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Standard Curve Analysis Module

USER GUIDE

for use with:

QuantStudio™ Design and Analysis Software v2

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Revision	Date	Description
C.0	5 July 2023	The document was updated for QuantStudio™ Design and Analysis Software v2.7 and the Standard Curve Analysis Module v1.7. • The list of compatible data files was removed. See the documentation for the main software for this information. • The instructions to set up a standard curve were updated. The starting quantity must be greater than 0.
B.0	15 April 2020	Changes for version 1.4: Remove send to the instrument run queue; add Replicate Group Table.
A.0	26 August 2019	New document.

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

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About the Standard Curve Analysis Module

The Standard Curve Analysis Module for QuantStudio™ Design and Analysis Software v2 is used to determine absolute target quantity in test samples.

For more information about standard curve analysis, see Chapter 5, “About standard curve analysis”.

2 Workflow: Standard curve analysis

For detailed instructions about setting up a plate file, or reviewing data in the **Quality Check** tab, see **Help ▸ Help Contents**.

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3 Set up a plate file for standard curve 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 Standard Curve Analysis Module” on page 13).

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, hover over the plate file, then click ··· **(Actions)**.

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

Confirm or edit the run method for standard curve analysis

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

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

Add samples 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 10.

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]	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 • Negative Control
Quantity (standard samples only)	Enter the quantity for the standard sample. **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 9).

[1] For more information, see "Sample types for standard curve analysis" on page 16.
[2] Each target requires its own standard curve.

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

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 10.

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
   - Manually add targets to the **Targets** table

- Manually add targets to wells in the plate layout
- Import TaqMan™ assay plate and card files

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 standard curve analysis. • Standard • 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 standard curve analysis" on page 16.

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

Set up the standard curve

Note:

- Multiple targets can be assayed using standard curve analysis, but each target requires its own standard curve.
- To import a standard curve from a different data file, see "(Optional) Edit standard curve analysis settings" on page 15.
- You can also set up the standard curve during sample setup (see "Add samples and assign to wells" on page 9).

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 if needed.
   - **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.

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)**.

   Note:
   - Click ··· **(Actions)** ▸ **Export Reagents** to export reagents.
   - Click ··· **(Actions)** ▸ **Import Reagents** to import reagents.
   - Click ··· **(Actions)** ▸ **Scan Reagents** to scan reagents.

3. Enter the following information for each reagent.
   - Name
   - Type
   - Barcode
   - Part Number
   - Lot Number
   - 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**.
   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 specifics on starting an instrument run, see the instrument documentation.

4 Perform standard curve 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 Standard Curve Analysis Module

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

Click **Analyze**, then review the results in the **Standard Curve** tab.

Review results in the Standard Curve Plot

1. In the **Standard Curve** tab, in the plot pane, select a target from the **Targets** dropdown list.
2. In the plot pane, click ⚙, then select an option from the **Color By** dropdown list: **Target**, **Sample**, or **Task**.
   The 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 17.
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.
6. In the **Replicate Group Table**, review the quantity mean and quantity SD if needed.

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

- Omit wells, then reanalyze (see "Identify and omit outliers from standard curve analysis" on page 14).
- Repeat the plate run, adjusting plate file setup and analysis settings to improve results.

Identify and omit outliers from standard curve 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 **Standard Curve** tab, in the plot pane, click on an outlier data point to highlight the well in the **Well Table**.

   Outlier wells can also be omitted in the **Quality Check** tab (see **Help ▸ Help Contents**).
2. In the **Well Table**, select **Omit** in the row of the outlier well.
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 **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 standard curve analysis settings

Open the Standard Curve Analysis Module.

1. Click **Actions** ▸ **Standard Curve Analysis Setting**.
2. In the **General Setting** tab, edit the analysis settings if needed.

Standard Curve Analysis Option	Description
On Plate Standard Curves	Select to use the standard curve from the current data file. Click **Export** to export the standard curve.
External Standard Curves	Select to use a standard curve that was previously exported from another data file for analysis in the current data file. The two data files must be from the same instrument type, block type, and run method. • Click **Import**, navigate to the standard curve file, then click **Open**. • Click **Delete** to delete an imported standard curve. • Click **Export** to export the standard curve.

3. Click **Apply**.

The data is reanalyzed using the updated analysis settings.

5 About standard curve analysis

Overview of standard curve analysis

Standard curve analysis is used to determine absolute target quantity in samples.

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

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

Sample types for standard curve analysis

Standard curve analysis includes the following sample types for each target of interest. Each unique target requires its own standard curve.

Sample type (Type column in Samples table)	Sample description	Automatic target task assignment[1] (Task column in Targets table)
Standard	A sample that contains known quantities or known relative quantities of the target • For known quantities—quantify the target in the standard sample using an independent method • For known relative quantities—generate a relative dilution series of the target standards **Note:** You must enter a quantity for each standard sample in the **Samples** table. Do not edit the quantity in the **Targets** table.	Standard
Unknown	Test sample	Unknown
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.

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.

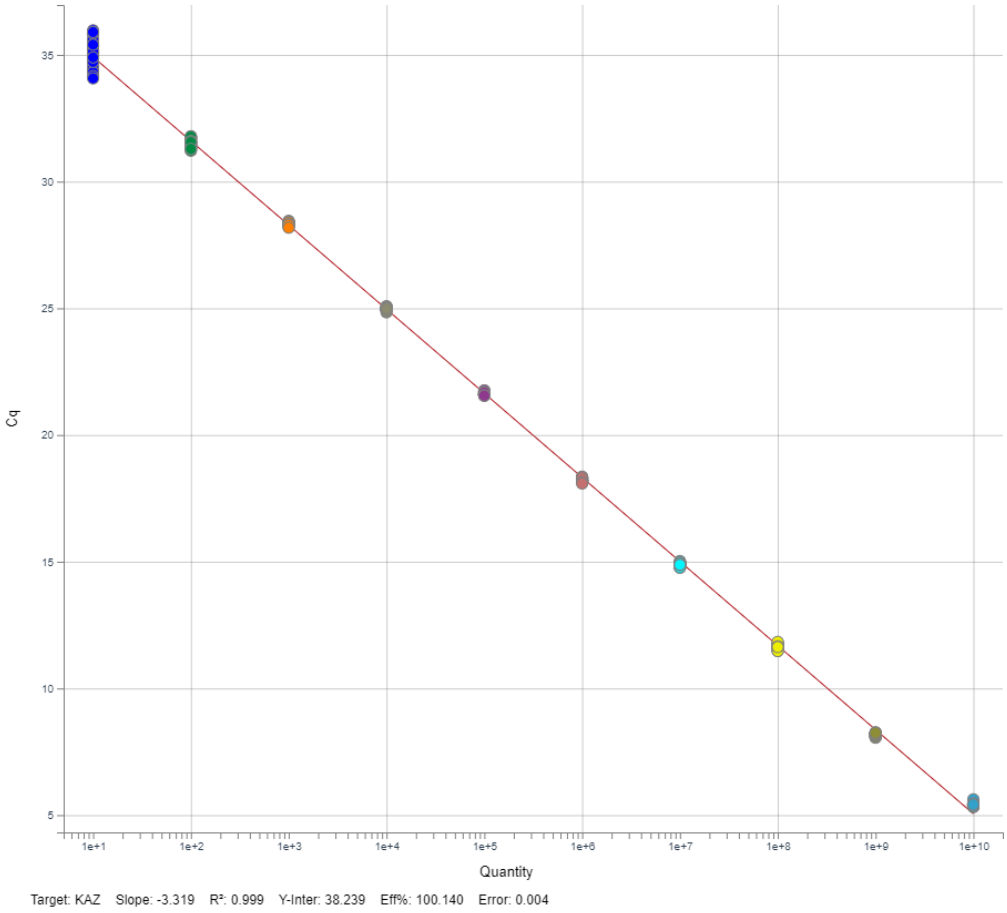

Figure 1 Example Standard Curve Plot

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.

A Documentation and support

Related documentation

Document	Publication number
QuantStudio™ Design and Analysis Software v2 User Guide	MAN0018200
QuantStudio™ Design and Analysis v2 User Guide (Thermo Fisher™ Connect Platform)	MAN0018202

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

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