

Relative Quantification Analysis Module

USER GUIDE

for use with:

Diomni™ Design and Analysis (RUO) Software 3

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

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About the Relative Quantification Analysis Module

The Relative Quantification Analysis Module for Diomni™ Design and Analysis (RUO) Software 3 is used to 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”.



Workflows for relative quantification analysis

Workflow: Relative standard curve analysis

①	Set up a plate file
	Select a system template or existing plate file to set up a new plate file (page 10)
	Confirm or edit the run method (page 11)
	Confirm or edit the plate setup (page 11)
	Review and save the plate file (page 17)

②

Perform relative quantification analysis

Review results in the Amplification Plot (page 19)

Select the Relative Quantification Analysis Module (page 19)

Review results in the Standard Curve Plot (page 19)

Review results in the Gene Expression Plot (page 20)

(Optional) Review results in the Endogenous Plot (page 21)





(Optional) Omit outliers from relative quantification analysis
(page 23)

(Optional) Review dye signal profile in the Multicomponent Plot
(page 23)

(Optional) Review signal profile in the Raw Data Plot (page 24)

(Optional) Edit relative quantification analysis settings (page 24)

Workflow: Comparative C_t analysis

①	Set up a plate file
	Select a system template or existing plate file to set up a new plate file (page 10)
	Confirm or edit the run method (page 11)
	Confirm or edit the plate setup (page 11)
	Review and save the plate file (page 17)

②

Perform relative quantification analysis

Review results in the Amplification Plot (page 19)

Select the Relative Quantification Analysis Module (page 19)

Review results in the Gene Expression Plot (page 20)

(Optional) Review results in the Endogenous Plot (page 21)

(Optional) Omit outliers from relative quantification analysis
(page 23)

(Optional) Review dye signal profile in the Multicomponent Plot
(page 23)

(Optional) Review signal profile in the Raw Data Plot (page 24)

(Optional) Edit relative quantification analysis settings (page 24)

For detailed instructions about setting up a plate file, see the primary user guides for the software. See “Related documentation” on page 35.

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 the primary user guides for the software.

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.

1. Click  **Run templates**.
The **Plate Gallery** is displayed.
2. Click the tab at the top of the **Plate Gallery** that is associated with the type of plate file to be used to set up a new plate file.

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

3. 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 setup or post-run analysis (see “Select the Relative Quantification Analysis Module” on page 19).

4. Open the plate file.

- Hover over the plate file, then click ... **(More Options)**.
The ... **(More Options)** menu for the plate file displays all of the options to open the plate file.
- Click the plate file.

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

Confirm or edit the run method

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

- 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 the primary user guides for the software.
- Click ... **(More Options)** ► **Filter Settings** to confirm or edit filter settings.

Confirm or edit the plate setup

For detailed instructions about plate setup, see the primary user guides for the software.

Add samples and assign to wells: relative standard curve analysis

For detailed instructions about plate setup, see the primary user guides for the software.

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
To assign a sample from the **Samples** table to the well, select a well in the plate layout, then select the checkbox associated with the sample in the **Samples** table.
- Manually add samples to wells in the plate layout
The sample is added to the **Samples** table.

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

Column	Description
Name	Sample name
Color	Sample color
Type ^[1]	<p>Relative standard curve analysis uses the following sample types.</p> <ul style="list-style-type: none"> • 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 24. • 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 29.

^[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 the primary user guides for the software.

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
To assign a sample from the **Samples** table to the well, select a well in the plate layout, then select the checkbox associated with the sample in the **Samples** table.
 - Manually add samples to wells in the plate layout
The sample is added to the **Samples** table.
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. <ul style="list-style-type: none">• Unknown—test and reference samples <p>Note: To identify a sample as the reference sample, see “(Optional) Edit relative quantification analysis settings” on page 24</p> <ul style="list-style-type: none">• Negative Control

^[1] For more information, see “Sample types for comparative Ct analysis” on page 30.

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 13).

-
1. In the **Plate Setup** tab, in the plate setup pane, click ⋮ (**More Options**) ▶ **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	<ol style="list-style-type: none"> 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 greater than 0.

- **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 the primary user guides for the software.

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. <ul style="list-style-type: none"> • 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 29.

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: <ul style="list-style-type: none"> • Samples table Biogroup dropdown list • Biogroup table
Add biogroups in the Biogroup table	a. Click Biogroup . b. 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 **⋮ (More Options) ▶ 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	<ol style="list-style-type: none"> a. In the custom attribute tab, click + (Add). b. 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

In the on-premise configuration, adding, editing, or deleting a reagent is a controlled function. The user role must have the permission of **Add/Edit/Delete Reagent**.

1. In the **Plate Setup** tab, in the **Targets/SNP Assays** table pane, click **Reagents**.
2. In the **Reagents** table, perform one of the following actions.
 - Click **+ (Add)**.
 - Click **⋮ (More Options) ▶ Export Reagents** to export reagents.
 - Click **⋮ (More Options) ▶ Import Reagents** to import reagents.
 - Click **⋮ (More Options) ▶ Scan Reagents** to scan reagents.

3. If you are manually adding reagents or editing reagents, enter the following information in the table.

- | | |
|-----------|-------------------|
| • Name | • Part Number |
| • Type | • Lot Number |
| • Barcode | • 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. If you are scanning the reagent barcode, in the **Scan Reagent** dialog box, select or deselect the **Enable automatic parsing** checkbox.

5. If you are scanning the reagent barcode, when the **Scan Reagent** dialog box is displayed, use a barcode scanner to scan the reagent label.

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.

The fields in the **Scan Reagent** dialog box are populated.

6. In the **Scan Reagent** dialog box, click **Add**.

7. (Optional) Click **✕ (Remove)** in the row of a reagent to delete it from the table.

Select a passive reference

In the on-premise configuration, editing the passive reference is a controlled function. The user role must have the permission of **Edit Passive Reference**.

The passive reference is set for the plate. The default passive reference is ROX™ dye.

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

In Thermo Fisher™ Connect Platform, instruments are connected by the InstrumentConnect application.


The QuantStudio™ 6 Pro Real-Time PCR Instrument and the QuantStudio™ 7 Pro Real-Time PCR Instrument can connect to the software.

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**.

This option allows you to create new plate files using the current plate file as a template.

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 more information about starting a run, see the documentation for the instrument.



Perform relative quantification analysis

Review results in the Amplification Plot

For detailed instructions about reviewing results in the **Amplification Plot**, see the primary user guides for the software.

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 31.


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 22).

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 23).
- 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 33.

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 34.

Select the endogenous controls for analysis (see “(Optional) Edit relative quantification analysis settings” on page 24).

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 24).
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
Name	The sample name or the biogroup name. The biogroup name is used if biogroups are defined for the run.
Target	Target name
EqC _q Mean	<p>The Equivalent C_q Mean is the arithmetic average of the Equivalent C_q values for technical replicates.</p> <p>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].</p> <p>Note: The EqC_q mean is available only for singleplex assays.</p> <p>The C_q Mean values in the Quality Check tab are not affected by the target efficiencies entered in the Relative Quantification Analysis Settings.</p>
Adjusted EqC _q Mean	<p>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].</p> <p>If this is included in the relative quantification calculation, wells with C_q scores greater than the maximum allowed value are adjusted to the specified C_q limit. Otherwise it is undetermined and it is not included in the relative quantification calculation.</p> <p>Note: The adjusted EqC_q mean is available only for singleplex assays.</p>
ΔEqC _q Mean	<p>ΔEqC_q Mean is the arithmetic average of the technical replicate Equivalent C_q values for the sample replicate group.</p> <p>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.</p>
ΔEqC _q SE	<p>ΔEqC_q SE is the standard error of the sample-replicate-group-level Equivalent C_q values.</p> <p>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.</p>
ΔΔEqC _q	ΔΔEqC _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	<p>View the minimum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings.</p> <p>Note: The minimum includes the variability associated with the endogenous control and targets in only the test samples.</p>

(continued)

Column	Description
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 24).

^[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 24).

(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 support C_q precision, consider omitting the outliers from analysis.

In relative quantification analysis, the outliers are omitted in the **Quality Check** tab.

For detailed information about setting up the plot in the **Quality Check** to omit outliers, see the primary user guides for the software.

1. In the **Quality Check** tab, select **Amplification Plot** from the dropdown list.
2. Omit outliers.
 - In the **Well Table**, select **Omit** in the row of the outlier well.
 - In the **Plate Layout**, select a well or multiple wells, then select **⋮ (More Options) ▶ Omit Wells**.
 - In the amplification plot, click and drag around the data to omit. The selected data are displayed in the **Well Table** and the **Plate Layout**. Omit the wells in the **Well Table** or the **Plate Layout**.
3. 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 the primary user guides for the software.

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 confirm 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 the primary user guides for the software.

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 the primary user guide for the software.

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	<p>Select the algorithm used to calculate the relative quantification minimum and maximum values (error bars).</p> <ul style="list-style-type: none"> • 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	<p>Turn on to automatically use the target that exceeds the designated C_q value as the default for analysis.</p> <p>Maximum Allowed C_q Mean is used to calculate the Adjusted EqC_q Mean (see “Review the results in the Well Table” on page 22).</p>
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	<p>Select to have the software algorithmically normalize the C_q scores for the relative quantification calculation.</p> <p>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</p> <p>Note: Global normalization is not supported for multiplex analysis.</p>
Skip normalization	<p>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.</p> <p>Note: We recommend target normalization for most analysis.</p>

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 22). 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 analysis.

7. Click **Apply**.

The data are reanalyzed using the updated analysis settings.

Export the results

In the on-premise configuration, the location to save the file is defined in the export settings. For users with the permission of **Edit Export Destination**, the location can be selected for a file download. For users without the permission of **Edit Export Destination**, the location cannot be selected.

In the Thermo Fisher™ Connect Platform, the results are exported to the downloads folder of the computer. The file name and location cannot be selected.

For more information about setting up the export location, see the primary user guides for the software.

1. In the table pane, click ... **(More Options) ▶ Export**.
In the Thermo Fisher™ Connect Platform, the file is downloaded.
2. *(Optional)* In the **Export CSV** dialog box, edit the file name in the **File Name** field.
The **File Name** field is populated with a default file name.
3. Click **Browse** to select a location to save the file.
The **Browse** button is not available in the on-premise configuration.
4. Click one of the following options.
 - Click **Download**, then select a location for the file download. This option is available for the on-premise configuration, for users who have the permission of **Edit Export Destination**.
 - Click **Save**. This option is available for the on-premise configuration, for users who do not have the permission of **Edit Export Destination**.
 - Click **Export**. This option is available in the desktop configuration.



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 C_t ” on page 28).

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 C_t ” on page 28.

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 C_t ” on page 28.

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.	<ul style="list-style-type: none"> • 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.	<ul style="list-style-type: none"> • 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 <ul style="list-style-type: none"> 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
	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 24.

- 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 24.

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.

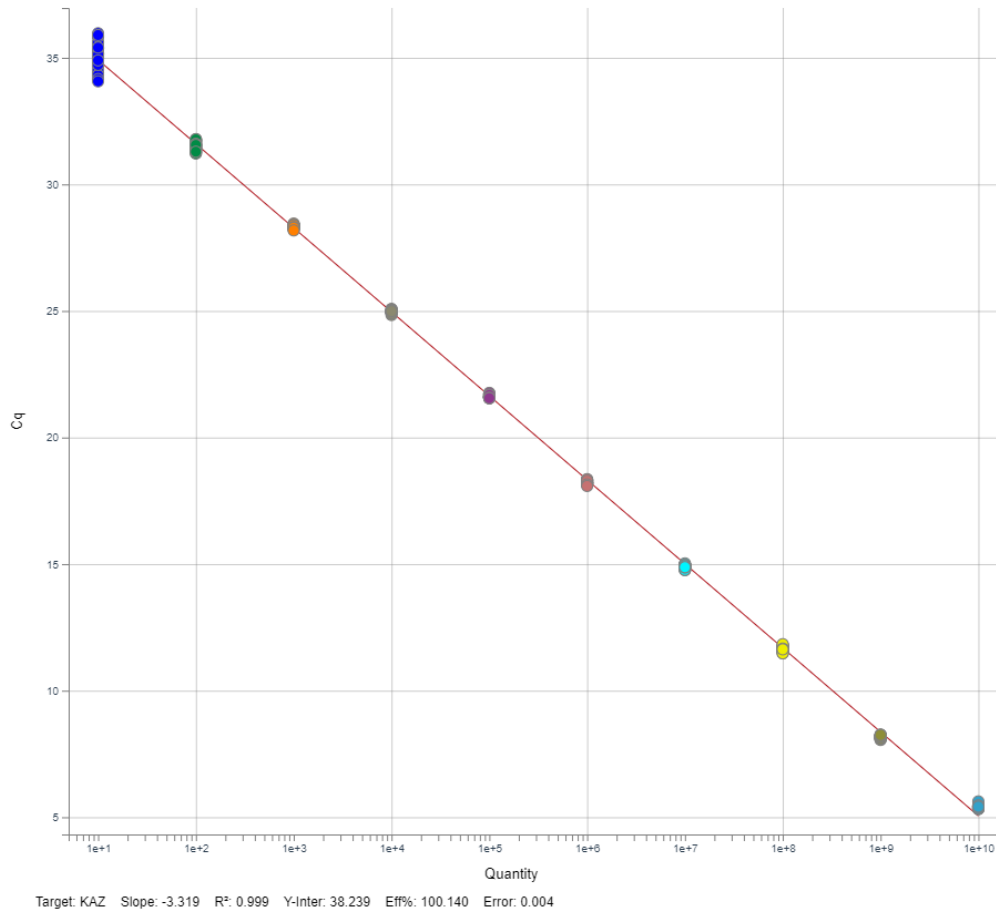


Figure 1 Example Standard Curve Plot

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.	<p>A slope close to -3.3 indicates optimal, 100% PCR amplification efficiency.</p> <p>Factors that affect amplification efficiency:</p> <ul style="list-style-type: none"> • 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: <ul style="list-style-type: none"> – 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.	<ul style="list-style-type: none"> • 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	<p>The standard error of the slope of the regression line in the standard curve.</p> <p>The error can be used to calculate a confidence interval (CI) for the slope and therefore the amplification efficiency.</p>	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.	<p>A C_q value > 8 and < 35 is desirable.</p> <ul style="list-style-type: none"> • 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.

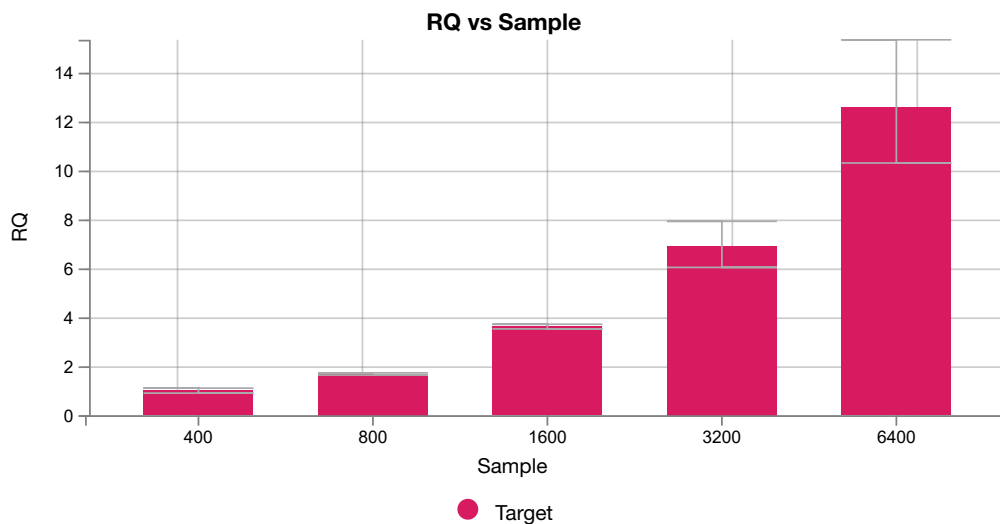


Figure 2 Example Gene Expression Plot In this example, there is one target of interest, and the reference sample (calibrator) is sample 400.

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.

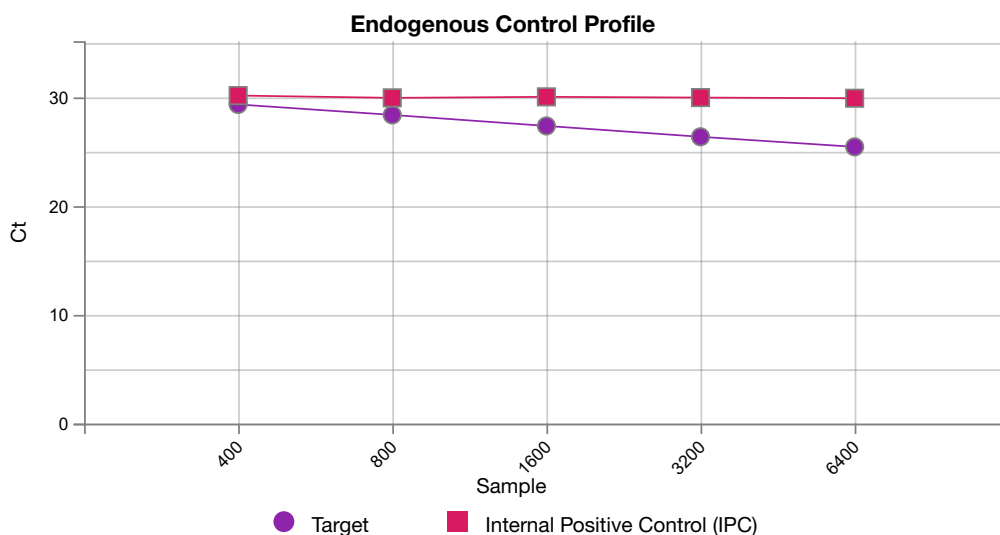


Figure 3 Example Endogenous Plot



Documentation and support

Related documentation

Document	Publication number
<i>Diomni™ Design and Analysis (RUO) Software 3 (Desktop) User Guide</i>	MAN0030162
<i>Diomni™ Design and Analysis (RUO) 3 User Guide (Thermo Fisher™ Connect Platform)</i>	MAN0030163
<i>Diomni™ Design and Analysis (RUO) Software 3 (On-Premise) User Guide</i>	MAN1000091

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