from the dropdown list.
Reference Group	Select the reference group from the dropdown list.
Reference Sample	Select the reference sample from the dropdown list.

[1] Only required for biogroup analsyis.

7. Click **Apply**.

The data is reanalyzed using the updated analysis settings.

5 About relative quantification analysis

Relative quantification analysis is used to determine the relative quantity of a target of interest in a test sample relative to a reference sample. You can use relative standard curve analysis or comparative C_t analysis for relative quantification (see “Relative quantification: relative standard curve vs. comparative Ct” on page 25).

Relative quantification analysis is commonly used for the following applications.

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

Overview of relative standard curve analysis

For relative standard curve analysis, the software performs the following tasks.

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

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

   The reference sample is used as the basis for relative quantification results (or 1× sample). For example, in a study of drug effects on gene expression, an untreated control is an appropriate reference sample.
2. The software generates standard curves for the target of interest and the endogenous control using data from the corresponding standard dilution series.
3. The software uses the standard curves to adjust the C_q value of each target proportionally to achieve equivalent target efficiencies.
4. The calculated equivalent C_q values for the target of interest are normalized to the endogenous control.
5. To determine the relative quantity of the target in test samples, the software compares the normalized ΔC_q for the sample to the normalized ΔC_q for the reference sample.

For a comparison of relative standard curve analysis to the comparative C_t ($\Delta\Delta C_t$) analysis, see "Relative quantification: relative standard curve vs. comparative Ct" on page 25.

Overview of comparative C_t analysis

For comparative C_t analysis, the software performs the following tasks.

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

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

   The reference sample is used as the basis for relative quantification results (or 1× sample). For example, in a study of drug effects on gene expression, an untreated control is an appropriate reference sample.
2. The measurements for the target of interest are normalized to the endogenous control.
3. To determine the relative quantity of the target in test samples, the software compares the normalized ΔC_q for the sample to the normalized ΔC_q for the reference sample.

For a comparison of comparative C_t analysis to relative standard curve analysis, see "Relative quantification: relative standard curve vs. comparative Ct" on page 25.

Relative quantification: relative standard curve vs. comparative C_t

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.

Characteristic	Relative standard curve	Comparative C_t
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.

Sample types for relative quantification analysis

Sample types for relative standard curve analysis

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

Sample type (Type column in Samples table)	Sample description	Automatic target task assignment[1] (Type column in Targets table)
Standard	A sample that contains known or known relative quantities of the target and is used to generate standard curves • For known quantities—quantify the target in the standard sample using an independent method. • For known relative quantities—generate a relative dilution series of the target standards.	Standard
Unknown	Test sample	Unknown

Sample type (Type column in Samples table)	Sample description	Automatic target task assignment[1] (Type column in Targets table)
Unknown	Reference sample—The sample that is used as the basis for relative quantification results[2]	Unknown
No-template control (NTC/ Negative Control)	Water or buffer No amplification of the target should occur in NTC wells.	Negative Control

[1] The software automatically assigns a task to the target in a well based on the sample type in that well.

[2] To identify a sample as the reference sample, see "(Optional) Edit relative quantification analysis settings" on page 21.

- The precision of quantification experiments improves as the number of replicate reactions increases. Set up the number of replicates appropriate for the analysis.
- 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). For best results, use a concentrated template, such as a plasmid or PCR product.

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

Sample types for comparative C_t analysis

Comparative C_t analysis includes the following sample types for the endogenous control target and each target of interest.

Sample type (Type column in Samples table)	Sample description	Automatic target task assignment[1] (Task column in Targets table)
Unknown	Test sample	Unknown
	Reference sample—The sample that is used as the basis for relative quantification results[2]	
No-template control (NTC/ Negative Control)	Water or buffer No amplification of the target should occur in NTC wells.	Negative Control

[1] The software automatically assigns a task to the target in a well based on the sample type in that well.

[2] To identify a sample as the reference sample, see "(Optional) Edit relative quantification analysis settings" on page 21.

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

Plots

Standard Curve Plot overview

The **Standard Curve Plot** displays the standard curve for samples designated as standards. The software calculates the quantity of a target in an unknown sample using the standard curve.

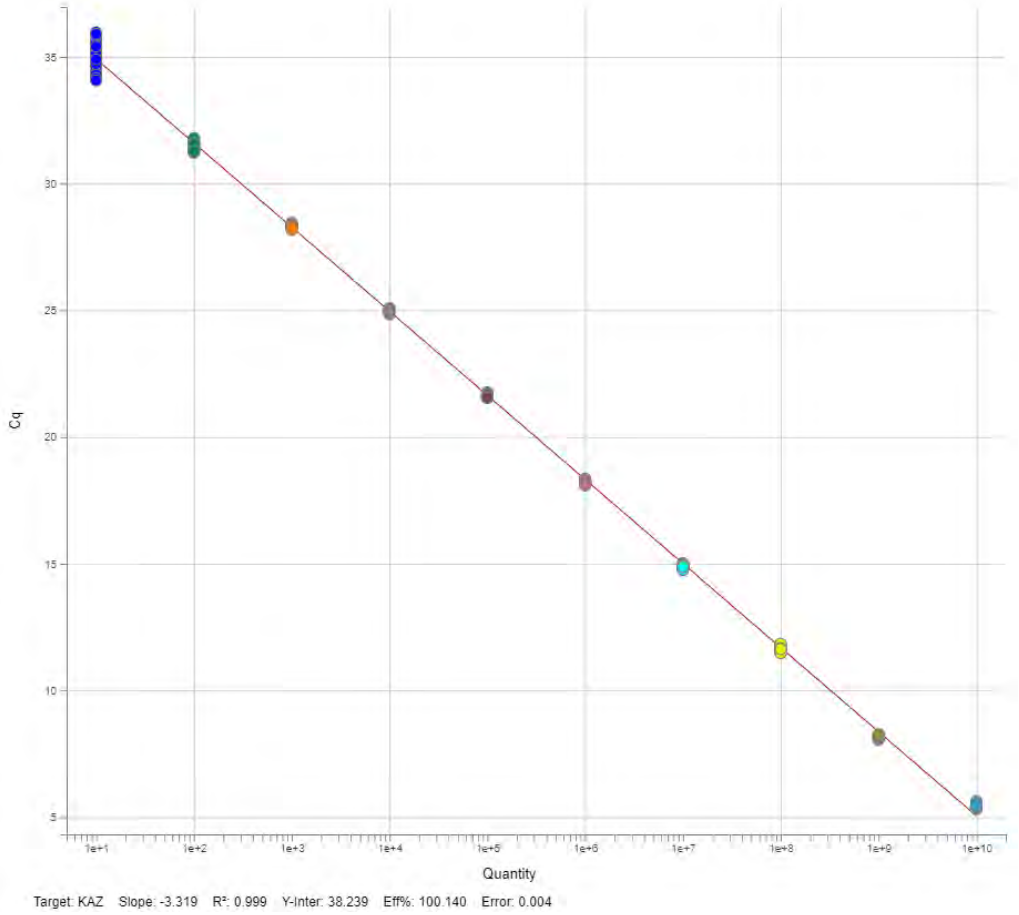

Figure 1 Example Standard Curve Plot

Table 1 Results or metrics to review in the Standard Curve Plot

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

Gene Expression Plot overview

The **Gene Expression Plot** displays the fold change in the target expression levels in test samples relative to the reference sample.

There are three gene expression plots available, depending on the analysis type.

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.
RQ vs. Biogroup	Groups the relative quantification (RQ) values by biogroup. Each target is plotted for each biogroup.

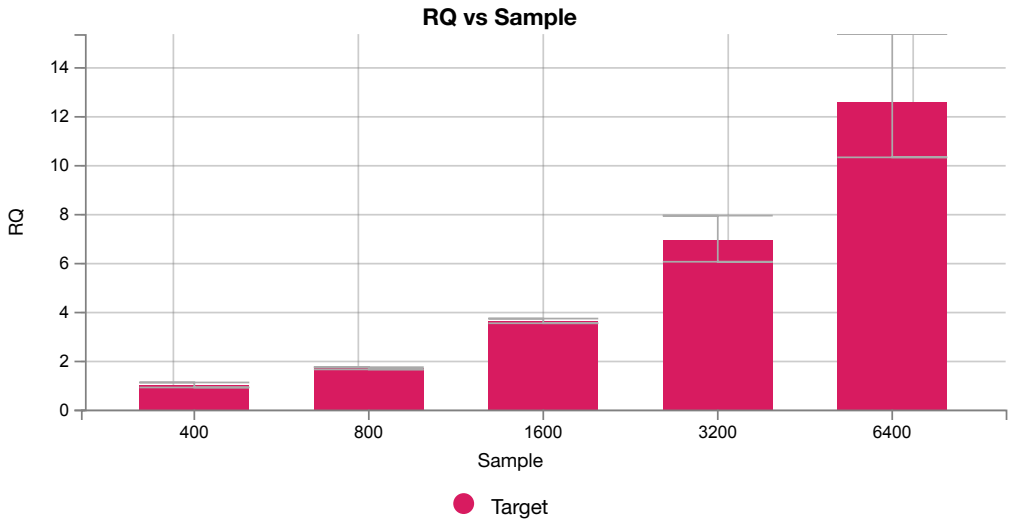

Figure 2 Example Gene Expression Plot

In this example, there is one target of interest, and the reference sample (calibrator) is sample 400.

Endogenous Plot overview

The **Endogenous Plot** is a visual display of the C_q values of potential endogenous control targets across all samples (endogenous control profile).

Use the **Endogenous Plot** to choose the best endogenous control for your analysis. Select the target with a quantity (indicated by C_q value) that does not change under experimental conditions.

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

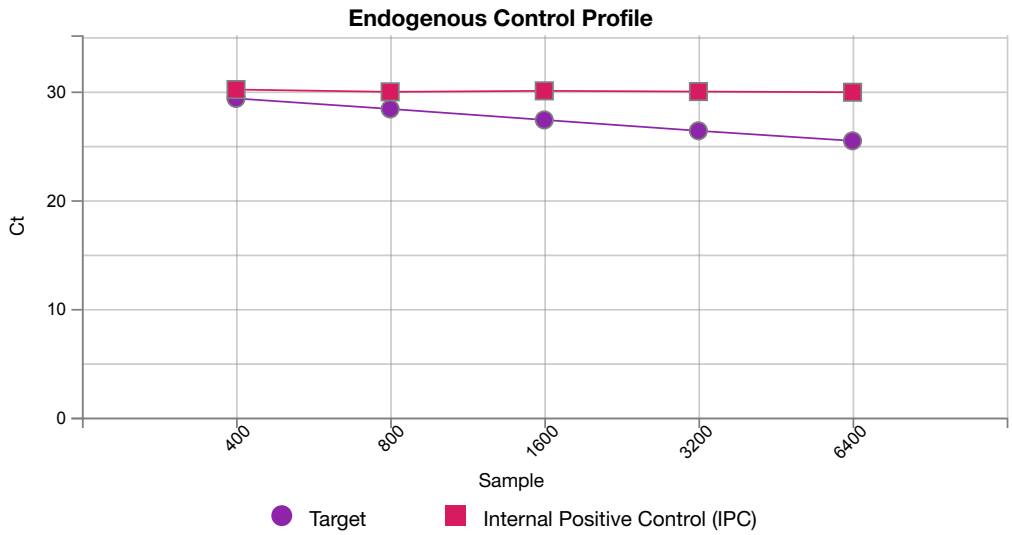

Figure 3 Example Endogenous Plot

Documentation and support

Related documentation

Document	Publication number
QuantStudio™ Design and Analysis Software v2 User Guide	MAN0018200
QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems User Guide	MAN0018045
QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems Site Preparation Guide	MAN0017992

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Index

S

T

W