Multi-Well Plates and Array Card Experiments

USER GUIDE

QuantStudio™ 12K Flex Real–Time PCR System

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Revision C

ThermoFisher SCIENTIFIC

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

Revision history: 4470050 C (English)

Revision	Date	Description
C	7 November 2025	The filepath was updated. The filepath is `<…>:\Program Files\Applied Biosystems\QuantStudio 12K Flex Software` for QuantStudio™ 12K Flex Software v1.7 and later. The filepath is `<…>:\Program Files (x86)\Applied Biosystems\QuantStudio 12K Flex Software` for QuantStudio™ 12K Flex Software v1.6 and earlier.
B	18 July 2024	• The software is not distributed on a CD. The example files are embedded in the software installer. • The default installation drive was updated to `<…>:\Program Files (x86)\Applied Biosystems\QuantStudio 12K Flex Software`, where <…> is the installation drive. The default installation drive is `C:` if the software is installed by the customer. The default installation drive is `D:` if the software is installed by a Thermo Fisher Scientific field service engineer. • Additional files were added to the **User Sample Files** folder (“Data files in the examples folder” on page 16). • The user documentation for the instrument was updated to *QuantStudio™ 12K Flex Real–Time PCR System v1.6 or later Maintenance and Administration Guide* (Pub. No. MAN0018832). • The ReadiApp templates were updated (“Create an experiment using the ReadiApp feature” on page 84). • The cDNA synthesis kit was updated to SuperScript™ VILO™ cDNA Synthesis Kit (Cat. No. 11754250). • The labels for call settings for genotyping experiments were updated (“Call settings” on page 303). • The option to specify a call cycle was added for genotyping experiments (“Call settings” on page 303). • The documentation and support appendix was updated (Appendix A, “Documentation and support”). – The list of materials was removed. For information about materials, see *QuantStudio™ 12K Flex Real–Time PCR System v1.6 or later Maintenance and Administration Guide* (Pub. No. MAN0018832). – The related documentation was updated. – The information about the software help system was updated. – The customer and technical support information was updated. – The limited product warranty was added.
A	1 March 2012	New document.

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

Contents

About this guide

IMPORTANT! Before using this product, read and understand the information the "Safety" appendix in *QuantStudio™ 12K Flex Real–Time PCR System v1.6 or later Maintenance and Administration Guide* (Pub. No. MAN0018832).

Purpose

This document functions as both a tutorial and as a guide for performing your own experiments using the 384-Well, 96-Well (01.ml and 0.2ml), and the TaqMan™ Array Card consumables on the QuantStudio™ 12K Flex Real–Time PCR System.

Prerequisites

This user guide is intended for personnel who have been specifically trained by Thermo Fisher Scientific. The manufacturer is not liable for damage or injury that results from use of this manual by unauthorized or untrained parties.

This guide uses conventions and terminology that assume a working knowledge of the Microsoft™ Windows™ operating system, the internet, and internet-based browsers.

Note: First-time users of the QuantStudio™ 12K Flex Real–Time PCR System, read this booklet thoroughly. The booklet provides information and general instructions that are applicable to all the experiments described in this document.

How to use these booklets as tutorials

Each booklet in this guide provides a tutorial for running an example experiment using QuantStudio™ 12K Flex Software and the example data provided with the software. The following booklets are provided:

- "Getting started" on page 17—Introductory information and experiment workflow common to all experiments.
- "Running standard curve experiments" on page 86—Designing, running, and analyzing a Standard Curve experiment.
- "Running relative standard curve experiments" on page 145 and "Running comparative Ct experiments" on page 198—Designing, running, and analyzing Relative Standard Curve and Comparative C_T experiments.
- "Running genotyping experiments" on page 254—Designing, running, and analyzing a Genotyping experiment.

- "Running presence absence experiments" on page 310—Designing, running, and analyzing a Presence/Absence experiment.
- "Running melt curve experiments" on page 361—Designing, running, and analyzing a Melt Curve experiment.
- Appendix A, "Documentation and support"—Additional documentation and support.

Note: In all parts, the term "experiment" refers to the entire process of performing an experiment, including setup, run, and analysis.

How to use the guides with your own experiments

Each booklet contains instructions specific to an example experiment provided with the software. However, you can use the booklets as guides for your own experiments. Tips for running your own experiments are provided at various points in each booklet.

Assumptions

This guide assumes that you have access to the example experiments provided with the software.

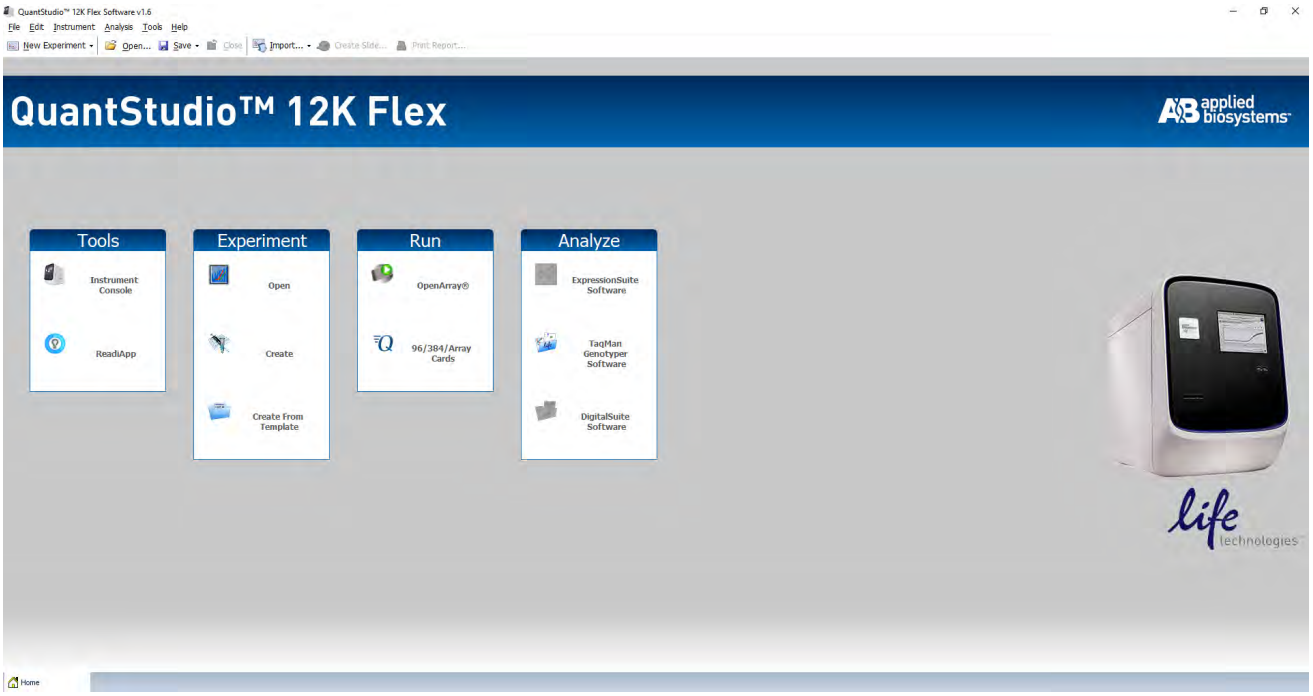
How to access an example experiment

Start the QuantStudio™ 12K Flex Software

Double-click (QuantStudio™ 12K Flex Software shortcut) to access the Home screen, shown below.

Note: You can customize the Home screen by importing an image of your choice. The image is displayed on the right side of the screen.

To personalize the Home screen, go to **Tools** ▸ **Select Welcome Image**. Browse to the image of your choice, then click **OK**.



Note: The icons in the **Analyze** menu appear active only if downloaded the software.

Open an example experiment

In the Home screen, click **Open**, then navigate to one of the following folders, where <…> is the installation drive. The default installation drive is `C:` if the software is installed by the customer. The default installation drive is `D:` if the software is installed by a Thermo Fisher Scientific field service engineer.

- QuantStudio™ 12K Flex Software v1.7 and later—`<…>:\Program Files\Applied Biosystems\QuantStudio 12K Flex Software\examples`
- QuantStudio™ 12K Flex Software v1.6 and earlier—`<…>:\Program Files (x86)\Applied Biosystems\QuantStudio 12K Flex Software\examples`

The `examples` folder contains folders for each type of experiment. Open the folder that corresponds to the experiment, then open the example experiment file.

Data files in the examples folder

- Gene Expression
  - Comparative C_T
  - Relative Standard Curve
- Genotyping
- Melt Curve
- Presence Absence
- Standard Curve

In addition to the above, the examples folder also contains the **User Sample Files** folder:

- `BarCode Template.txt`
- `Custom Sample Properties Example.xls`
- `Human miRNA OA Plate Example.csv`
- `Open Array Plate Format.csv`

User attention words

Five user attention words may appear in this document. Each word implies a particular level of observation or action as described below:

Note: Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! Provides information that is necessary for proper instrument operation or accurate chemistry kit use.

CAUTION! Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury.

Except for IMPORTANT, the safety alert words in user documentation appear with an open triangle figure that contains a hazard symbol. These hazard symbols are identical to the hazard symbols that are affixed to the instrument. See the “Safety” appendix in *QuantStudio™ 12K Flex Real–Time PCR System v1.6 or later Maintenance and Administration Guide* (Pub. No. MAN0018832) for descriptions of the symbols.

Part I

Getting started

1 General information and instructions

Note: For more information about any of the topics discussed in this guide, access the Help from within QuantStudio™ 12K Flex Software by pressing F1, clicking in the toolbar, or selecting **Help ▸ QuantStudio 12K Flex Software**.

Set up an experiment

Define experiment properties

All experiments require the same general setup tasks. Individual booklets supply specific parameters. The following procedures outline general steps to take to set up an experiment.

Access QuantStudio™ 12K Flex Software, then from the **Experiment** menu, click **Create**. Click **Experiment Properties** to access the **Experiment Properties** screen.

Define experiment name and type

1. Enter a unique experiment name in the Experiment Name field. The default is a date and time stamp, which you can change. For example, 2011-12-08 123517.
   - Enter a name that is descriptive and easy to remember. You can enter up to 100 characters.
   - You can only use the alpha-numeric, period (.), hyphen (-), underscore (_) and spaces ( ) characters.

   Note: Make sure each experiment name is unique. If you have named two different experiments with the same name, you cannot run them on the same instrument. You will receive the following error message:

   If you do not want to delete the existing experiment, rename the duplicate experiment and then proceed to the run.

2. *(Optional)* Enter or scan the barcode on the reaction plate. You can enter up to 100 characters in the Barcode field.

3. *(Optional)* Enter a user name to identify the owner of the experiment. You can enter up to 100 characters in the User Name field.

4. *(Optional)* Enter comments to describe the experiment.

5. Select the block type you are using to run the experiment
   - 384-Well
   - Array Card
   - 96-Well (0.2mL)
   - Fast 96-Well (0.1mL)

6. Select the experiment type:
   - Standard Curve
   - Relative Standard Curve
   - Comparative C_T ($\Delta\Delta C_T$)
   - Melt Curve
   - Genotyping
   - Presence/Absence

Select the reagent

Select the reagent you are using to detect the target sequence:

- TaqMan™ Reagents
- SYBR™ Green Reagents
- Other

Note: If you select SYBR™ Green as the reagent, then you have the option of including a melt curve for that experiment.

Define the instrument run properties

1. Select the ramp speed for the experiment:
   - Standard
   - Fast

2. For Genotyping and Presence/Absence experiments, select the options for the data collection to include in the experiment run:
   - **Pre-PCR Read**—To include data before amplification occurs. Use the data collected during pre-PCR read to normalize florescence data collected during post-PCR read.
   - **Amplification**—To include real-time data.
   - **Post-PCR Read**—To include data after amplification has taken place.

3. *(Optional)* For real-time data collection, you can change the default analysis settings in the Preferences for the following:
   - Automatic analysis
   - Automatic save
   - Baseline settings

   Go to **Tools ▸ Preferences**. Click the Experiment tab. Select the Auto Analysis and Auto Save check boxes for the QuantStudio™ 12K Flex Software to automatically analyze and save experiment results. You can also edit the following default baseline settings:

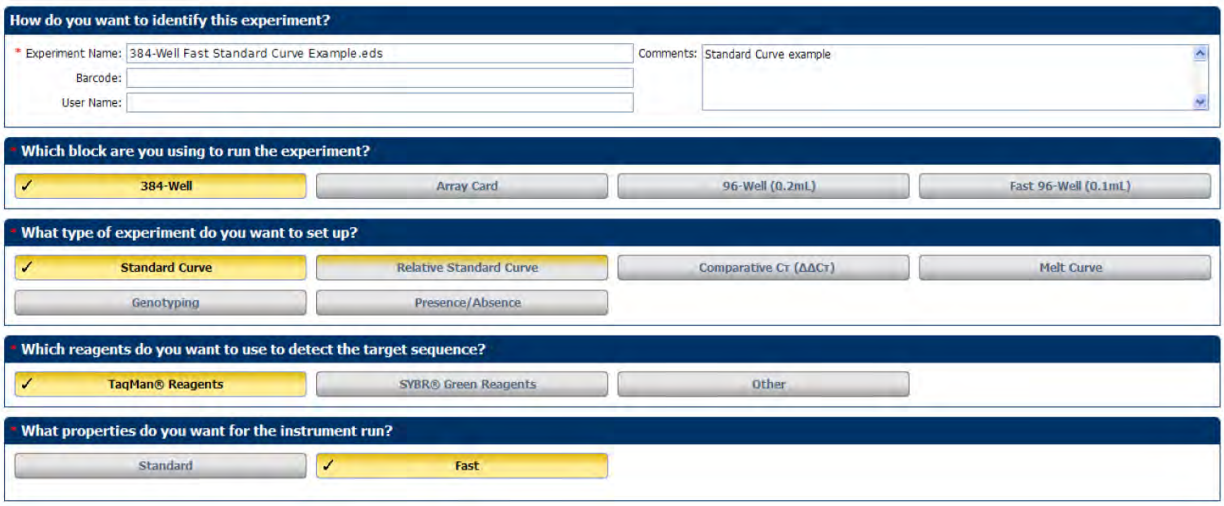
Field	Entry
Start Cycle Number	3 (default)
End Cycle Number	15 (default)

Note: By default, the Auto Analysis and Auto Save check boxes are selected.

4. For the Melt Curve experiment, select the **Include PCR** check box, to include PCR.

5. Save the experiment. The default file name (.eds extension) is the experiment name that you entered when you set up the experiment and saved it for the first time. Changes to the experiment name after the first save do not update the file name. To change the file name, select **File ▸ Save As**.

The **Experiment Properties** screen for a Standard Curve experiment is shown in the following graphic:



Define targets, samples, and biological replicate groups

Use the **Define** screen to define targets, samples and biological replicates for your experiment. For Genotyping experiments, use this screen to specify the number of SNP assays to include in the experiment.

Note: You can start a run without these definitions, but there will be no real-time data in the amplification plots (the amplification plots can be seen only after you have set up the plate).

1. Click **Define** to access the **Define** screen.
2. Define targets.
   a. Click **New** to add targets and define them.
   b. In the target table, click a cell in the **Target Name** column for the target, then enter your target name. The default name is Target 1.
   c. Select the **Reporter** and **Quencher** from the respective drop-down menu.

      Note: The default reporter and quencher dyes used depend on the reagent selected during experiment setup. For example, if TaqMan™ is the selected reagent, the default reporter FAM™ and default quencher is **NFQ-MGB.**

   d. Select the target **Color** from the drop-down menu.
   e. *(Optional)* Click **Save to Library** to save the newly added or existing edited targets to the target library.

      Note: Use the targets from the Target Library to avoid re-entering the information. See "(Optional) Use libraries when designing your own experiments" on page 30 for information on target libraries.

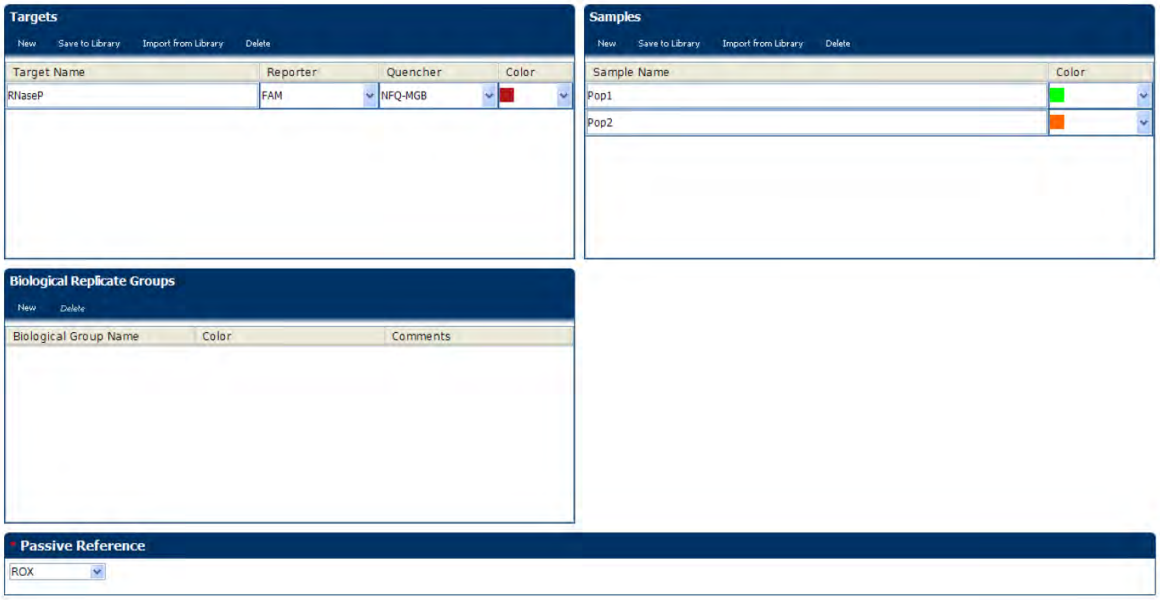
   f. Click **Import from Library** to add targets from the target library.
3. Define samples.
   a. Click **New** to add samples and name them.
   b. In the samples table, click a cell in the **Sample Name** column for the sample to define and enter your sample name. The default sample name is Sample 1.
   c. Select the sample **Color** from the drop-down menu.
   d. *(Optional)* Click **Save to Library** to save the newly added or existing edited samples to the sample library.

      Note: Use the samples from the Sample Library to avoid re-entering the information. See "(Optional) Use libraries when designing your own experiments" on page 30 for information on sample libraries.

   e. Click **Import from Library** to add samples from the sample library.

4. Define biological replicates.
   a. In the **Define Biological Replicates Groups** table, click **New** to add biological replicate group and name them. You can enter up to 100 characters in this field.
   b. Select the **Color** from the drop-down menu.
   c. Click in the **Comments** column to add comments for that biological replicate group.
5. Select the **Passive Reference** from the drop-down menu.

   The **Define** screen for a Standard Curve experiment is shown in the following graphic:



Assign targets, samples, and biological replicate groups

Use the **Assign** screen to assign targets, samples, and biological replicate groups to wells in the reaction plate. For Genotyping experiments, use this screen to assign SNP assays.

Note: You can start a run without these assignments, but there will be no real-time data in the amplification plots (the amplification plots can be seen only after you have set up the plate).

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1. Click **Assign** to access the **Assign** screen.
2. Assign targets.
   a. Select wells using the plate layout or the well table on the **Assign** screen.

b. Select a target and assign its task, in the plate, from the drop-down menu. Depending on the experiment type, options are:

Experiment type	Legend	Tasks
Standard Curve	U	Unknown
	S	Standard
	N	Negative Control
Relative Standard Curve	U	Unknown
	S	Standard
	N	Negative Control
Comparative CT	U	Unknown
	N	Negative Control
Genotyping	U	Unknown
	1/1	Positive Control Allele 1/Allele 1
	2/2	Positive Control Allele 2/Allele 2
	1/2	Positive Control Allele 1/Allele 2
	N	No Template Control
Presence/Absence	U	Unknown
	I	Internal Positive Control
	N	Negative Control
	ł	Blocked Internal Positive Control
Melt Curve	U	Unknown
	N	Negative Control

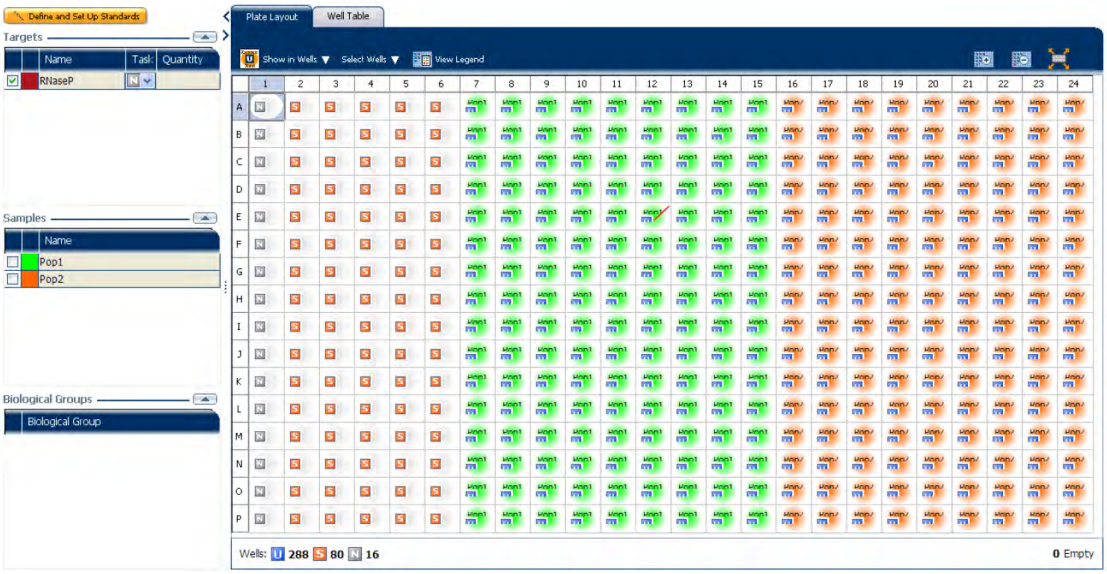
3. Assign Samples.
   a. Select wells using the plate layout or the well table on the **Assign** screen.
   b. Select the check box next to the sample to assign to the selected wells.

   Note: You can assign only one sample to a well.

4. Assign Biological Replicate Groups.
   a. Select wells using the plate layout or the well table on the **Assign** screen.

b. Select the check box next to the biological replicate group to assign to the selected wells.

The **Assign** screen for a Standard Curve experiment is shown in the following graphic:

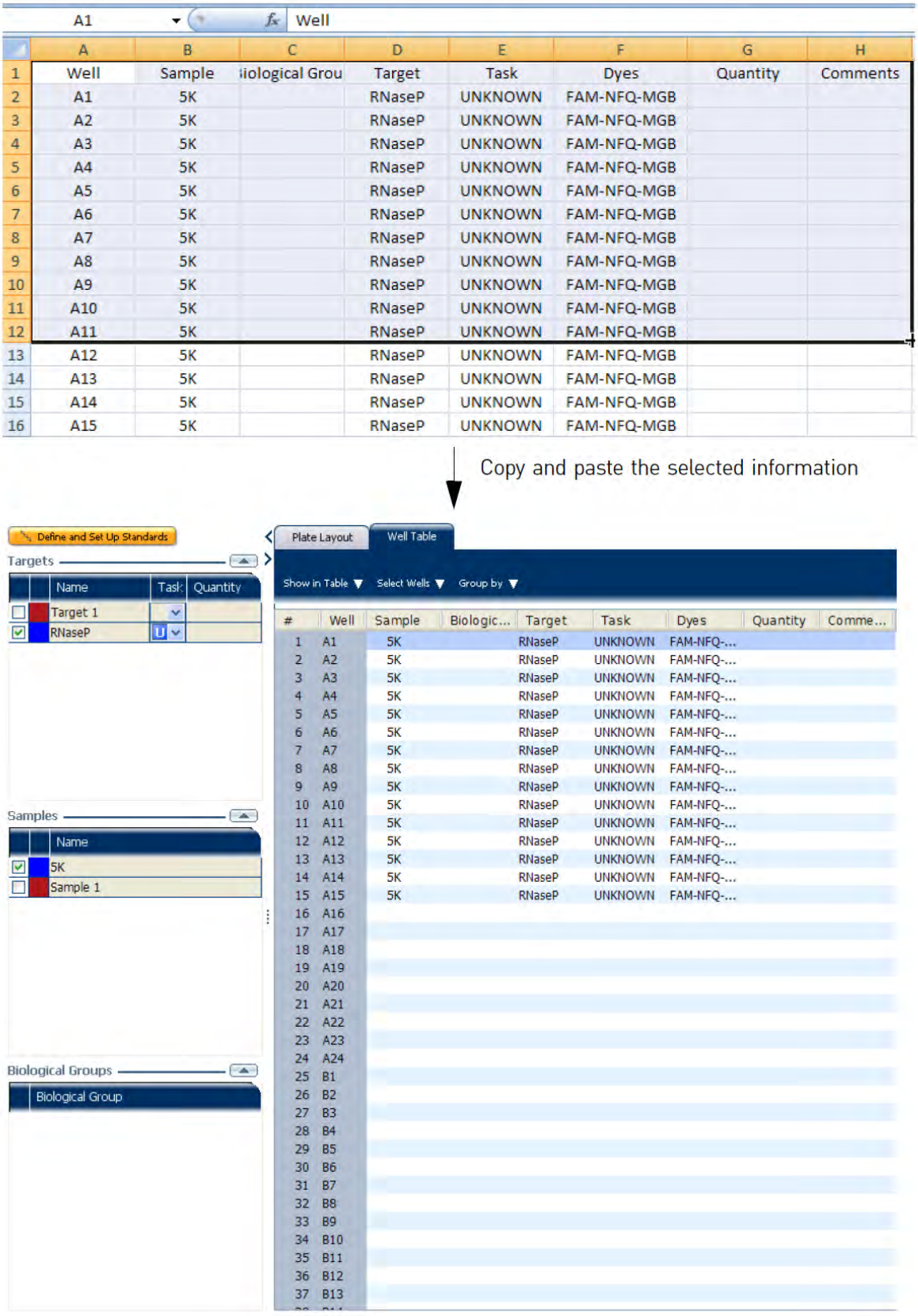


Assign targets, samples, and biological replicate groups - alternate procedure

As shown in the following graphics, you can also paste assignment information from an * **.xls** file into the plate layout of the QuantStudio™ 12K Flex Software for wells with single targets.

Note: You must select the header, and the **Well Number** and **Well Position** columns while copying information from the * **.xls** file.

Note: Any of the columns not copied are treated as NULL values for those columns.



	A	B	C	D	E	F	G	H
1	Well	Sample	iiological Grou	Target	Task	Dyes	Quantity	Comments
2	A1	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
3	A2	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
4	A3	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
5	A4	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
6	A5	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
7	A6	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
8	A7	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
9	A8	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
10	A9	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
11	A10	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
12	A11	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
13	A12	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
14	A13	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
15	A14	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		
16	A15	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB		

#	Well	Sample	Biologic...	Target	Task	Dyes	Quantity	Comme...
1	A1	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
2	A2	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
3	A3	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
4	A4	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
5	A5	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
6	A6	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
7	A7	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
8	A8	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
9	A9	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
10	A10	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
11	A11	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
12	A12	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
13	A13	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
14	A14	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
15	A15	5K		RNaseP	UNKNOWN	FAM-NFQ-...		
16	A16							
17	A17							
18	A18							
19	A19							
20	A20							
21	A21							
22	A22							
23	A23							
24	A24							
25	B1							
26	B2							
27	B3							
28	B4							
29	B5							
30	B6							
31	B7							
32	B8							
33	B9							
34	B10							
35	B11							
36	B12							
37	B13							

Define the run method

Use the **Run Method** screen to set up the run method for your own experiments in the QuantStudio™ 12K Flex Software.

1. Click **Run Method** to access the **Run Method** screen.

   Note: You can save multiple run methods to the Run Method Library for later use. See "(Optional) Use libraries when designing your own experiments" on page 30 for information on run method libraries.

2. Enter a number from 1 to 20 for the reaction volume per well. The instrument supports the following maximum reaction volumes for the consumables listed below:
   - MicroAmp™ Optical 384-Well Reaction Plate - 30 µL
   - Applied Biosystems™ Array Card - 1 µL
   - MicroAmp™ Optical 96-Well Reaction Plate (0.2 mL)- 200 µL
   - MicroAmp™ Optical 96-Well Reaction Plate (0.1 mL)- 100 µL
   - MicroAmp™ Optical 8-Tube Strip with cap (0.2 µL)- 200 µL
   - MicroAmp™ Fast 8-Tube Strip with cap(0.1 µL)- 100 µL
   - MicroAmp™ Optical Reaction Tube without cap (0.2 µL)- 200 µL
   - MicroAmp™ Fast Reaction Tube without cap (0.1 µL)- 100 µL

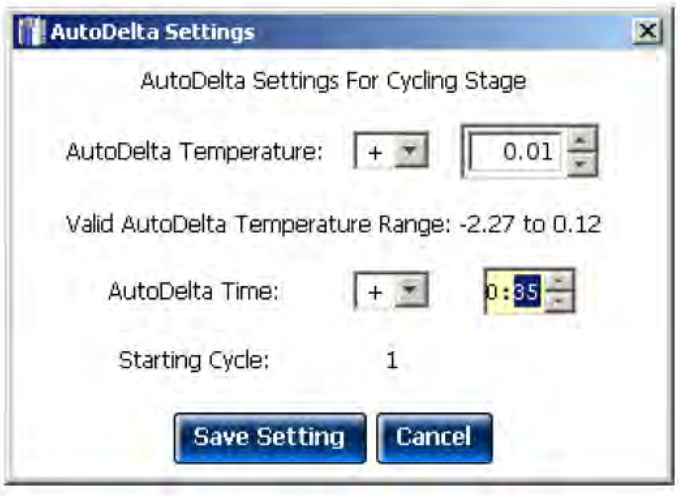
3. In the **Graphical View** tab, review and, if necessary, edit the run method.
   - Make sure that the thermal profile is appropriate for your reagents.
   - Edit the default run method or replace it with one from the run method library included in the QuantStudio™ 12K Flex Software.
   - Enable data collection by clicking  .

     Note: Enabling data collection is especially useful when you later need to analyze data collected in real-time during the various stages.

   - Edit the ramp rate. You can increase or decrease the ramp rate for a stage.

     Note: Ramp rates are decimal numbers from 0.015–3.4.

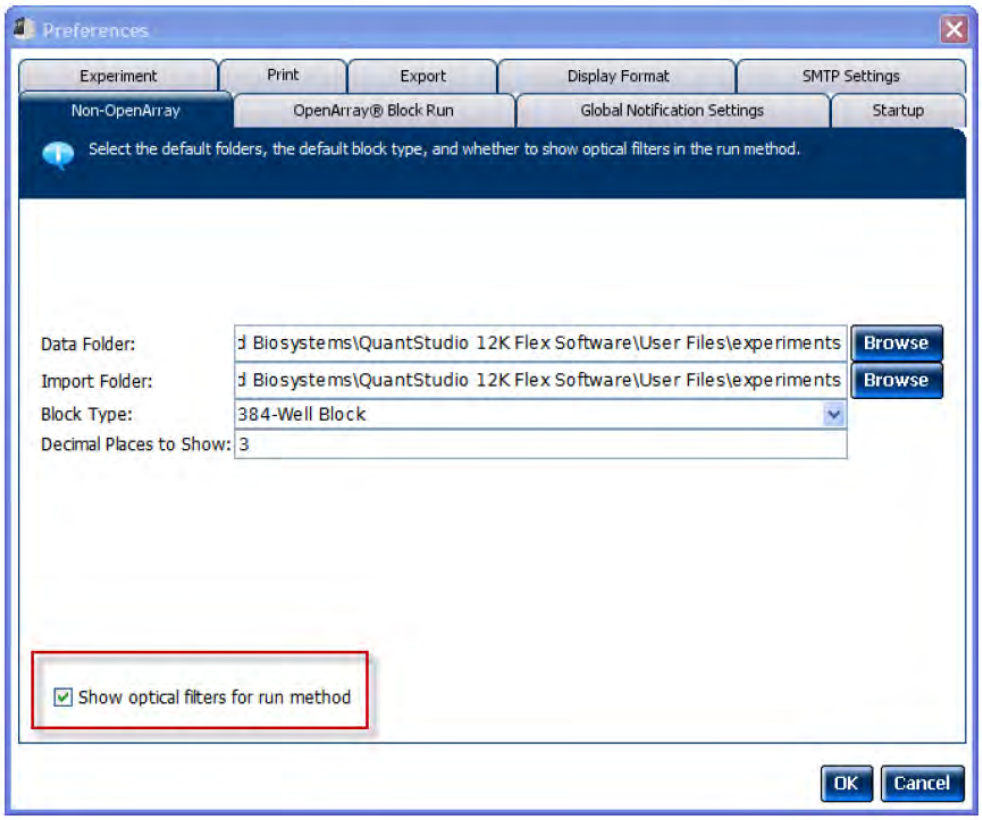
   - Edit the PCR Stage.
   - Change the Number of Cycles for the PCR stage.
   - Select the **Enable AutoDelta** checkbox, to increase or decrease the temperature and/or hold time for each subsequent cycle or to change the Starting Cycle for AutoDelta. Enabling AutoDelta displays the ▲ icon. Click the AutoDelta Off ▲ icon to change the AutoDelta settings for the cycling stage in the AutoDelta Settings dialog box. Then, click **Save Setting** to display the AutoDelta On ▲ icon.



Note: If you selected SYBR™ Green as the reagent, the Melt Curve stage automatically appears in the **Run Method** screen. If you delete the Melt Curve Stage section from the protocol, then the melt curve is active in the **Add Stage** drop-down menu.

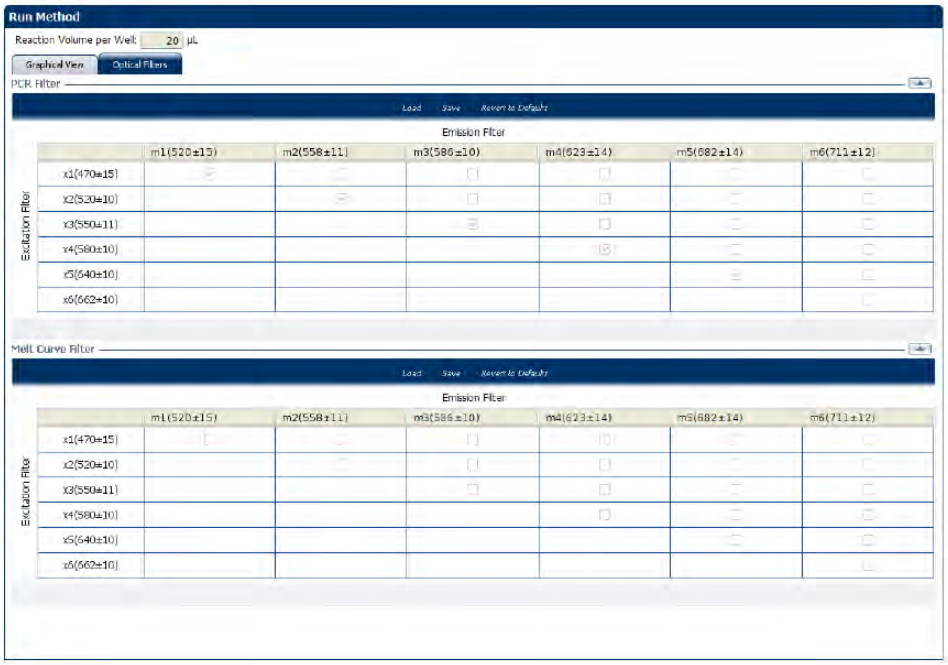
4. Complete the tasks on the **Optical Filters** tab:

   By default, the **Optical Filters** tab is not visible. To show the **Optical Filters** tab, go to **Tools** ▸ **Preferences**, and select the **Show optical filters for run method** checkbox under the **Non-OpenArray** tab.



- To add a new filter set to the filter set library, click **Save**.
- To load a saved filter set, click **Load**.

- To go back to the original filter set combinations, click **Revert to Defaults**.



Note: Select the filter set that matches the profile of the dye you have added to the plate. See *QuantStudio™ 12K Flex Real–Time PCR System v1.6 or later Maintenance and Administration Guide* (Pub. No. MAN0018832) for information on the emission spectrum for each dye, and the filter at which each dye is read.

(Optional) Use libraries when designing your own experiments

The QuantStudio™ 12K Flex Software allows you to save information to libraries, so you can easily use the information again when setting up an experiment. The libraries include:

- Targets library
- Samples library
- SNP Assay library (only available for Genotyping experiments)
- Run Method library

Target, sample, and SNP assay libraries

You can access the Targets, Samples, and SNP Assay libraries from the **Tools** menu to add, edit, delete, and import or export items. You can also access a library by clicking **Import from Library** in the **Define** screen when you are setting up an experiment.

Run Method library

You can use the Run Method library from the **Run Method** screen to:

- Save a new run method for later use.
- To select an existing run method for an experiment.

To add a run method to the Run Method Library:

1. Click **Save Run Method** in the toolbar of the **Graphical View** tab on the **Run Method** screen.
2. Enter a name and description *(optional)* for the run method, then click **Save**.

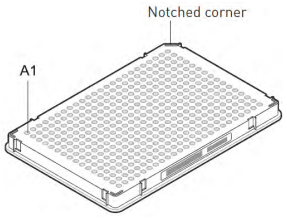
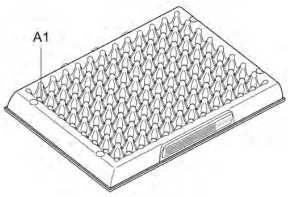
To select a run method from the Run Method Library

Click **Open Run Method** in the **Run Method** screen, and select one from the saved run methods.

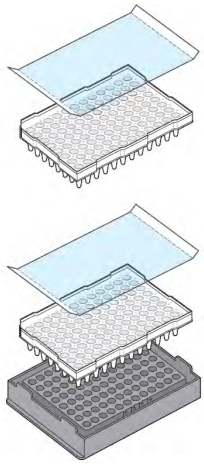
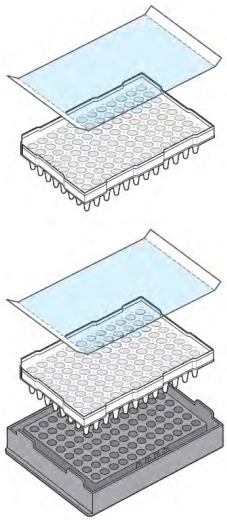
Prepare reactions

Supported consumables

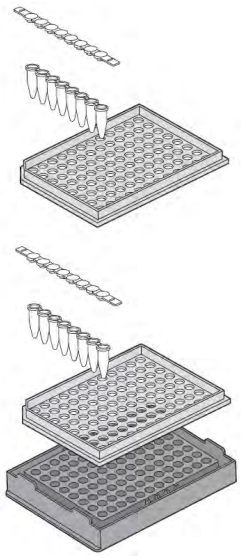
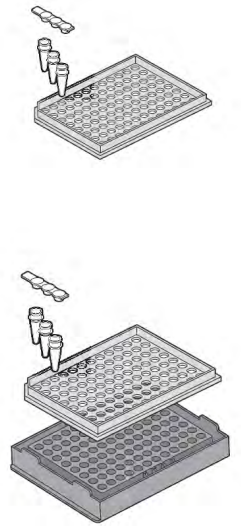
The QuantStudio™ 12K Flex Real-Time PCR Instrument is optimized for Applied Biosystems™ consumables. These can be ordered from the Thermo Fisher Scientific website. Use the consumables appropriate for the sample block of your instrument.

Sample block	Consumable	Consumable description	Maximum reaction volume supported	Recommended reaction volume
384-Well Plate		• MicroAmp™ Optical 384-Well Reaction Plate • MicroAmp™ Optical Adhesive Film	30 µL	5-20 µL
Array Card		Applied Biosystems™ Array Card	1 µL	1 µL

(continued)

Sample block	Consumable	Consumable description	Maximum reaction volume supported	Recommended reaction volume
96-Well Plate (0.2 mL)		• MicroAmp™ Optical 96-Well Reaction Plate • MicroAmp™ Optical Adhesive Film • MicroAmp™ 96-Well Support Base (only used during sample preparation) • QuantStudio™ 12K Flex System 96-Well Plate Adaptor	200 µL	10-100 µL
Fast 96-Well Plate (0.1 mL)		• MicroAmp™ Fast Optical 96-Well Reaction Plate • MicroAmp™ Optical Adhesive Film • MicroAmp™ 96-Well Support Base (only used during sample preparation) • QuantStudio™ 12K Flex System Fast 96-Well Plate Adaptor	100 µL	10-30 µL

(continued)

Sample block	Consumable	Consumable description	Maximum reaction volume supported	Recommended reaction volume
96-Well Plate (0.2 µL) and Fast 96-Well plate (0.1 µL)		• MicroAmp™ Optical 8-Cap Strip • MicroAmp™ Optical 8-Tube Strip (0.2 µL)/MicroAmp™ Fast 8-Tube Strip (0.1 µL) • MicroAmp™ 96-Well Tray/Retainer Set (Blue) (0.2 µL)/MicroAmp™ 96-Well Tray (Black) (0.1 µL) • MicroAmp™ 96-Well Support Base (only used during sample preparation) • QuantStudio™ 12K Flex System 96-Well Tube Adaptor/QuantStudio™ 12K Flex System Fast 96-Well Tube Adaptor	• 100 µL for Fast 96-well plate • 200 µL for 96-well plate	• 10-30 µL for Fast 96-well plate • 10-100 µL for 96-well plate
96-Well Plate (0.2 µL) and Fast 96-Well plate (0.1 µL)		• MicroAmp™ Optical Tube without cap (0.2 µL)/MicroAmp™ Fast Reaction Tube without Cap (0.1 µL) • MicroAmp™ Optical 8-Cap Strip • MicroAmp™ 96-Well Support Base (only used during sample preparation) • MicroAmp™ 96-Well Tray/Retainer Set (Blue) (0.2 µL)/MicroAmp™ 96-Well Tray (Black) (0.1 µL) • QuantStudio™ 12K Flex System 96-Well Tube Adaptor/QuantStudio™ 12K Flex System Fast 96-Well Tube Adaptor	• 100 µL for Fast 96-well plate • 200 µL for 96-well plate	• 10-30 µL for Fast 96-well plate • 10-100 µL for 96-well plate

IMPORTANT! Make sure that you use the flat caps for 0.2 µL tubes and 0.1 µL tubes. Use of rounded caps damages the heated cover.

Supported reagents

The reagents listed below can be used for experiments performed on the QuantStudio™ 12K Flex Real–Time PCR System.

Experiment	Reagent
Standard Curve	• TaqMan™ Reagents • SYBR™ Green reagents • Other reagents
Relative Standard Curve	• TaqMan™ Reagents • SYBR™ Green reagents • Other reagents
Comparative C_T ($\Delta\Delta C_T$)	• TaqMan™ Reagents • SYBR™ Green reagents • Other reagents
Melt Curve	• SYBR™ Green reagents • Other reagents
Genotyping	• TaqMan™ Reagents • Other reagents
Presence/Absence	• TaqMan™ Reagents • Other reagents

Note: TaqMan™ Fast Universal PCR Master Mix is not recommended to be used with the 96-well (0.2 µL) reaction plates or reaction tubes and tube strips sealed with caps.

Reagent detection process

TaqMan™ reagents

TaqMan™ reagents use a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles.

Advantages

- Increased signal specificity with the addition of a fluorogenic probe.
- Multiplex capability.
- Optional preformulated assays, optimized to run under universal thermal cycling conditions, are available.
- Can be used for either 1- or 2-step RT-PCR.

Limitations

Require synthesis of a unique fluorogenic probe.

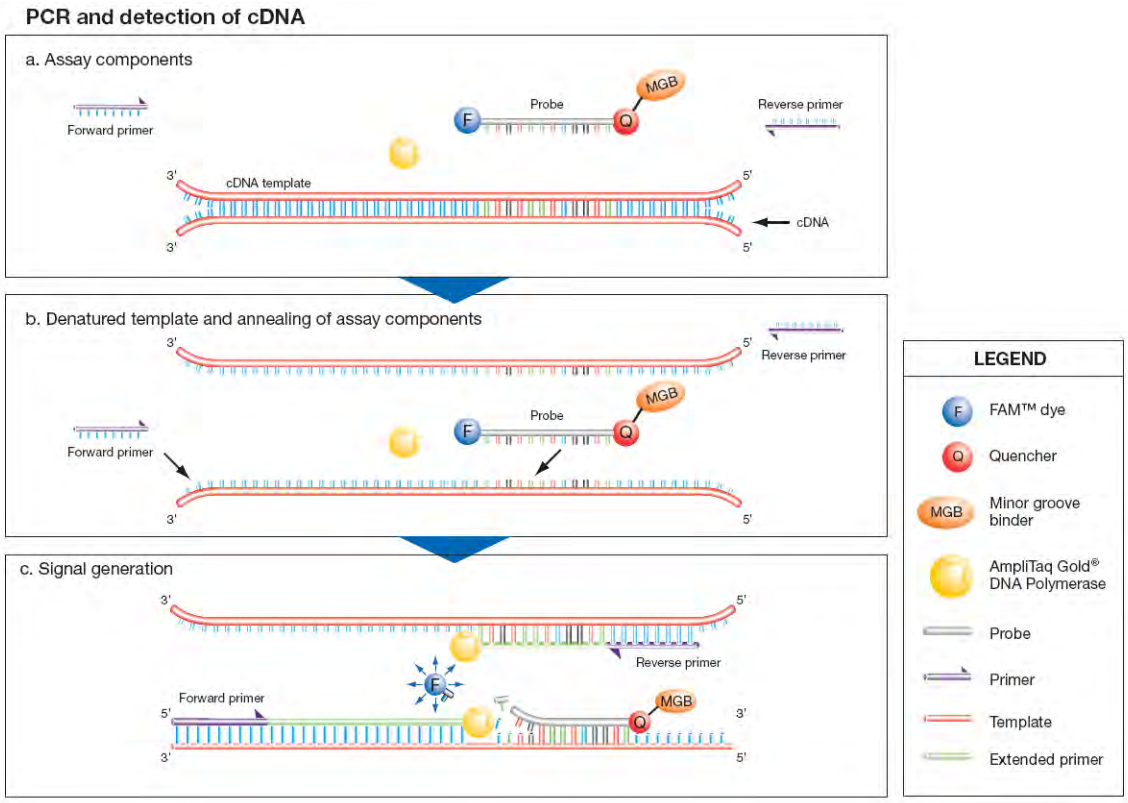


Figure 1 TaqMan™ reagents detection process

SYBR™ green reagents

SYBR™ Green reagents use SYBR™ Green I dye, a double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles.

Advantages

- Economical (no probe needed).
- Allow for melt curve analysis to measure the Tm of all PCR products.
- Can be used for either 1- or 2-step RT-PCR.

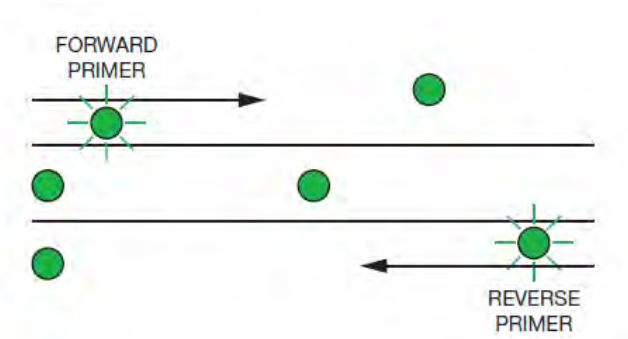
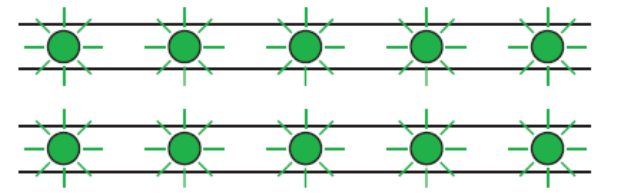
Limitations

Bind nonspecifically to all double-stranded DNA sequences. To avoid erroneous information signals, check for nonspecific product formation using melt curve or gel analysis.

Table 1 SYBR™ Green detection process

Method	Description
	**Step 1: Reaction setup** The SYBR™ Green I dye fluoresces when bound to double-stranded DNA.

Table 1 SYBR Green detection process *(continued)*

Method	Description
	**Step 2: Denaturation** When the DNA is denatured into single-stranded DNA, the SYBR™ Green I dye is released and the fluorescence is drastically reduced.
	**Step 3: Polymerization** During extension, primers anneal and PCR product is generated.
	**Step 4: Polymerization completed** SYBR™ Green I dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the instrument.

Precautions while preparing reactions

- Make sure that you do not prepare the reactions on a wet table. Wet surfaces lead to contamination of your reactions.
- Wear appropriate protective eyewear, clothing, and powder-free gloves.
- Make sure that you use the appropriate consumables. The quality of pipettors and tips and the care used in measuring and mixing dilutions affect data accuracy.
- Make sure that you perform dilutions exactly as instructed. Mistakes or inaccuracies in making the dilutions directly affect the quality of results.
- Use a permanent marker or pen to mark a tube and the side of a plate or array card. Do not use fluorescent markers.
- Make sure that the arrangement of the PCR reactions matches the plate layout displayed in the QuantStudio™ 12K Flex Software.

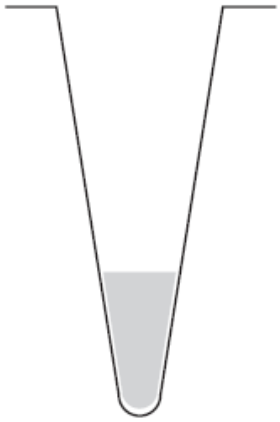
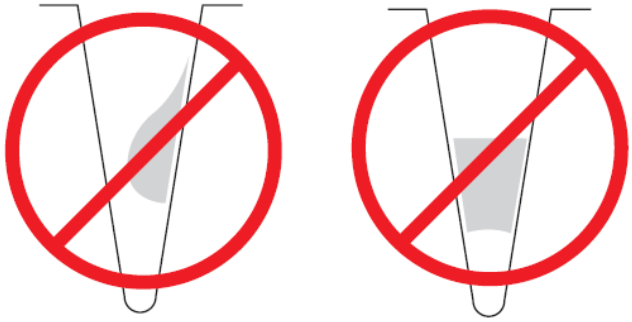
Materials required while preparing the dilutions

- DI water or DEPC water
- Microcentrifuge tubes
- Pipettors
- Pipette tips

- Vortex mixer
- Centrifuge
- Sample stock
- Standard stock
- Reaction mix components
- Plate or array card

Guidelines for preparing the dilutions, reaction mix, and plate

- Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers.
- Use TE buffer or water to dilute the standards and samples.
- Prepare the reagents according to the manufacturer's instructions.
- Keep the dilutions and assay mix protected from light, in the freezer, until you are ready to use it. Excessive exposure to light may affect the fluorescent probes or dyes.
- Prior to use:
  - Mix the master mix thoroughly by swirling the bottle.
  - Resuspend the assay mix by vortexing, then centrifuge the tube briefly.
  - Thaw any frozen samples by placing them on ice. When thawed, resuspend the samples by vortexing, then centrifuge the tubes briefly
- Do not allow the bottom of the reaction plate to become dirty. Fluids and other contaminants that adhere to the bottom of the reaction plate can contaminate the sample block(s) and cause an abnormally high background signal.

Correct	Incorrect
	
Liquid is at the bottom of the well.	Not centrifuged with enough force *Or* Not centrifuged for enough time

- For Genotyping experiments, prepare the reactions for each SNP separately.
- Place the reaction plate or array card at 4°C and in the dark until you are ready to load it into the instrument

Seal the reaction plate

If you use optical adhesive film to seal your reaction plates, seal each reaction plate as follows:

Note: The sealing instructions are applicable to 384-well and 96-well reaction plates.

1. Load the reaction plate using the plate layout described in "Assign targets, samples, and biological replicate groups" on page 22.

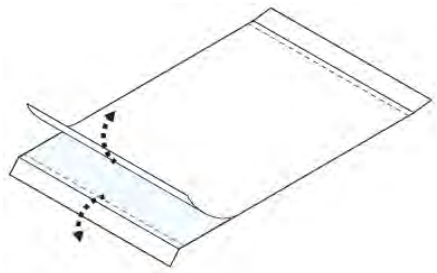
   Note: For 96-well reaction plates, place the reaction plate onto the center of the 96-well base, then perform this step. Be sure that the reaction plate is flush with the top surface of the 96-well base.

   Note: You can also use the MicroAmp™ Optical 8-Cap Strip to seal the 96-well reaction plates.

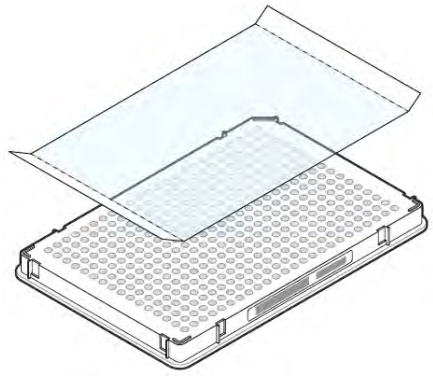
2. Remove a single optical adhesive film from the box. Bend both end-tabs upward. Hold the film backing side up.

3. In one swift movement, peel back the white protective backing from the center sealing surface. Do not touch the center sealing surface.

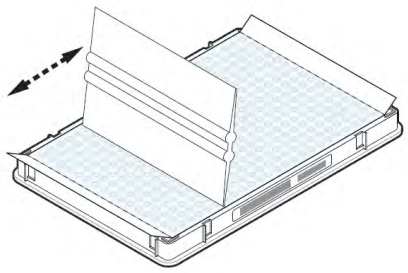
   IMPORTANT! Improper peeling of the optical adhesive film may result in haziness, but it will not affect results. Haziness disappears when the film comes into contact with the heated cover in the instrument.



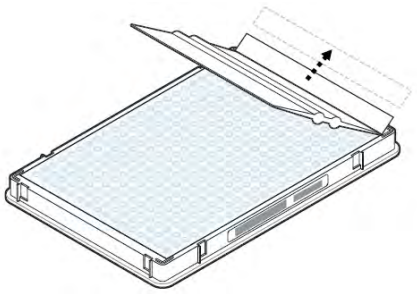
4. Holding the film by the end-tabs, lower the film onto the reaction plate (adhesive side facing the reaction plate). Make sure that the film completely covers all wells of the reaction plate.



5. Applying firm pressure, move the applicator slowly across the film, horizontally and vertically, to ensure good contact between the film and the entire surface of the reaction plate.



6. Using the applicator to hold the edge of the film in place, grasp one end of the end-tab and pull up and away sharply. Repeat for the other end-tab.



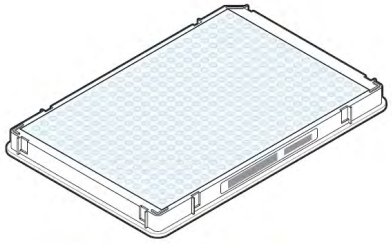
Note: Ensure clean removal of both end-tabs from the dotted line. Improper peeling of the end-tab can cause sticking of plate on the heated cover assembly.

7. To ensure a tight, evaporation-free seal, repeat step 5 on page 38.

   Applying firm pressure, run the edge of the applicator along all four sides of the outside border of the film.

Note: Optical adhesive films do not adhere on contact. The films require the application of pressure to ensure a tight, evaporation-free seal.

8. Inspect the reaction plate to be sure that all wells are sealed. You should see an imprint of all wells on the surface of the film. Check for the perforated tab to be completely torn off to avoid plates from sticking to the instrument after a run.



IMPORTANT! Remove all excess adhesive from the perimeter of the optical adhesive cover. When the film is applied, the glue from the optical adhesive cover can adhere to the edges of the plate. If the excess glue is not removed, the plate may adhere to sample block of the QuantStudio™ 12K Flex Real-Time PCR Instrument.

Fill and seal the array card

Fill and spin the array card

IMPORTANT! Wear powder-free gloves while preparing the array cards.

1. Remove an array card from its box and place it on a clean, dry surface.
2. Using a permanent marker, mark the side of the empty array cards.
3. Transfer the experiment-related chemistries and solutions into the port of the array card.

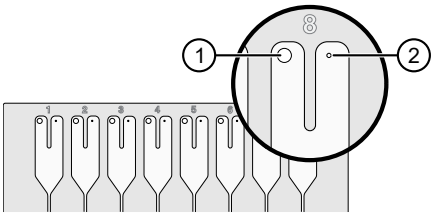
   For each transfer:

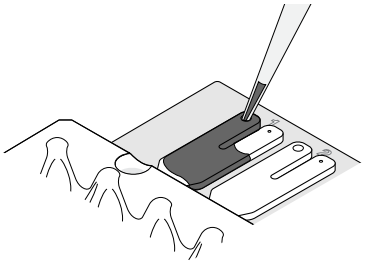
   a. Place the array card on a lab bench, with the foil side down.

   b. Load 100 µL of fluid into a pipette.

c. Hold the pipette in an angled position (approximately 45 degrees) and place the tip into the fill port. There is a fill port on the left arm of each fill reservoir – it is the larger of the two holes.

Do not allow the tip to contact and possibly damage the coated foil beneath the fill port.

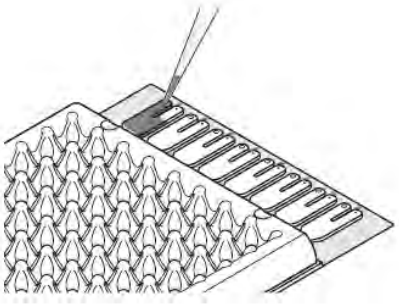




① Fill port
② Vent port

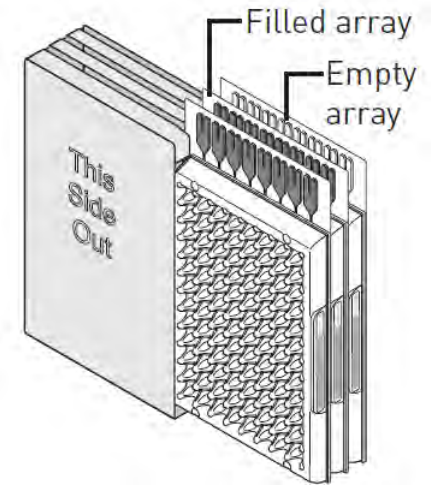
d. Dispense the fluid so that it sweeps in and around the fill reservoir toward the vent port. Pipette fluid into the fill reservoir, but **do not** go past the first stop of pipettor plunger when pipetting the reagents into the array card, or you may blow the solution out of the port.

IMPORTANT! Do not allow the tip to contact and possibly damage the coated foil beneath the fill port.

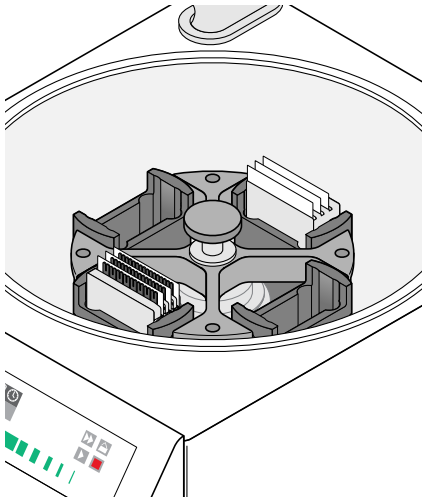


4. Place the filled array card(s) into a centrifuge array card carrier clip and place empty array card(s) in the remaining slots. Make sure that the labels on the buckets and clips face the same way.

IMPORTANT! Make sure to balance the loads in opposite buckets in the centrifuge.



5. Place the filled carrier clips into the centrifuge buckets. Make sure that the array-card fill reservoirs and bucket and clip labels face outward when loaded into the centrifuge. Balance the loads in opposite buckets.



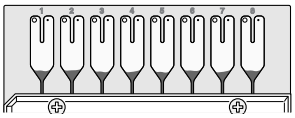
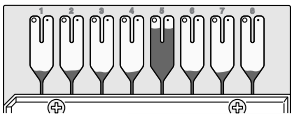
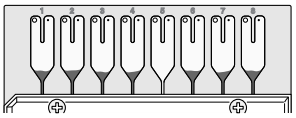
IMPORTANT! You must run the centrifuge with all four buckets in place and each of the two carriers filled with the array card. Place empty array cards into unfilled slots.

6. Close the centrifuge cover, then spin the array card(s) for 1 minute at 1200 rpm.

7. When the run is finished, stop the centrifuge, then spin the array card(s) again for 1 minute at 1200 rpm.

IMPORTANT! Do not try to save time by doing one spin for 2 minutes. The two sets of ramps are important for a good fill into the array card.

8. When the second run is finished, open the centrifuge and check that the fluid levels in the reservoirs of each array card have decreased by the same amount. Also, check for the formation of bubbles in all wells and note possible problems.

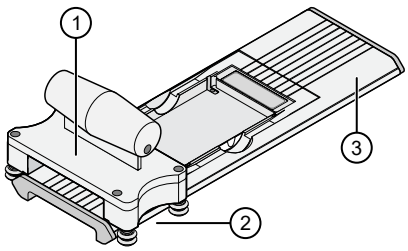
Consistent filling	Inconsistent filling	
		

9. If necessary, centrifuge the array card(s) for an additional minute to fill any unfilled wells. Do not exceed three 1-minute runs or centrifuge the array card for longer than 1 minute at a time.

Seal the array card

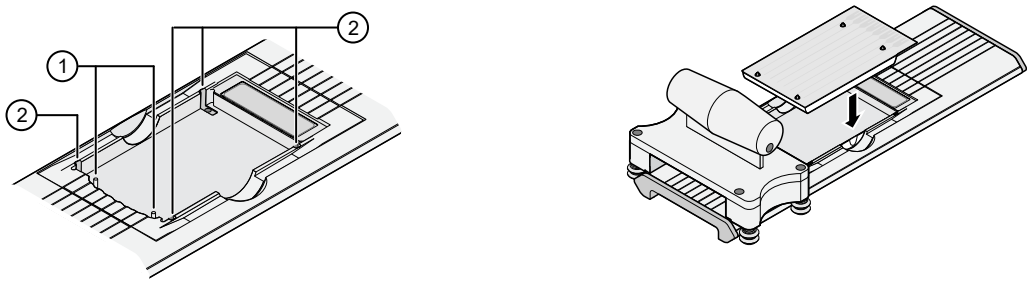
1. With the carriage (roller assembly) of the TaqMan™ Array Card Sealer in the Start position, place a filled array card into the fixture with the foil side up so that the fill reservoirs are the farthest away from the carriage.

2. Press down on all four corners of the array card to ensure that it is fully seated within the fixture.



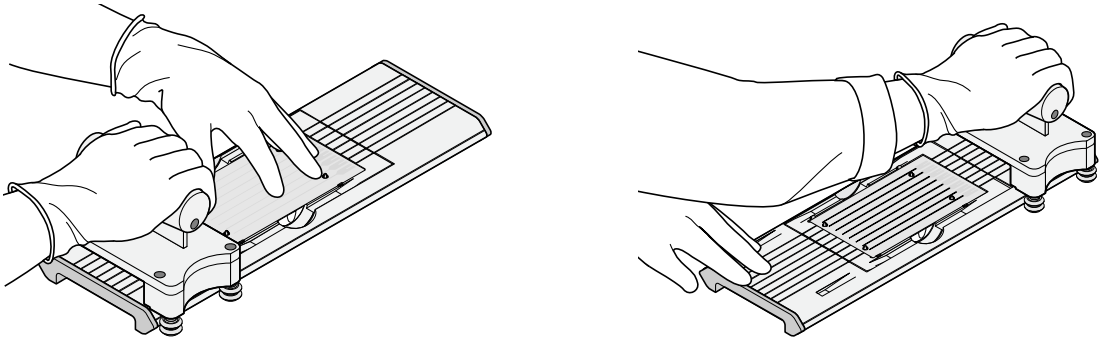
① Carriage
② Carriage starting position
③ Carriage ending position

3. Use the two alignment pins in the fixture to position the array card correctly.

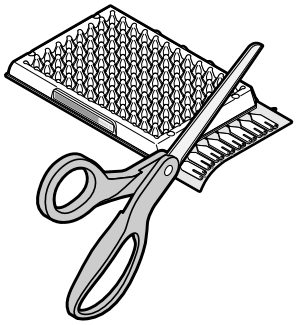


① Alignment pins
② Spring clips

4. Seal the array card by running the carriage slowly over it, in one direction only. Do not apply downward force on the carriage as you move it forward over the card.



5. Remove the sealed array card from the fixture and trim the fill reservoirs from the array card assembly using scissors. Trim the foil array card so that the edge is even with the plastic carrier.



IMPORTANT! Completely remove the fill reservoirs from the array card so that the edge is free of residual plastic. The plastic from the fill reservoirs that extends beyond the edge of the card can prevent the card from seating properly on the sample block and affect amplification.

Correct trim	Incorrect trim
	

IMPORTANT! As you seal the remaining filled array cards, store them in a dark place until you are ready to use them. The fluorescent dyes in the array card are photosensitive. Prolonged exposure to light can diminish the fluorescence of the dye.

Capping and uncapping the 96-well reaction tubes and tube strips

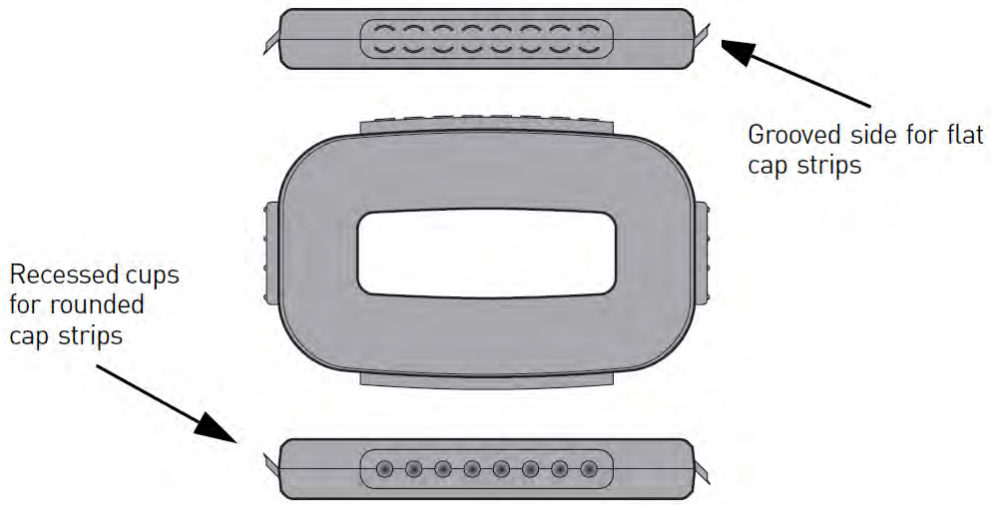
Note: Make sure that you secure the caps on the tubes and tube-strips tightly to avoid sample evaporation.

If you use the 96-well MicroAmp™ Optical 8-Tube Strips or MicroAmp™ Optical Tubes without Cap, use the MicroAmp™ Cap Installing Tool (Cat. No. 4330015) and follow the instructions below for:

- Applying the MicroAmp™ Optical 8-Cap Strip or MicroAmp™ Optical Tubes without Cap to the tubes
- Removing a cap string from a plate

Required materials:

- MicroAmp™ Cap Installing Tool
- MicroAmp™ Optical 8-Tube Strips or MicroAmp™ Optical Tubes without cap
- MicroAmp™ Optical 8-Cap Strip



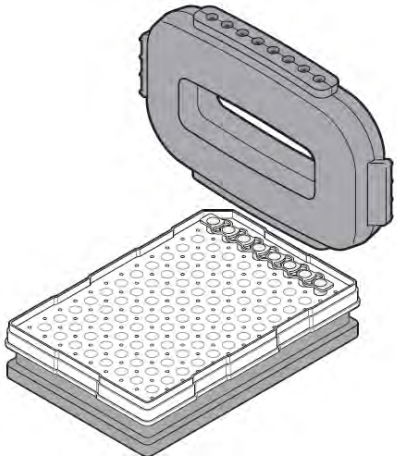
MicroAmp® Cap Installing Tool

Apply the MicroAmp™ Optical 8-cap strip (flat)

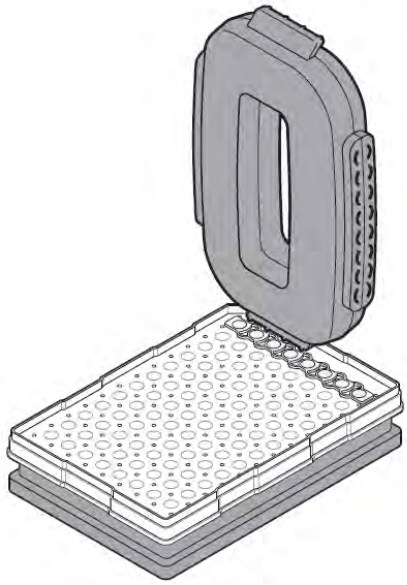
1. Grasp the MicroAmp™ Cap Installing Tool so that the grooved side is exposed.
2. Hold the strip of caps over the tube strip or the row of tubes.
3. Use the grooved side of the tool to push and seat each cap firmly in place. Use a rocking motion to properly seat each cap.

Remove a cap string from a plate

The MicroAmp™ Cap Installing Tool is also used for removing the MicroAmp™ Optical 8-Cap Strip from the 96-well optical plates and tray/retainer assemblies.



1. Insert the small protrusions on the side of the tool under the webbing between the caps on a cap strip.
2. Slowly pry the strip from the plate or tray/retainer assembly.

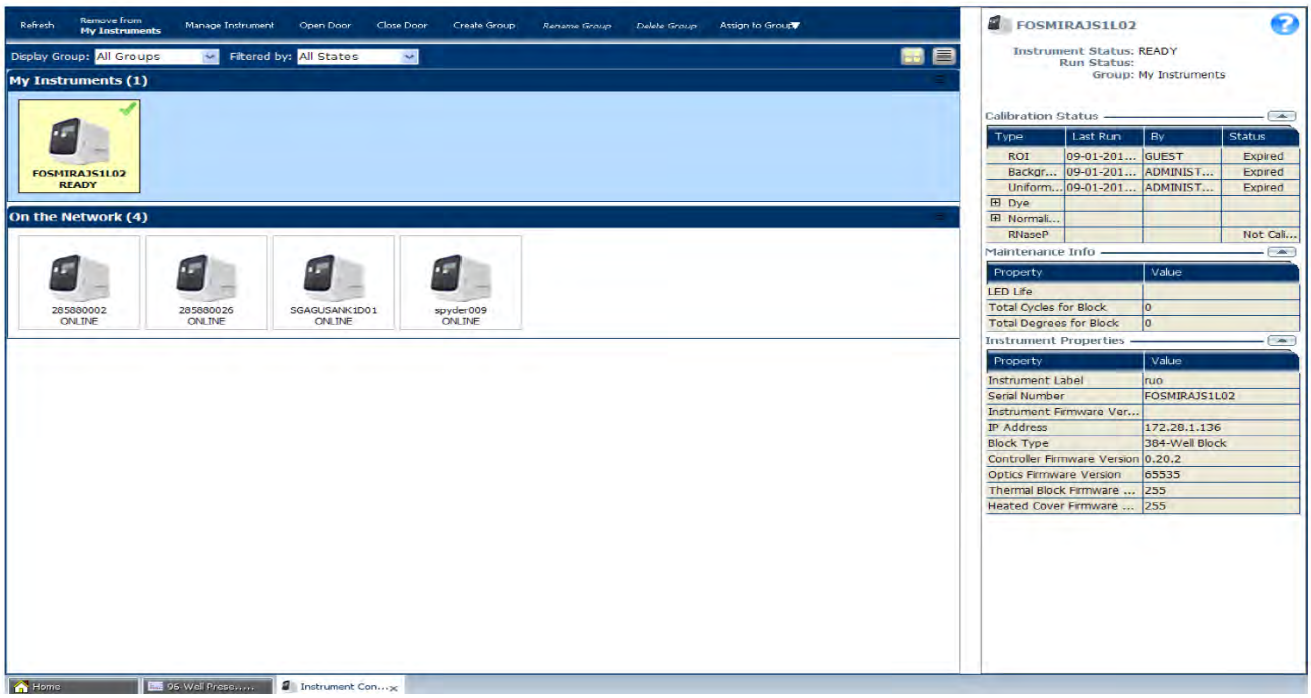


Start the experiment

1. Access the **Instrument Console** (see “Instrument Console screen” on page 45).
2. Load the reaction plate or array card into the instrument (see “Load the reaction plate or array card into the instrument” on page 48).
3. Run the experiment (see “Run the experiment” on page 50).

Instrument Console screen

The **Instrument Console** screen displays all the QuantStudio™ 12K Flex Real-Time PCR Instruments discovered on a network.



Left pane

The features on the left pane of the **Instrument Console** screen allow the following items:

- Instrument access: Open and close the instrument door from the software user interface.
- Group management:
  - Create, rename, and delete groups and assign instruments to the groups.
  - Add and remove instruments to and from the **My Instruments** pane.

    Note: To add instruments, select the icon of the instrument that you want to add to the **My Instruments** pane. Click **Add to My Instruments**. Similarly, click **Remove from My Instruments** to remove an instrument from the **My Instruments** pane. You can also drag and drop the instrument icon into the **My Instruments** pane or into the group created by you.

  - Display instrument groups from the **Display Group** dropdown list according to their activity. Select the status from the **Filtered By** dropdown list. For more information on the status of an instrument, see "Monitor the experiment" on page 52.
- Instrument management:
  - Monitor experiments (check the run status or monitor a temperature plot or amplification plot during a run). For more information on monitoring experiments, see "Monitor the experiment" on page 52.
  - Maintain instruments (check the calibration status of instruments and perform different calibrations). For more information about instrument maintenance, see *QuantStudio™ 12K Flex Real–Time PCR System v1.6 or later Maintenance and Administration Guide* (Pub. No. MAN0018832).
  - Manage files (upload setup files; download completed experiments; and create, rename, and delete experiment files and plate setup folders).

    Note: Completed experiments are downloaded into the default folder **Completed Experiments**.

    Note: To manage files, click **Manage Instrument**.

    Use the **File Manager** tool to create, rename or delete folders for holding setup files for starting a run or completed experiments for analysis. To move setup files from one folder to the other, click **Move**, then select the setup folder you want to shift the setup file into.

    IMPORTANT! To manage and monitor, you must move instruments from the **On the Network** pane to the **My Instruments** pane or a custom group. You can start a run and calibrate instruments present only in the **My Instruments** pane or the custom group(s) that you created.

Right pane

The right pane of the **Instrument Console** screen displays the following information:

- The name of the instrument whose instrument icon is selected.
- The run status of the selected instrument.
- The group the instrument belongs to.
- The calibration status, maintenance reminders and instrument properties of the selected instrument.

The calibration status is indicated by the ⚠ icon. The icon appears in the **Status** column of the **Calibration Status** table after the last reminder date before the calibration expires.

Status icons

You can monitor the instrument status and view calibration and other information in the **Instrument Console** screen.

Instrument status icon

The status of an instrument is represented by an icon in the top-right corner of the thumbnail representation of the instrument in the **Instrument Console** screen. An instrument displays the status when you place the instrument icon in the **My Instruments** pane or in the groups that you created.

1. In the **Home** tab (🏠), select **Instrument Console**. If you do not see an instrument, click **Refresh** in the instrument console toolbar.

2. If needed, move the instrument from the **On the Network** group to a group which can be monitored.
   a. Click the instrument of interest, then click **Assign to Group** in the instrument console toolbar.

   b. Select the **My Instruments** or a personal group in the drop-down list. The instrument is now monitored.

Icon	Instrument status
	Ready
[No icon]	Available on the network but cannot be monitored because that instrument is not in the **My Instruments** pane or a group you created.
	Run in process (The time remaining for the run is shown to the left of the icon.)

(continued)

Icon	Instrument status
	Unavailable
	Incompatible firmware version
	No longer connected to the network
	Error occurred during run

Load the reaction plate or array card into the instrument

CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the temperature of the sample block(s) can exceed 100°C. Keep your hands away until the sample block(s) reaches room temperature.

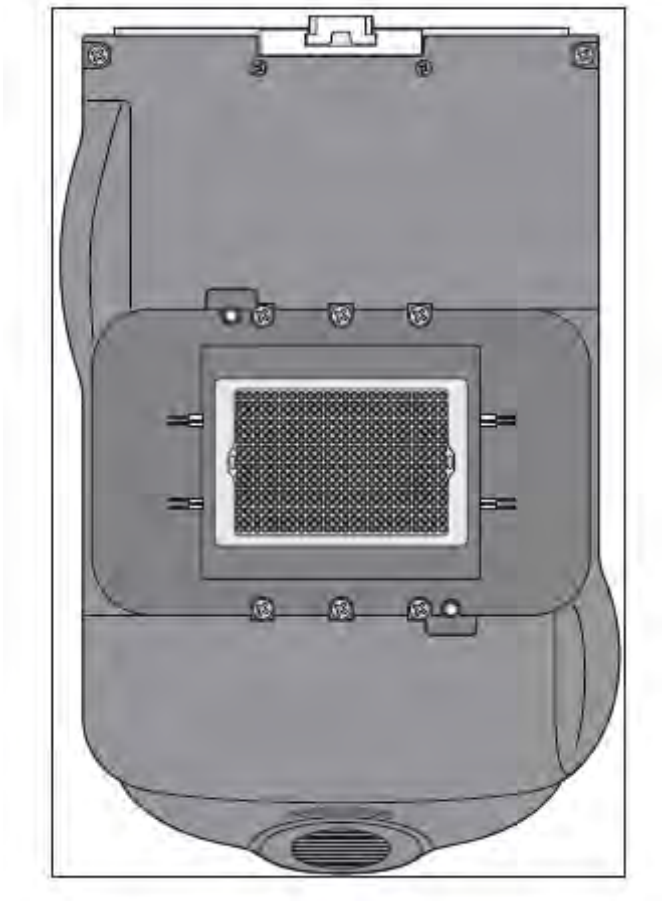
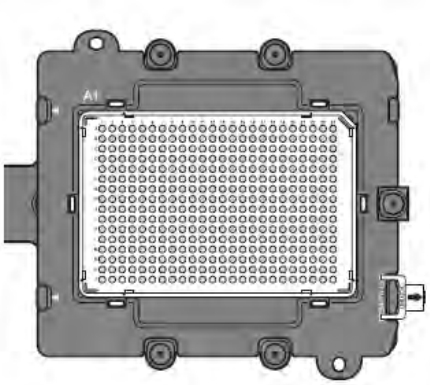


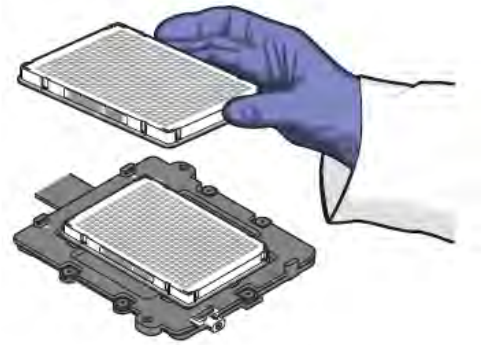
Figure 2 Sample block

IMPORTANT! Wear powder-free gloves when you handle the reaction plate or array card.

IMPORTANT! Plates and array cards should be loaded and unloaded by operators who have been warned of the moving parts hazard and have been adequately trained.

1. Touch on the instrument touchscreen or click **Open Door** in the **Instrument Console** screen of the software to allow the plate adapter to come out from the instrument side.
2. Place the reaction plate or array card on the plate adapter. Ensure that the reaction plate or array card is properly aligned in the holder.
   - Make sure the well A1 is positioned at the top-left of the tray for any of the plate formats.
   - Make sure the barcode (for any of the plate formats) is facing the front of the instrument.





- If using reaction tubes or tube strips, make sure you use adaptors. The adaptors are attached to the plate transport arm. The tray containing the tubes or tube strips must be placed on the adaptor and not into the sample block directly.

IMPORTANT! For optimal performance with partial loads, load at least 16 tubes and arrange them in:

Adjacent columns of 8 tubes, using rows A through H. For example, use wells in columns 6 and 7 (rows A through H).

Or

Adjacent rows of 8 tubes, using columns 3 through 10. For example, use wells in row D (columns 3 through 10) and row E (columns 3 through 10).

IMPORTANT! Make sure that you use the flat caps for the 0.2µL tubes and 0.1µL tubes. Use of rounded caps damages the heated cover.

3. Touch on the instrument touchscreen or click **Close Door** in the **Instrument Console** screen of the software to retract the plate adapter back into the instrument.

Enable or change the notification settings

You can configure the QuantStudio™ 12K Flex Software to alert you by email when the instrument begins and completes a run, or if an error occurs during a run.

Note: For details on using the notification settings feature, see *QuantStudio™ 12K Flex Real–Time PCR System v1.6 or later Maintenance and Administration Guide* (Pub. No. MAN0018832).

Run the experiment

You can run the experiment in either of the following two ways:

- From the software (“Start a run from the software” on page 51)
- From the instrument touchscreen (“Start a run from the instrument touchscreen” on page 51)

Note: The example experiments in each of the getting started guide booklets start a run from the software.

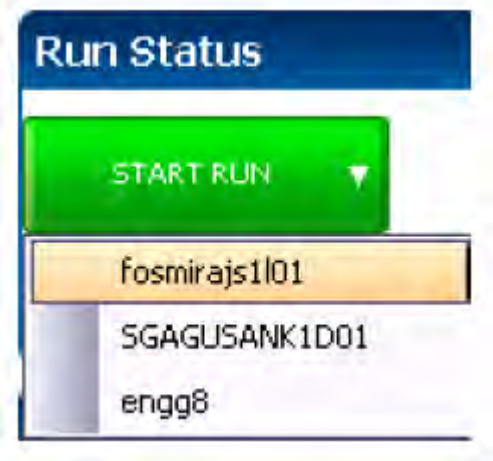
IMPORTANT! Make sure that instrument calibration is up-to-date. If a calibration has expired, you get a warning when you start a run. For information on calibrating the instrument, see *QuantStudio™ 12K Flex Real–Time PCR System v1.6 or later Maintenance and Administration Guide* (Pub. No. MAN0018832).

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the instrument is in operation.

Start a run from the software

1. In the navigation pane of the QuantStudio™ 12K Flex Software, click .
2. Click **START RUN**. Select the instrument for the run from the drop-down menu of the instruments placed under **My Instruments**.

   IMPORTANT! Make sure that the instrument to run the experiment on is in **My Instruments** or the custom group, and that it is ready to run an experiment. If the preferred instrument is not present under **My Instruments** or the custom group, or if it is unavailable, clicking **START RUN** does not display instrument names in the drop-down menu.



Start a run from the instrument touchscreen

1. Touch the instrument touchscreen to awaken it.

   Note: If the touchscreen is not at the **Main Menu** screen, touch .
2. In the **Main Menu** screen, touch **Browse Experiments**.
3. In the **Browse** screen, touch **(Folders)** to display the folders containing the experiment setup files.
4. Touch any of the folder names to display the experiments in that folder.

5. In the **Experiments** screen, select the desired experiment, then touch  to view or edit the experiment before starting the run.

   Note: You can start a run immediately by clicking  , then go to step 8 on page 52.

6. *(Optional)* Modify the experiment parameters as needed. You can use the following options:
   - Use the  button and the  button to add and delete a stage or step to the thermal profile.
   - Use the  button to add a melt curve to the thermal profile.
   - Use the  button to save the experiment you modify.

7. In the **Save Experiment** screen, touch each field to edit the items.
   - Experiment name
   - Folder to save the experiment
   - Reaction volume
   - Barcode Number
   - Notes

   When finished, touch **Save & Start Run** to start the experiment.

8. In the **Start Run** screen, touch each field as needed to modify the associated parameter, then touch **Start Run Now** to start the experiment.

   Note: When the run is complete, touch  to unload the plate from the instrument. You can download the results of the experiment from a computer if the instrument is connected to a network, or copy the data to a USB device as explained in “Transfer experiment results” on page 59.

Monitor the experiment

Note: If there is loss of connection during an experiment, remove the instrument from the **My Instruments** list. Add the instrument back to the **My Instruments** list. You can then resume monitoring the experiment.

You can monitor an experiment run in three ways:

- From the instrument touchscreen, in the same way that you run the experiment (see “Start a run from the instrument touchscreen” on page 51).
- From the **Run** screen of the software, while the experiment is in progress, as shown below.
- From the **Instrument Console** screen of the software (to monitor an experiment started from another computer or from the QuantStudio™ 12K Flex Instrument touchscreen), as described in “Monitor a run from the software Instrument Console screen” on page 53.

Monitor an experiment from the software Run screen

1. Click **Amplification Plot** from the **Run Experiment** menu to monitor the amplification plot of the experiment you are running.

   Note: For melt curve experiments, click **Melt Curve Plot** from the **Run Experiment** menu.

2. Click **Temperature Plot** from the **Run Experiment** menu to monitor the temperature plot of the experiment you are running.

Monitor a run from the software Instrument Console screen

1. In the **Instrument Console** screen, select the icon of the instrument that you are using to run the experiment.
2. Click **Manage Instrument**.
3. In the **Instrument Manager** screen, click **Monitor Running Instrument**.

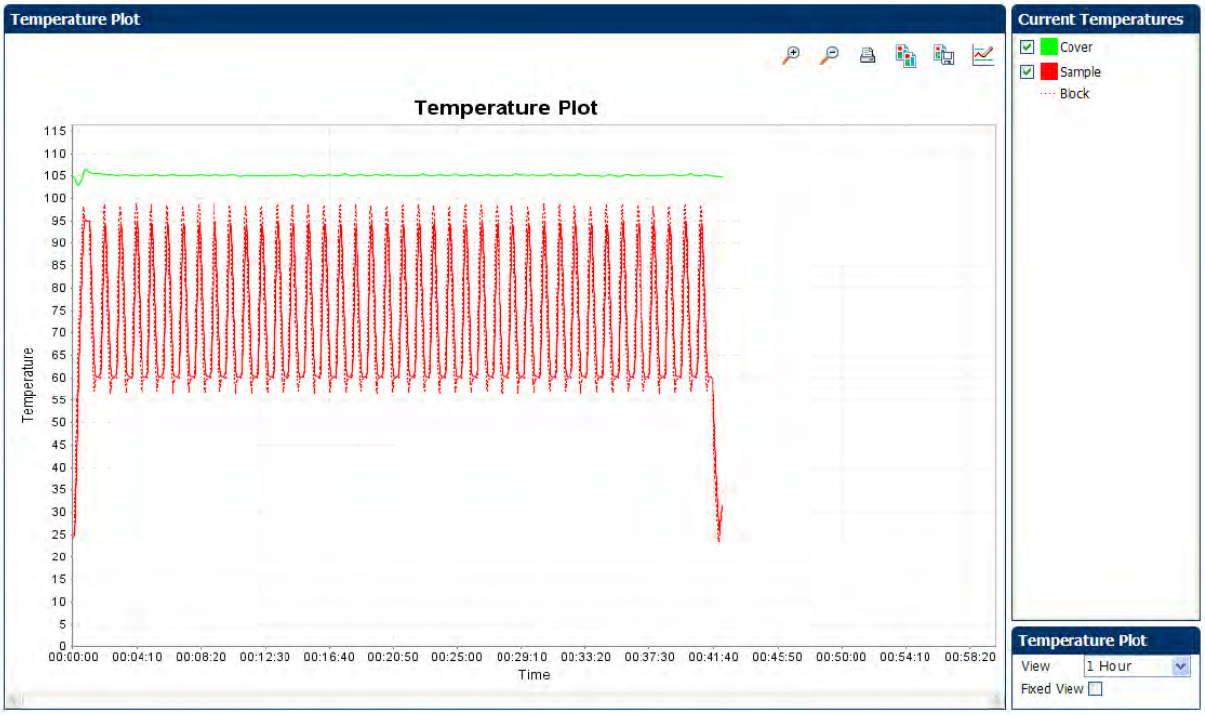
You can view the progress of the run in real time from the **Run** screen. During the run, periodically view the Amplification Plot, Temperature Plot, and Run Method for potential problems.

Action	Instructions
Stop the run	• In the software, click **STOP RUN**. • In the **Stop Run** dialog box, click one of the following options: – Click **Stop Immediately** to stop the run immediately. – Click **Cancel** to continue the run.
View amplification data in real time	Select **Amplification Plot**. See "To monitor the Amplification Plot" on page 54.
View temperature data for the run in real time	Select **Temperature Plot**. See "To monitor the Temperature Plot" on page 54.
View progress of the run in the **Run Method** screen	Select **Run Method**. See "To monitor the Run Method" on page 55.
Enable or disable the notification settings	Select or deselect **Enable Notifications**. See "Enable or change the notification settings" on page 50.

Note: The individual experiment booklets provide illustrations of the different experiments in real time.

Note: For melt curve experiments, click **Melt Curve Plot** from the **Run Experiment** menu.

The **Run** screen for a standard curve experiment run looks like this:



To monitor the Amplification Plot

To view data in the Amplification Plot, click **Amplification Plot** from the **Run Experiment** menu, select the **Plate Layout** tab, then select the wells to view.

The **Amplification Plot** screen allows you to view sample amplification as your instrument collects fluorescence data during a run. If a method is set up to collect real-time data, the **Amplification Plot** screen displays the data for the wells selected in the **Plate Layout** tab. The plot contrasts normalized dye fluorescence (ΔRn) and cycle number.

The **Amplification Plot** screen is useful for identifying and examining abnormal amplification, including:

- Increased fluorescence in negative control wells.
- Absence of detectable fluorescence at an expected cycle (determined from previous similar experiments run using the same reagents under the same conditions).

Note: If you notice abnormal amplification or a complete absence of signal, troubleshoot the error as explained in the software help (click or press **F1).**

To monitor the Temperature Plot

To view data in the **Temperature Plot** screen, click **Temperature Plot** from the **Run Experiment** menu.

During a run, the **Temperature Plot** screen displays the temperatures of the sample block(s), the heated cover, and samples (calculated) in real-time.

Action	Instructions
Add or remove temperature plots	Select **Cover** or **Sample Block** to view the presence of the associated data in the plot.
Change the time to display in the plot	From the **View** drop-down list, select the amount of time to display in the plot.
Display a fixed time window during the instrument run If the entire plot does not fit in the screen, the screen is not updated as the run progresses. For example, if you select 10 minutes from the **View** drop-down list, the plot will show data for 10 minutes. If the **Fixed View** is: • Deselected, the plot updates as the run progresses even after 10 minutes. • Selected, the plot does not update as the run progresses even after 10 minutes.	Select **Fixed View**.

The **Temperature Plot** screen can be useful for identifying hardware failures. When monitoring the **Temperature Plot** screen, observe the **Sample** plot and the **Block** plot for abnormal behavior.

- The **Sample** plot and the **Block** plot should mirror each other approximately. A significant deviation of the plots can indicate a problem.
- The **Cover** plot should maintain the constant temperature specified in the method. A departure from the constant temperature may indicate a problem.

Note: If you notice abnormal temperature plot, troubleshoot the error as explained in the software help (click ? or press F1).

To monitor the Run Method

To view data in the **Run Method** screen, click **Run Method** from the **Run Experiment** menu.

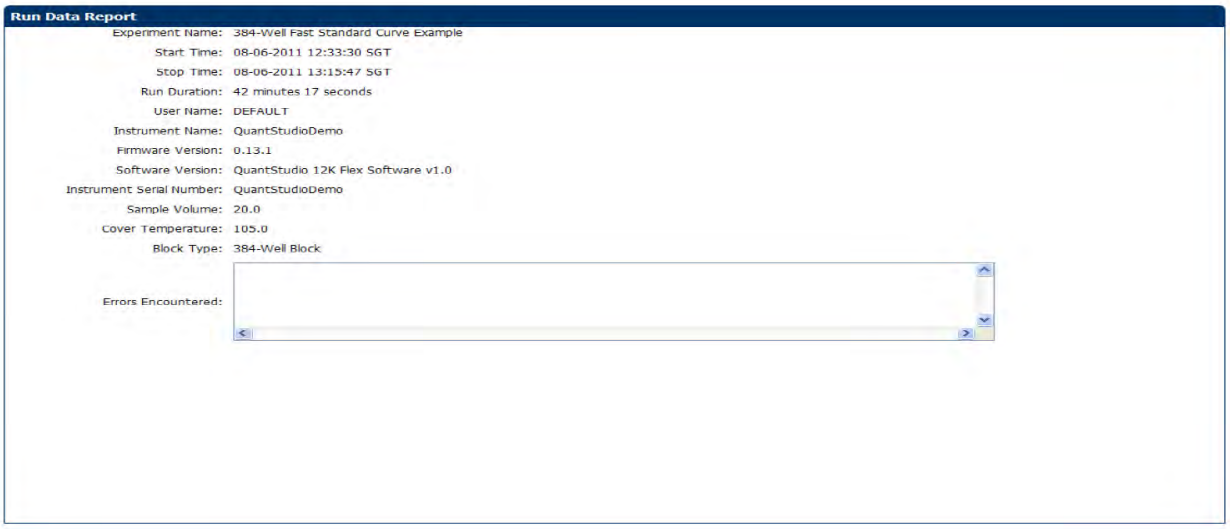
The **Run Method** screen displays the run method selected for the run in progress. The software updates the **Run Status** field throughout the run.

Action	Instructions
Change the number of cycles	In the **Adjust # of Cycles** field, enter the number of cycles to apply to the **Cycling Stage**.
Add a melt curve stage to the end of the run	Select **Add Melt Curve Stage to End**.
Add a Hold stage to the end of the run	Select **Add Holding Stage to End**.
Add an indefinite hold to the end of the run	Select **Add Infinite Hold to End**.
Apply your changes	Click **Send to Instrument**.

If an alert appears, click the error for more information and troubleshoot the problem as explained in the software help (click ? or press **F1).**

To view the run data

After a run is complete, you can view a run report by clicking **View Run Data**. The **View Run Data** screen displays information about the completed run, as in the following example from a Standard Curve experiment:



The run report data helps in:

- Comparing two experiments of the same type run on two different instruments.
- Troubleshooting. For example, after a firmware upgrade, you can compare an experiment run before and after the upgrade to determine if the upgrade affected performance.

Monitor a run from the instrument touchscreen

The touchscreen displays the method for the experiment, the date and time at which the run started, the time remaining in the run, and other information.

Action	Instructions
Display a graphical view of the run	Touch **Experiment View**.
Show the Amplification Plot for the run	Touch **Plot View**, then touch **Experiment View** to return to the **Run Method** screen.
Display the time elapsed and the time remaining in the run	Touch **Time View**, then touch **Experiment View** to return to the **Run Method** screen.
Stop the run	Touch **STOP** to stop the protocol run immediately.
View the events log	Touch the status bar to display the events log.

The run method on the instrument touchscreen is shown in the following graphics:

Figure 3 Experiment View tab

Figure 4 Time View tab

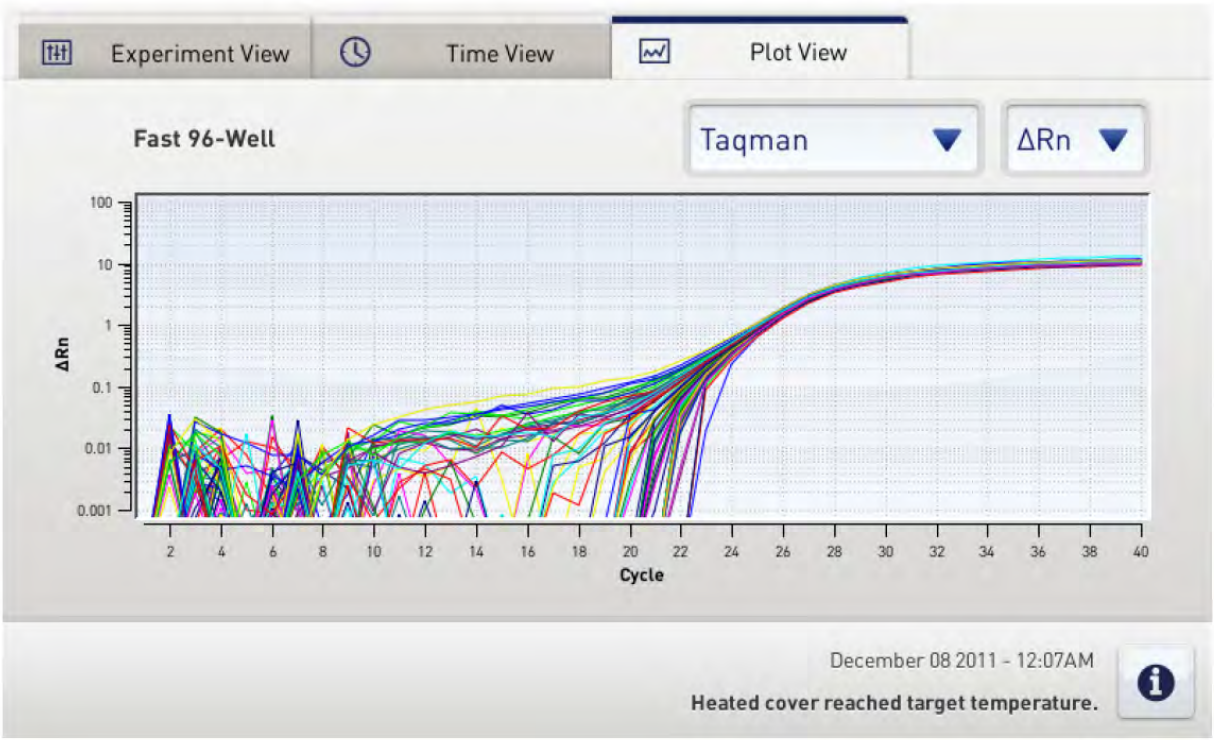


Figure 5 Plot View tab

The **Plot View** tab displays the Amplification Plot in real time. You can change the plot using the drop-down menus present below the Plot View tab.

Action	Instructions
Change the data displayed on the y axis. Select either **Rn** (normalized reporter) or **ΔRnΔ** (baseline-corrected normalized reporter).	Touch Rn.
Change the reporter dye displayed in the plot. Only dyes used in your experiment are shown.	Touch FAM.
View the run events that occurred during the run. Touch ℹ again to close the event list.	Touch ℹ.

Unload the instrument

When the instrument displays the **Main Menu** screen, unload the reaction plate from the instrument and transfer the experiment data to the computer for analysis.

Unload the reaction plate or array card

CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the temperature of the sample block(s) can exceed 100°C. Keep your hands away until the sample block(s) reaches room temperature.

1. Open the access door.
   - On the instrument touchscreen, touch .
   - In the **Instrument Console** screen of the software, click **Open Door**.
2. Remove the reaction plate or array card from the plate adapter.
3. Retract the plate adapter back into the instrument.
   - On the instrument touchscreen, touch .
   - In the **Instrument Console** screen of the software, click **Close Door**.
4. If the instrument does not eject the plate, perform the following steps.
   a. Power off the instrument.
   b. Wait for 15 minutes, then power on the instrument.
   c. Eject the plate.
   d. If the plate does not eject, power off and unplug the instrument, then open the access door.
   e. Wearing powder-free gloves, reach into the instrument, remove the plate from the heated cover, then close the access door.
   f. Perform a background calibration to confirm that the sample block has not been contaminated.

Transfer experiment results

Transfer the experiment results in one of the following ways:

- Download the experiment over the network (see “Download the experiment from the instrument over the network” on page 60)
- Transfer the experiment by a USB drive (see “Transfer the experiment from the instrument to the computer with a USB drive” on page 61)

Download the experiment from the instrument over the network

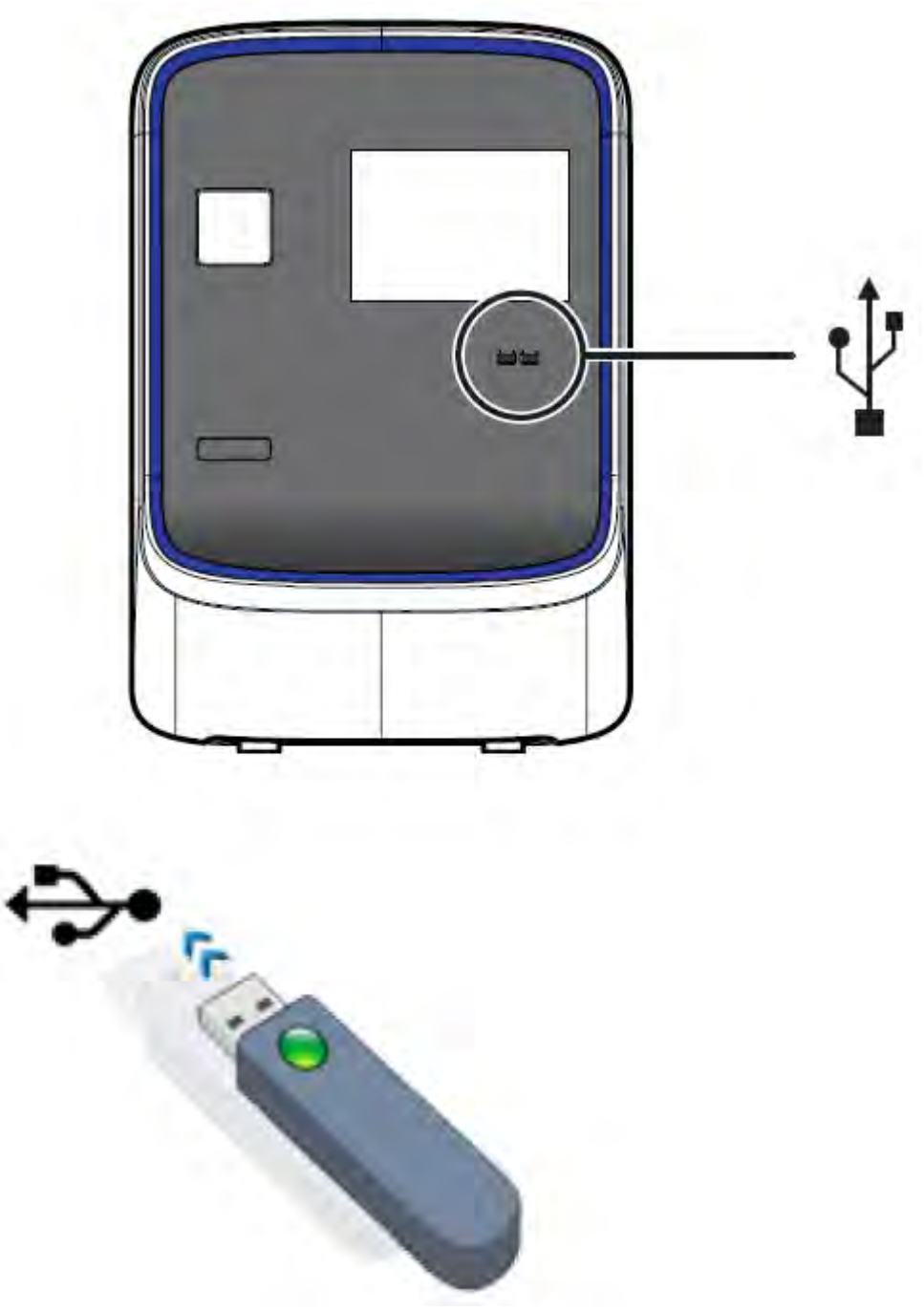
1. In the software, click **Instrument** ▸ **Instrument Console**.
2. Select the instrument icon of the instrument you just used to run the experiment from the **My Instruments** list.
3. Click **Manage Instrument** to open the **Instrument Manager** screen.
4. In the **Instrument Manager** screen, click **Manage Files**.
5. In the **Experiments** panel, select the experiment to download, then click **Download**.
6. In the **Save** dialog box, select the folder to hold the experiment results, then click **Save**.

   The experiments folder is located at `<…>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments`, where <…> is the installation drive.

   The default installation drive is `C:` if the software is installed by the customer. The default installation drive is `D:` if the software is installed by a Thermo Fisher Scientific field service engineer.

Transfer the experiment from the instrument to the computer with a USB drive

1. If not already connected to the instrument, connect a USB drive to the USB port.



2. Touch the instrument touchscreen to awaken it.
3. If the touchscreen is not at the **Main Menu** screen, touch .
4. In the **Main Menu** screen, touch **(Collect Results)** to save the data to the USB drive.

5. Touch one or multiple experiments, then touch **(Save to USB)** to copy selected experiment or experiments to the USB drive.

   Note: If your instrument cannot find the USB drive, remove the USB drive, then try again. If the instrument still does not recognize the USB drive, try another USB drive.

6. Touch to return to the **Main Menu** screen.

7. Remove the USB drive from your instrument, then connect it to one of the USB ports on your computer.

8. In the computer desktop, use the Windows™ explorer to open the USB drive.

9. Copy the example experiment file to `<…>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments`, where <…> is the installation drive.

   The default installation drive is `C:` if the software is installed by the customer. The default installation drive is `D:` if the software is installed by a Thermo Fisher Scientific field service engineer.

Review experiment results

About analysis results

Immediately after a run, the software automatically analyzes the data using the default analysis settings, then displays the **Amplification Plot** screen.

Note: For auto-analysis of data, after a run, go to **Tools** ▸ **Preferences** ▸ **Experiment**, then select the **Auto Analysis** checkbox.

Note: For Genotyping experiments, the software displays the **Allelic Discrimination Plot** screen.

To reanalyze the data, select all the wells in the plate layout, then click **Analyze**.

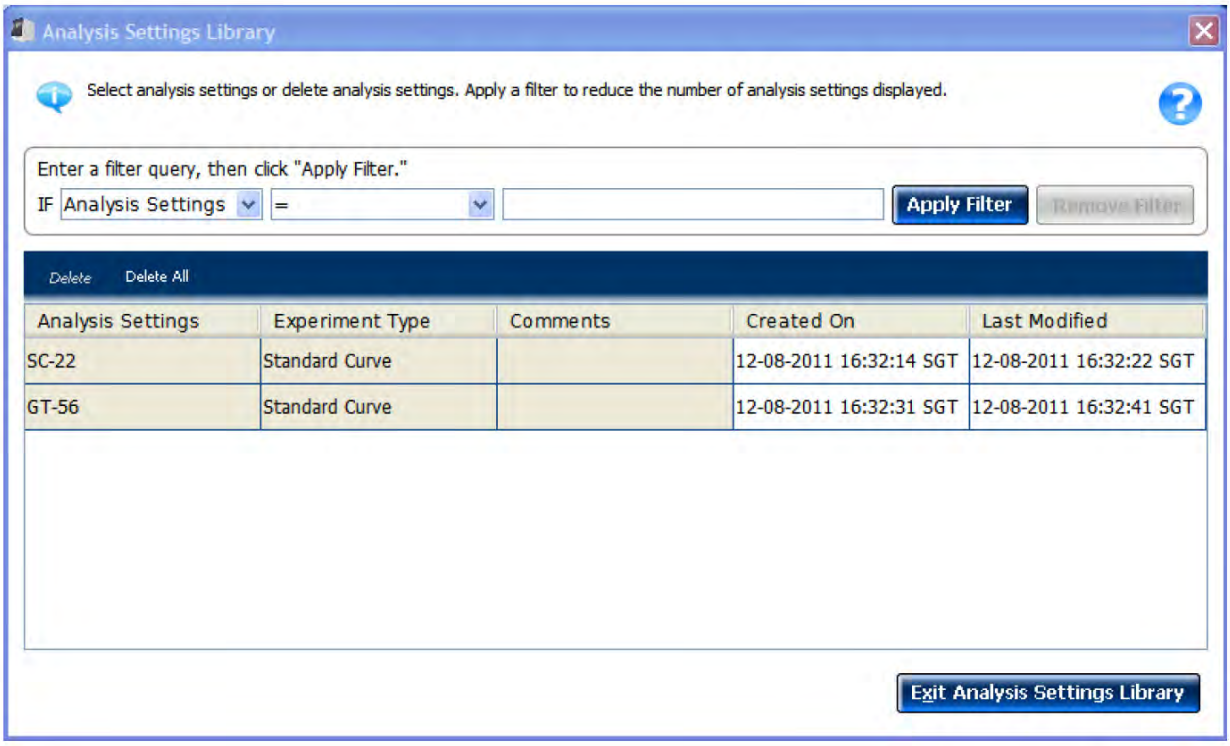
About the analysis settings library

Analysis settings are different for each experiment type. If the default analysis settings in the software are not suitable for your own experiment, you can change the settings in the **Analysis Settings** dialog box, then reanalyze your experiment.

You can save the changed analysis settings to the **Analysis Settings Library** so that you can use them in other experiments.

In the **Analysis Settings Library** dialog box you can apply a filter to reduce the number of settings protocols displayed.

You can access the **Analysis Settings Library** from the **Tools** menu. The **Analysis Settings Library** dialog box looks like this:



To change the analysis settings and to save them to the **Analysis Settings Library**:

1. From the **Experiment Menu** pane, select **Analysis**.
2. On the **Analysis** screen, click **Analysis Settings** to open the **Analysis Settings** dialog box.
3. Change the analysis settings as per your requirement.
4. Click **Save to Library** to save the changes you have made to the **Analysis Settings Library**.

You can import the analysis settings you have previously saved to the Analysis Settings Library, by clicking **Load from Library** in the Analysis Settings dialog box.

Override calibration

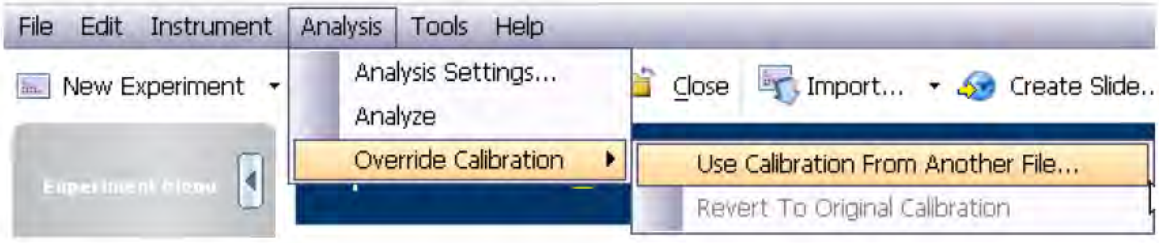
Each experiment file (.eds) stores the calibration data from the QuantStudio™ 12K Flex Real-Time PCR Instrument that it was run on. The calibration data can affect the analysis results of an experiment.

If you have run multiple experiments on different instruments and prefer the analysis results from a particular QuantStudio™ 12K Flex Real-Time PCR Instrument, then you can choose to use the calibration data from another instrument.

Use the calibration data from another experiment

1. Open the experiment file (.eds), in which you want to import the calibration data from another QuantStudio™ 12K Flex Real-Time PCR Instrument, in the QuantStudio™ 12K Flex Software.

2. Click **Analysis** ▸ **Override Calibration** ▸ **Use Calibration From Another File**.



3. Browse to experiment file (.eds) from which you want to use the calibration data.

Note: You can choose to override the calibration data in an experiment with the calibration data of only the same experiment type.

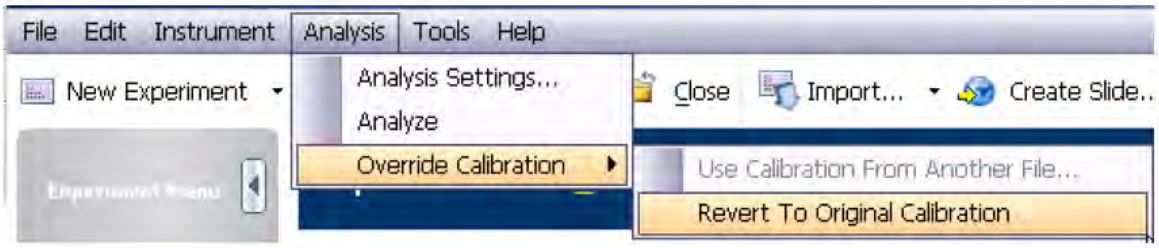
---

4. Click **Open**.

Revert to the original calibration data

1. Open the experiment file (.eds), in which you want to import the original calibration data, in the QuantStudio™ 12K Flex Software.

2. Click **Analysis** ▸ **Override Calibration** ▸ **Revert To Original Calibration**.



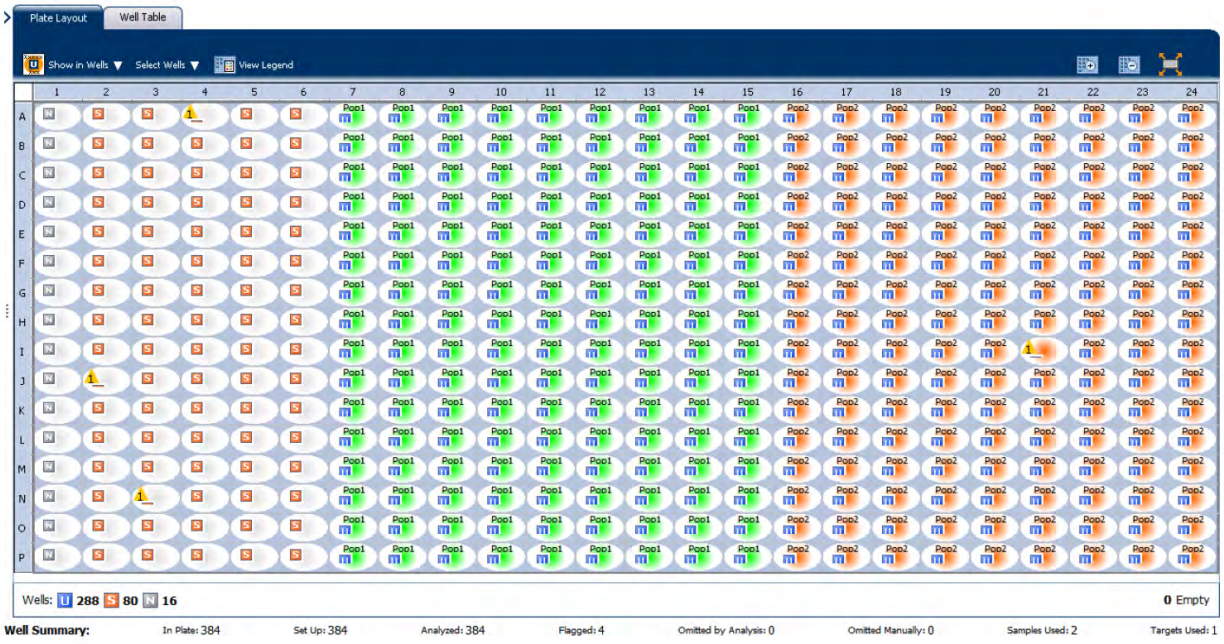
The experiment file displays analysis results as per the calibration data of the instrument that the experiment was run on.

Display wells

To display specific wells in the analysis plots, select the wells in the **Plate Layout** tab:

- To select wells of a specific type, use the **Select Wells** dropdown list. Select **Sample**, **Target**, or **Task**, then select the sample, target, or task name.
- To select a single well, click the well in the plate layout.
- To select multiple wells, click and drag over the desired wells, press control-click, or press shift-click in the plate layout.
- To select all the wells, click the upper left corner of the plate layout.

The plate layout for a Standard Curve experiment is shown in the following graphic:

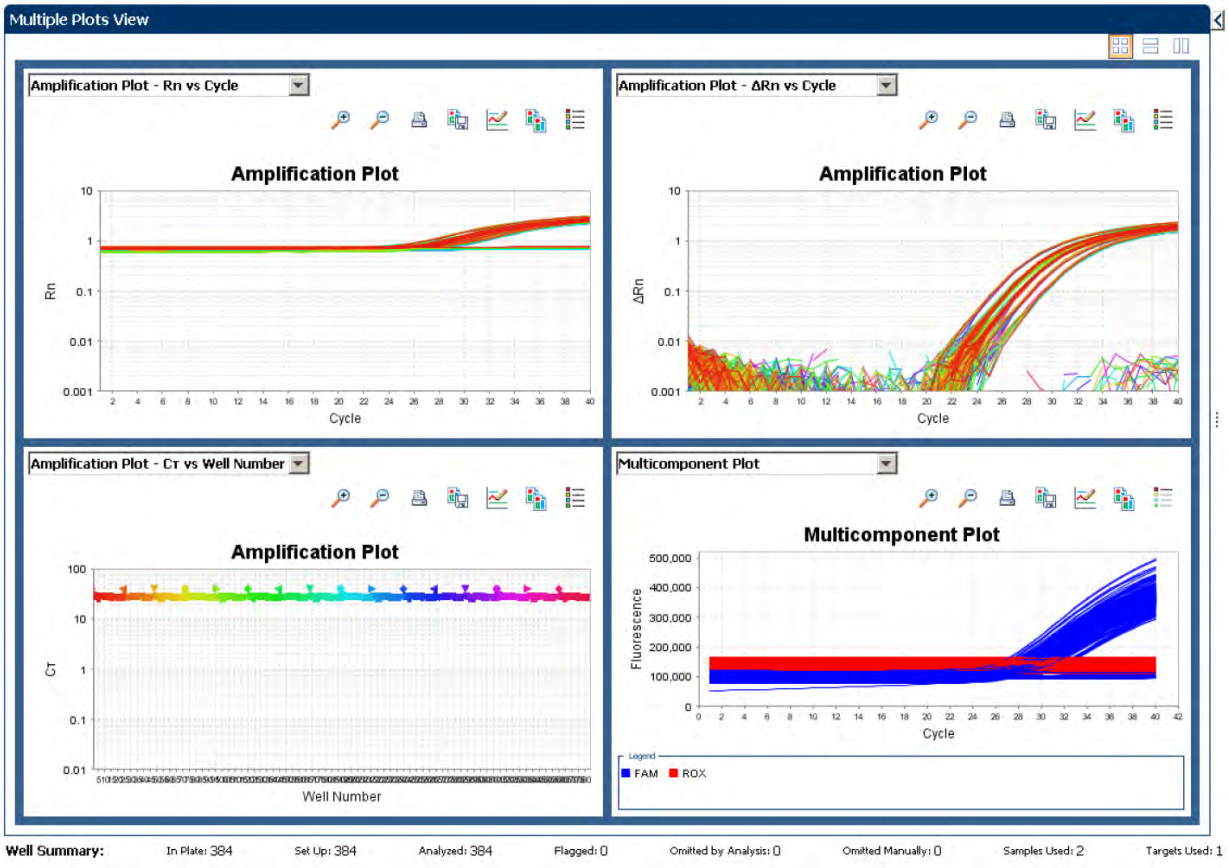


Display multiple plots

Use the **Multiple Plots View** screen to display up to four plots simultaneously. To navigate within the **Multiple Plots View** screen, from the **Experiment Menu** pane, select **Analysis ▸ Multiple Plots View**.

- To display four plots, click **(Show plots in a 2 × 2 matrix)**.
- To display two plots in rows, click **(Show plots in two rows)**.
- To display two plots vertically, click **(Show plots in two columns)**.
- To display a specific plot, select the plot from the dropdown list above each plot display.

The **Multiple Plots View** screen for a Standard Curve experiment is shown in the following graphic:



Display an expanded view of a plot or wells

- Click ⟩| to expand the view of a plot, displayed on the left-hand side of the screen.
- Click |⟨ to expand the view of the Plate Layout or Well Table displayed on the right-hand side of the screen.

Edit plot properties

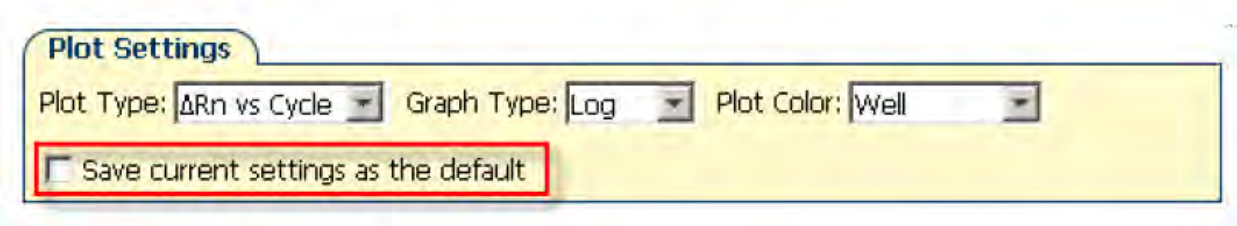
Use the **Plot Properties** dialog box on the **Analysis** screen to edit plot settings such as the font and color of the plot text, and the labels on the X axis and Y Axis.

1. Click on the **Analyze** screen (the icon appears above the plot) to open the **Plot Properties** dialog box
2. Edit the settings under the **General** tab, the **X Axis** tab, and the **Y Axis** tab.
   - Click the **General** tab to edit the plot title text, font, or color. You can also select whether to show the plot title.
   - Click the **X Axis** tab to edit the x axis label text, font, or color; select the tick marks and tick mark labels to display; and select the range to display.
   - Click the **Y Axis** tab to edit the y axis label text, font, or color; select the tick marks and tick mark labels to display; and select the range to display.
3. Click **OK**.

Save current settings as default

You can change the Plot Settings for the different analysis plots, and save them as defaults.

Select the **Save current settings as the default** checkbox on the respective plot screens under the **Analysis** menu.



Publish the analyzed data

Action	Instructions
Save a plot as an image file	Click
Print a plot	Click
Copy a plot to the clipboard	Click
Print a report	Click Print Report...
Export data	Click

Action	Go to	Then
Print the plate layout	**File ▸ Print**	Select the background color, then click **Print**.
Create slides	**File ▸ Send to PowerPoint**	Select the slides for your presentation, then click **Create Slides**.
Print a report	**File ▸ Print Report**	Select data for the report, then click **Print Report**.

Export an experiment

About exporting an experiment

The export feature of the QuantStudio™ 12K Flex Software allows you to export:

- Plate setup files for future experiments.

  Plate setup files contain setup information such as the well number, sample name, sample color, target name, dyes, and other reaction plate contents.
- Analyzed data in different formats for further analysis.

  The data can be exported in the QuantStudio™ 12K Flex format, the 7900 SDS format, and the RDML format.

  The 7900 format is applicable only to Standard Curve, Relative Standard Curve, Genotyping, Presence/Absence, and Melt Curve experiments.

  The RDML export format is applicable only to Standard Curve, Relative Standard Curve, Comparative C_T, and Melt Curve experiments. The RDML format is available only in a single file format.

  For Standard Curve experiments, you can also export the analyzed data from the QuantStudio™ 12K Flex Software to the external application, CopyCaller™ Software, if it is installed on your computer before the QuantStudio™ 12K Flex Software is installed. The application appears in the **Tools** menu.
- Gene Expression studies to carry out a comparative analysis.

Export procedure

Note: If you choose the **Auto Export** option during experiment setup or before running an experiment, the data is automatically exported to the location you specified. If you did not set the **Auto Export** option, the analyzed data is not exported automatically.

1. Open the experiment file that contains the data to export, and from the **Experiment Menu**, click **Export**.

2. Select the format for exported data.

   - **QuantStudio 12K Flex format**—Supports TXT, XLS, and XLSX file formats
   - **7900 format**—Supports only the TXT file format
     Single experiments are exported in the SDS 2.4 detector centric export format of the 7900 Sequence Detecting System.
   - **RDML format**—Supports only XML data (Real Time Data Markup Language)

3. Select the option to export all data in one file or in separate files for each data type.

   - **One File**—All data types are exported in one file.
     - If you select the XLS format, a worksheet is created for each data type.
     - If you select the TXT format, the data are grouped by data type.
   - **Separate Files**—Each data type is exported in a separate file. For example, if you select three different data types Results, Amplification, and Multicomponent to export, three separate files (one each for Results, Amplification, and Multicomponent) are created. You can select the type of file (XLS or TXT) to export from the **File Type** dropdown list.

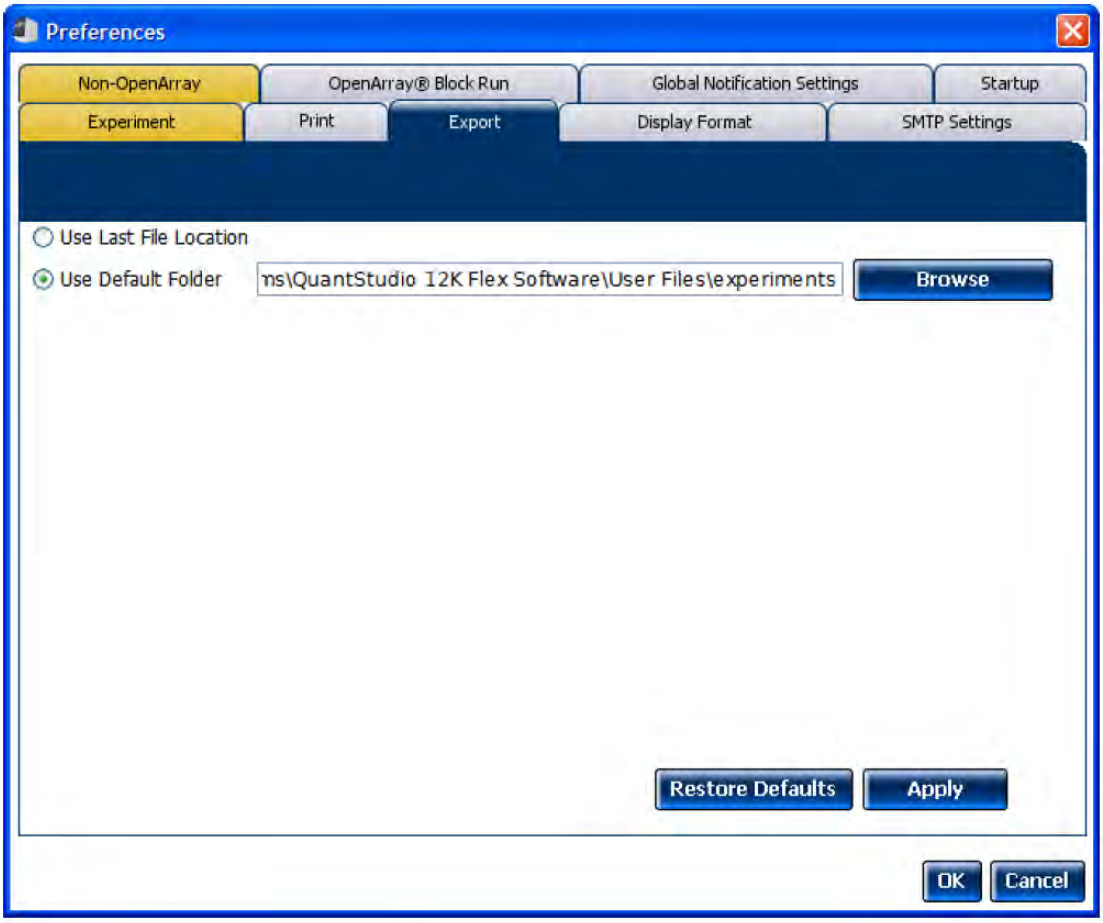
   Note: You cannot use an exported XLS file or an exported XLSX file when importing plate setup information.

4. *(Optional)* Select the **Open file(s) when export is complete** checkbox to automatically open the file when export is complete.

5. Enter a file name and location.
   a. Enter a name for the export file in the **Export File Name** field.

b. Enter the **Export File Location** information. Click **Browse** if you do not want to save the export file in the default export folder.

Note: To set up the location, go to **Tools** ▸ **Preferences**, then select the **Export** tab. You can select the **Use Last File Location** radio button or the **Use Default Folder** radio button.



6. Select the data to export.

Select	Export
Sample setup	Well, sample name, sample color, and target name of samples in the plate
Raw data	Raw fluorescence data for each filter, for each cycle
Amplification data	Amplification results, such as C_T values, Rn, or ΔRn
Multicomponent data	Fluorescence data for each dye, for each cycle
Results	Results information, such as C_T values, Rn, or calls
Technical Replicate Results (Tech. Rep. Results)	Technical replicates information, such as Sample name, Target name, Task, or RQ
Biological Replicate Results (Bio. Rep. Results)	Biological replicates information, such as Biogroup name, Target name, Task, or RQ

Note: Results data are not available for export until the run status is complete and the data are analyzed.

Note: The Technical Replicate Results and Biological Replicates Results are available only in Relative Standard Curve and Comparative C_T experiments.

7. *(Optional)* For Standard Curve experiments, select the external application, CopyCaller™ Software, if the software is installed on your computer.

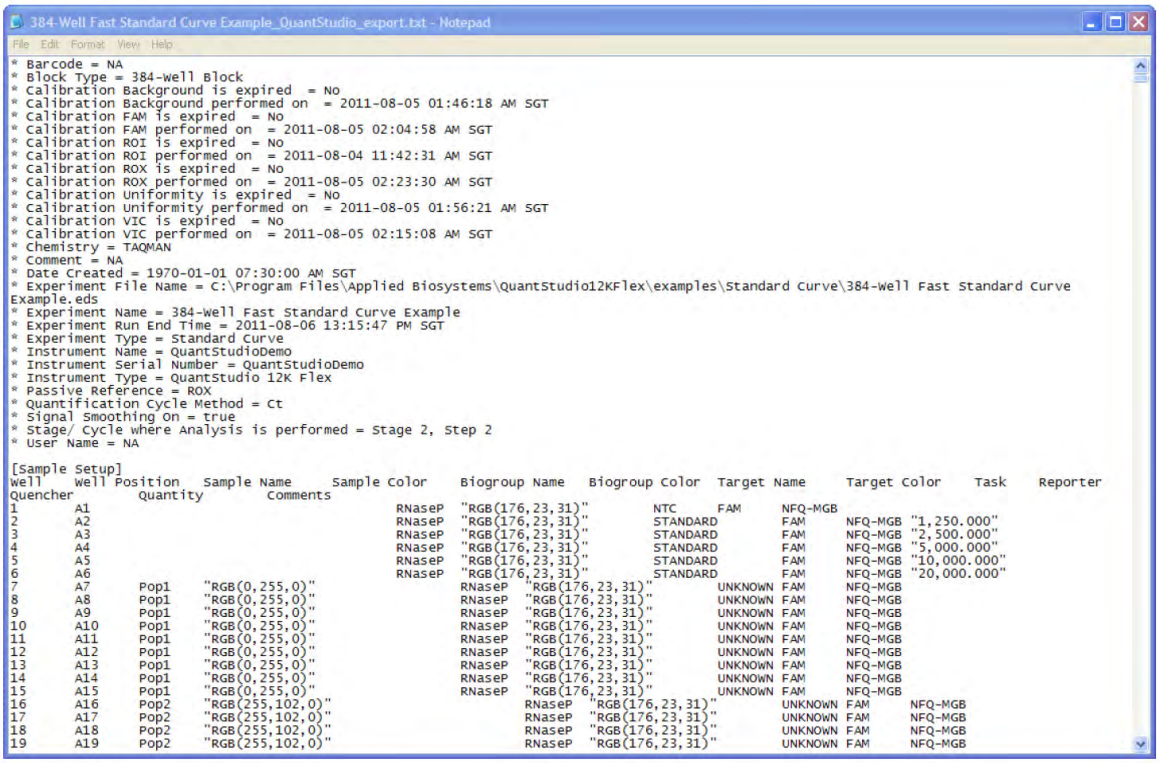
8. *(Optional)* After you have defined the export properties or after moving the table headings order, you can save those export settings as an export set by clicking **Save Export Set As**. Later you can import the heading order into another file by clicking **Load Export Set**. You can also delete export settings by clicking **Delete Export Set**.

   Note: It is advisable to keep the default order of the table headings if you are using CopyCaller™ Software for further analysis.

9. Click **Start Export**.

   The **Export** screen for a Standard Curve experiment is shown in the following graphic:

The exported file when opened in Notepad appears as shown in the following graphic:



384-Well Fast Standard Curve Example_QuantStudio_export.txt - Notepad

File Edit Format View Help

```
* Barcode = NA
* Block Type = 384-Well Block
* Calibration Background is expired  = No
* Calibration Background performed on  = 2011-08-05 01:46:18 AM SGT
* Calibration FAM is expired  = No
* Calibration FAM performed on  = 2011-08-05 02:04:58 AM SGT
* Calibration ROI is expired  = No
* Calibration ROI performed on  = 2011-08-04 11:42:31 AM SGT
* Calibration ROX is expired  = No
* Calibration ROX performed on  = 2011-08-05 02:23:30 AM SGT
* Calibration Uniformity is expired  = No
* Calibration Uniformity performed on  = 2011-08-05 01:56:21 AM SGT
* Calibration VIC is expired  = No
* Calibration VIC performed on  = 2011-08-05 02:15:08 AM SGT
* Chemistry = TAQMAN
* Comment = NA
* Date Created = 1970-01-01 07:30:00 AM SGT
* Experiment File Name = C:\Program Files\Applied Biosystems\QuantStudio12KFlex\examples\Standard Curve\384-Well Fast Standard Curve
Example.eds
* Experiment Name = 384-Well Fast Standard Curve Example
* Experiment Run End Time = 2011-08-06 13:15:47 PM SGT
* Experiment Type = Standard Curve
* Instrument Name = QuantStudioDemo
* Instrument Serial Number = QuantStudioDemo
* Instrument Type = QuantStudio 12K Flex
* Passive Reference = ROX
* Quantification Cycle Method = Ct
* Signal Smoothing On = true
* Stage/ Cycle where Analysis is performed = Stage 2, Step 2
* User Name = NA

[Sample Setup]
Well    Well Position   Sample Name     Sample Color    Biogroup Name   Biogroup Color  Target Name     Target Color    Task    Reporter
Quencher        Quantity        Comments
1       A1                                      RNaseP  "RGB(176,23,31)"        NTC     FAM     NFQ-MGB
2       A2                                      RNaseP  "RGB(176,23,31)"        STANDARD        FAM     NFQ-MGB "1,250.000"
3       A3                                      RNaseP  "RGB(176,23,31)"        STANDARD        FAM     NFQ-MGB "2,500.000"
4       A4                                      RNaseP  "RGB(176,23,31)"        STANDARD        FAM     NFQ-MGB "5,000.000"
5       A5                                      RNaseP  "RGB(176,23,31)"        STANDARD        FAM     NFQ-MGB "10,000.000"
6       A6                                      RNaseP  "RGB(176,23,31)"        STANDARD        FAM     NFQ-MGB "20,000.000"
7       A7      Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
8       A8      Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
9       A9      Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
10      A10     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
11      A11     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
12      A12     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
13      A13     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
14      A14     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
15      A15     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
16      A16     Pop2    "RGB(255,102,0)"                        RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
17      A17     Pop2    "RGB(255,102,0)"                        RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
18      A18     Pop2    "RGB(255,102,0)"                        RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
19      A19     Pop2    "RGB(255,102,0)"                        RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
```

2 Experiment shortcuts

This chapter provides you with shortcuts to use in the QuantStudio™ 12K Flex Software after you have learned experiment basics.

You can reuse experiment settings and plate setup information by: directly importing and editing a template, using the QuickStart feature with a template, importing experiment setup information, or importing a sample definition file; you can also prepare several experiments at once or create a new experiment using the ReadiApp feature.

Create an experiment from a template

You can use a template to create a new experiment. Templates are useful when you want to create many experiments with the same experiment parameters.

You can create an experiment from a template from the QuantStudio™ 12K Flex Software and from the instrument touchscreen.

Note: To access the software example templates, navigate to one of the following folders, where `<...>` is the installation drive. The default installation drive is `C:` if the software is installed by the customer. The default installation drive is `D:` if the software is installed by a Thermo Fisher Scientific field service engineer.

- QuantStudio™ 12K Flex Software v1.7 and later—`<...>:\Program Files\Applied Biosystems\QuantStudio 12K Flex Software\templates`
- QuantStudio™ 12K Flex Software v1.6 and earlier—`<...>:\Program Files (x86)\Applied Biosystems\QuantStudio 12K Flex Software\templates`

Create a template

1. In the QuantStudio™ 12K Flex Software **Home** screen, open an existing experiment, or create a new experiment.

   Note: To create a new experiment using the Experiment Setup, see “Set up an experiment” on page 18.

2. Select **File** ▸ **Save As Template**.

3. Enter a file name, select a location for the template, then click **Save**.

4. Click **Close**.

You can use that experiment as a template for similar experiments.

Create a new experiment using a template

1. From the **Home** screen, click **Create From Template**.
2. Locate and select the template file, then click **Open**.
   A new experiment is created using the setup information from the template.
3. Edit the experiment properties, plate definitions, plate assignments, and run method before you prepare the reactions and run the experiment.
4. Proceed to preparing reactions, running the experiment, and analyzing the data.

Create an experiment using a template on the instrument touchscreen

You can run experiments using templates from the instrument touchscreen by importing the templates from the QuantStudio™ 12K Flex Software instrument console or a USB drive. You can also modify the experiment parameters in the templates as per your requirement.

Run a pre-existing template

1. In the **Home** screen of the instrument touchscreen, touch **View Template**.
2. Select a pre-existing template from the templates list on the **View Templates** screen.
3. Touch **View** to see the run profile before you start a run.
4. After confirming the template setup is correct, touch **(Back)** to go back to **View Templates** screen.
5. Touch **Start Run**.

Edit a template before running the experiment

1. Touch **New** on the **View Templates** screen to create a new experiment from the existing template.

   Note: Select a template before you touch **New**.

2. Edit the experiment parameters in the **Create New Experiment** screen.
3. Select one of the following options.
   - Touch **Save & Exit** to save and exit the experiment.
   - Touch **Save & Start Run** to save and start the experiment run.

Use the QuickStart feature to start an experiment

You can use a template to run an experiment with the QuantStudio™ 12K Flex Software **QuickStart** feature.

Use the QuickStart feature from the QuantStudio™ 12K Flex Software

1. Prepare the reactions.
2. Log in to the QuantStudio™ 12K Flex Software.
3. In the **Home** screen, click **96/384/Array Cards** to access the **Run 96/384/Array Cards** dialog box.
4. Enter or select the following information.
   a. Instrument icon of the instrument to perform the run on.
   b. Experiment name.
   c. Experiment location.
   d. Experiment template file.
   e. *(Optional)* Barcode, User Name, and Comments for the experiment.

5. *(Optional)* To review the experiment or to make changes to any of the experiment parameters, click **Experiment Setup**.

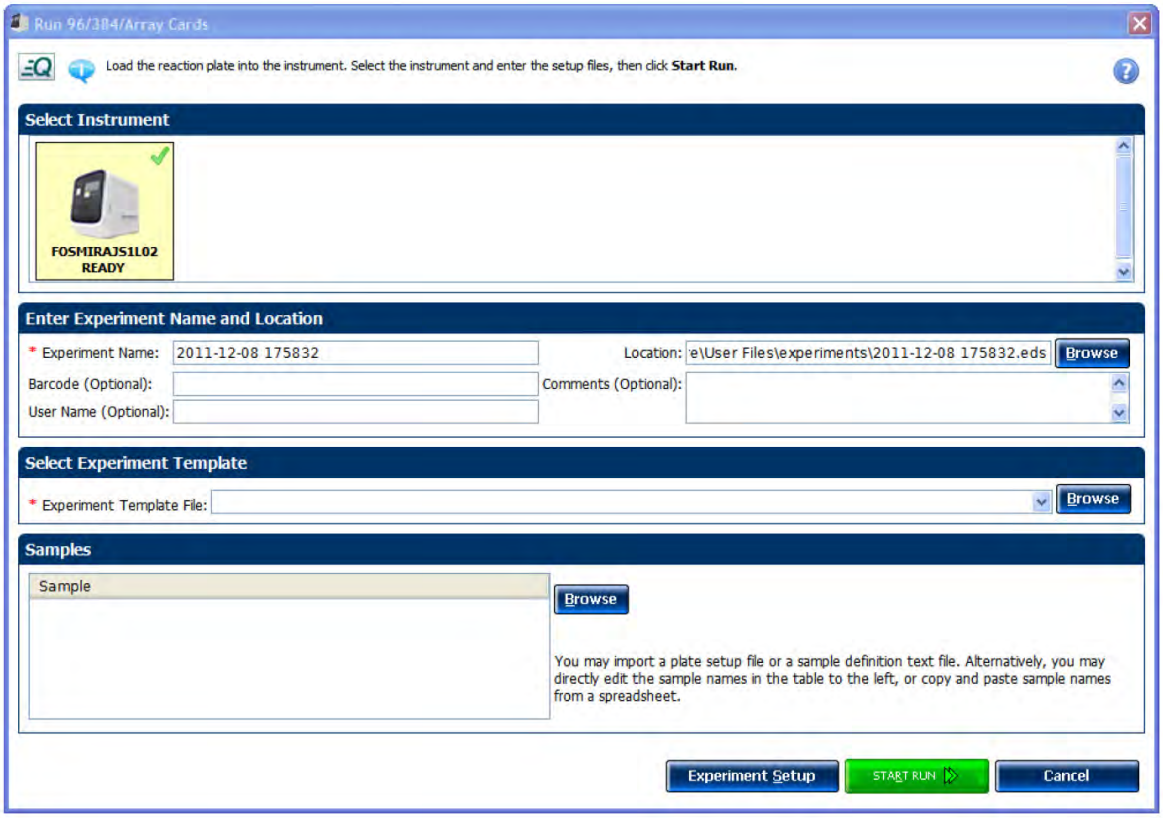


Figure 6 96/384/Array Cards dialog box

6. Proceed to running the experiment and analyzing the data.

QuickStart feature from the instrument touchscreen

You can use the **QuickStart** feature to start an experiment from the instrument touchscreen in the following ways:

- Start an experiment using a pre-defined template
- Start an experiment with a pre-defined short-cut button

Start an experiment using a pre-defined template

You can use a pre-existing template from the default experiments folder or use a custom template from another folder to start a run.

Start an experiment with a pre-defined short-cut button

The instrument touchscreen displays up to 18 shortcut buttons to templates or folders that contain experiments to be run. The shortcut buttons are present under **My Shortcuts** on the **Home** screen. To start a run, touch any of the predefined experiment or folder buttons.

1. Touch **Settings** to open the **Settings** menu.
2. Touch **Set Up Shortcuts** to list the shortcut targets.
3. On the **Shortcut Targets** screen, select an existing template **Shortcut Target** button or an unused button.
4. Touch **Set Shortcut**. If you selected an unused button, then touching **Set Shortcut** will list out the templates and folders to set the shortcut for.
5. Under the **From Templates** tab, select the templates for which you are creating the shortcut button.
6. *(Optional)* Create a shortcut button to show the templates or experiments in a particular folder for quick access, from those listed under the **From Folders** tab. You can touch **Edit** to create or edit shortcut buttons.

Import plate setup for an experiment

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

- TXT—Text format
- XML—XML format
- CSV—Comma separated values format
- SDT—Sequence detecting system (sds) template files format
- SDS—7900 v2.3 format

IMPORTANT! Make sure the file you select contains only plate setup data and that the experiment types match.

Note: For instructions on exporting an experiment, see “Export an experiment” on page 68.

1. Create a new experiment or open an existing experiment.
2. In the **Experiment Setup** screen, select **File ▸ Import Plate Setup** or access the **Import** dropdown list in the toolbar, then select **Import Plate Setup**.

3. Click **Browse**, locate and select the file to import, then click **Select**.

4. Click **Start Import**. The setup data from the exported text file is imported into the open experiment.

Note: If your experiment already contains plate setup information, the software asks if you want to replace the plate setup with the data from the import file. Click **Yes** to replace the plate setup.

5. After importing plate setup information, use **Experiment Setup** to set up your experiment, and then run the experiment.

Note: You can import plate setup information from a 96-well plate into a 384-well plate, provided that the file you are importing the information from is a TXT file.

Import sample information

You can import sample information from a sample definition file to include in the plate setup for your experiment. A sample definition file is a comma-delimited file (CSV) or a tab-delimited text file (TXT) that contains the following setup information: well number, sample name, and custom sample properties.

Note: Make sure that the sample definition file you select contains only sample information.

Create a sample definition file

1. Open a text editing program, for example, Notepad.
2. Enter the following column headers in the first row (press the Tab key between each entry if you are saving the file as TXT or enter a comma between each entry if you are saving the file as CSV):
   - ***Well***
   - ***Sample Name***
   - *(Optional)* Column header names for up to six user-defined custom fields (for example, ***Custom 1***, ***Custom 2***, etc.)

3. For each subsequent row, enter the well number, press the Tab key or enter a comma, then enter the sample name. Optionally, press the Tab key, then enter the custom properties for the sample.

4. Save the file with the TXT or CSV file extension.

   An example sample definition, saved with the CSV extension, file looks like this:

	A	B	C	D	E	F	G	H
1	Well	Sample Name	ID	Age	Sex	Weight	HairColor	Smoker
2	1	Sample 1	1	22	Female	25	black	Yes
3	2	Sample 2	2	25	Male	26	brown	No
4	3	Sample 3	3	45	Female	50	blonde	Yes
5	4	Sample 4	4	31	Male	33	red	Yes
6	5	Sample 5	5	29	Female	46	grey	No
7	6	Sample 6	6	26	Male	35	black	No
8	7	Sample 7	7	31	Female	33	black	Yes
9	8	Sample 8	8	32	Male	67	black	No
10	9	Sample 9	9	32	Female	55	brown	Yes
11	10	Sample 10	10	33	Male	44	blonde	Yes
12	11	Sample 11	11	34	Female	25	red	No
13	12	Sample 12	12	34	Male	26	grey	No
14	13	Sample 13	13	35	Female	50	black	Yes
15	14	Sample 14	14	35	Male	33	black	No
16	15	Sample 15	15	36	Female	46	black	Yes
17	16	Sample 16	16	36	Male	35	brown	Yes
18	17	Sample 17	17	37	Female	33	blonde	No
19	18	Sample 18	18	37	Male	67	red	No
20	19	Sample 19	19	38	Female	55	grey	Yes
21	20	Sample 20	20	38	Male	44	black	No

Import sample information from a sample definition file

1. Create a new experiment or open the experiment to receive the setup data (select **File** ▸ **Open**, select the file to open, then click **Open**).

2. From the open experiment, select **File** ▸ **Import Plate Setup**.

3. Click **Browse** to browse your computer for a sample definition text file (CSV). After you locate the file and select it, click **Select**.

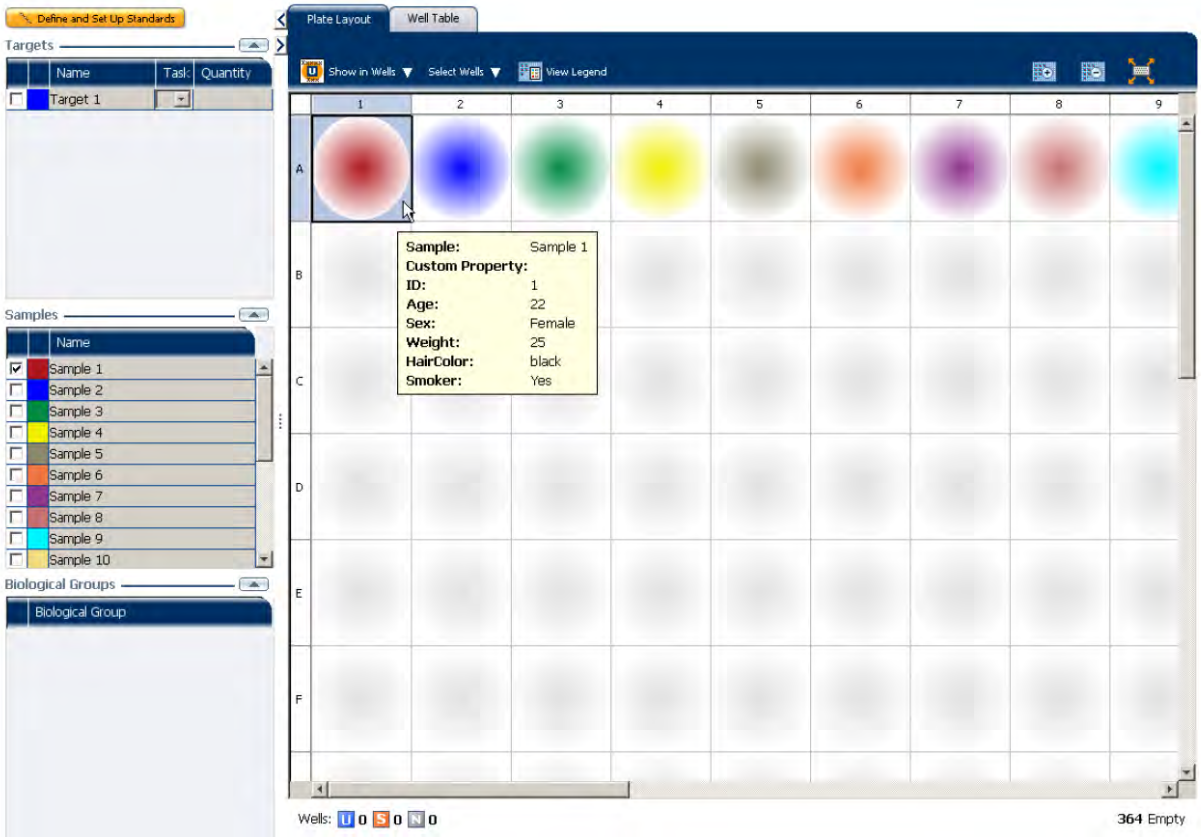
4. Click **Start Import**.

5. If your experiment already contains plate setup information, the software asks you if you want to replace the plate setup with the data from the file. Click **Yes** to replace the plate setup information.

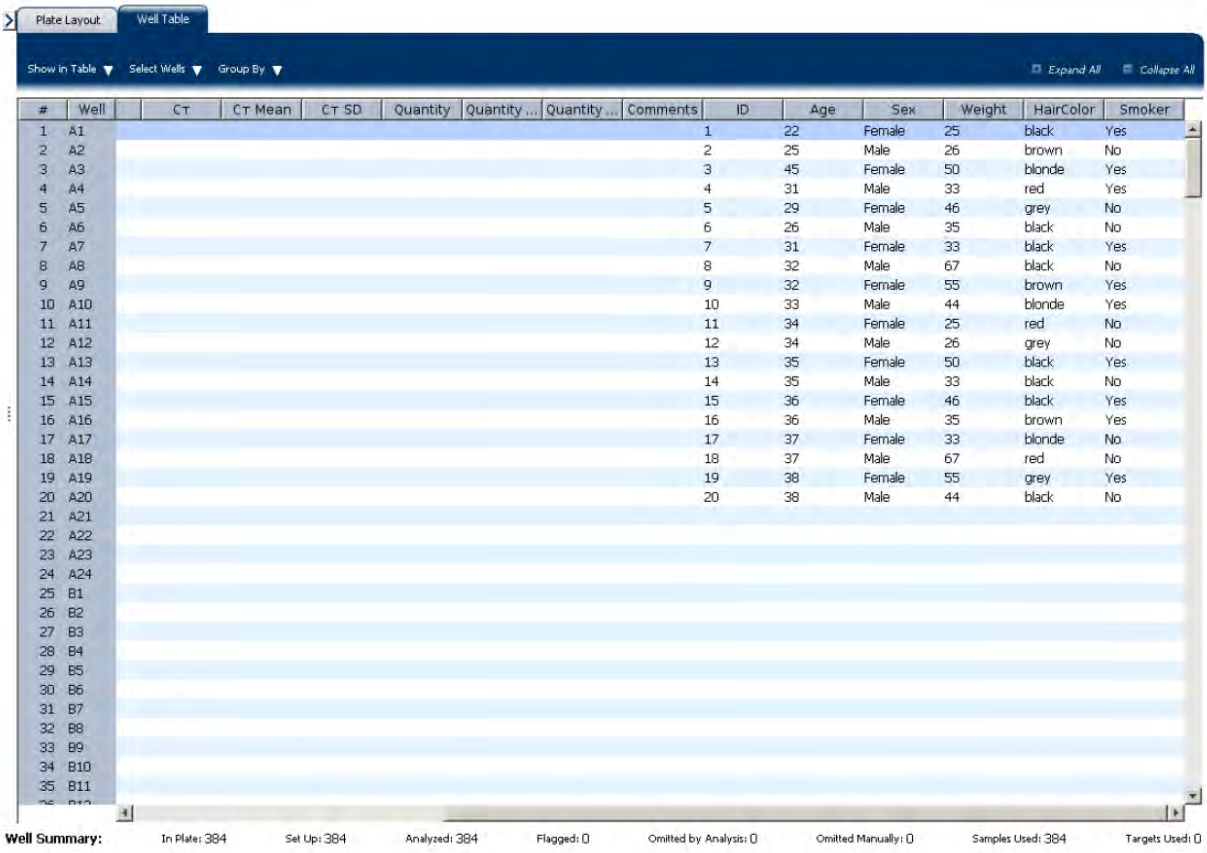
The samples appear in the **Samples** table for the experiment. All samples and well assignments in the experiment are replaced with those in the file. If defined, the custom sample properties also appear in the **Well Table** of the **Analysis** section, and also in the **Plate Layout** tooltips in both the **Setup** screen and the **Analysis** screen. The custom fields can be exported with the results data.

Note: You cannot edit the custom sample properties from within the **Well Table**. To modify this information, edit the custom fields in the sample definition file and import the file again. All of the sample information in the experiment is replaced with the information in the new file.

The **Assign** screen with information from the above sample definition file looks like this:



The **Well Table** in the **Analysis** section looks like this:



#	Well		Ct	Ct Mean	Ct SD	Quantity	Quantity ...	Quantity ...	Comments	ID	Age	Sex	Weight	HairColor	Smoker
1	A1									1	22	Female	25	black	Yes
2	A2									2	25	Male	26	brown	No
3	A3									3	45	Female	50	blonde	Yes
4	A4									4	31	Male	33	red	Yes
5	A5									5	29	Female	46	grey	No
6	A6									6	26	Male	35	black	No
7	A7									7	31	Female	33	black	Yes
8	A8									8	32	Male	67	black	No
9	A9									9	32	Female	55	brown	Yes
10	A10									10	33	Male	44	blonde	Yes
11	A11									11	34	Female	25	red	No
12	A12									12	34	Male	26	grey	No
13	A13									13	35	Female	50	black	Yes
14	A14									14	35	Male	33	black	No
15	A15									15	36	Female	46	black	Yes
16	A16									16	36	Male	35	brown	Yes
17	A17									17	37	Female	33	blonde	No
18	A18									18	37	Male	67	red	No
19	A19									19	38	Female	55	grey	Yes
20	A20									20	38	Male	44	black	No
21	A21														
22	A22														
23	A23														
24	A24														
25	B1														
26	B2														
27	B3														
28	B4														
29	B5														
30	B6														
31	B7														
32	B8														
33	B9														
34	B10														
35	B11														

Use a template to create a batch of experiments

Use the batch experiment utility to create multiple experiment files from the same template without using the Experiment Setup.

1. In the menu bar, select **Tools ▸ Batch Experiment Setup**.

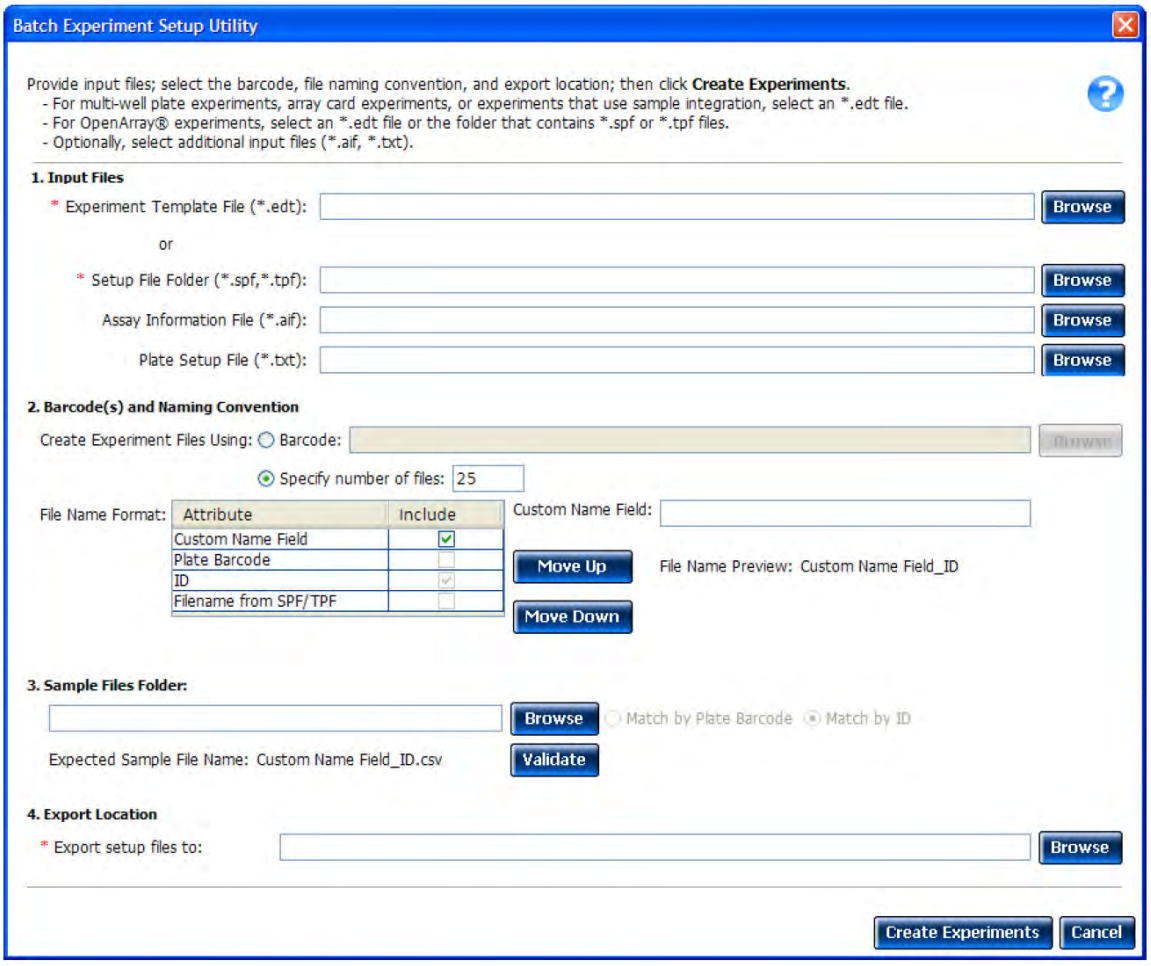


Figure 7 Batch Experiment Setup Utility dialog box

2. Select the file or the files to use to create the new experiments.
   a. For multi-well plate, array card experiments, or experiments that use sample integration, click **Browse** in the **Experiment Template File** field.
   b. Locate an EDT file to import, then click **Select**.
   c. For OpenArray™ experiments, click **Browse** in the **Experiment Template File** field or in the **Setup File Folder** field.
   d. Locate either an EDT file (template) or an SPF or a TPF file to import, then click **Select**.

e. *(Optional)* Repeat **steps 2a** on page 82 and **2b** on page 82 for the remaining setup file types to import Assay Information File (AIF), Plate Setup File (TXT)).

3. Select the option to create experiment files. The selected option determines the number of experiment files created.
   - **Specify Number of Files** field—Enter a number from 1 to 100.
   - **Barcode** radio button—Click **Browse**, then select a barcode file (TXT) to import. The software automatically adds the **Plate Barcode** attribute to the file name format. The number of experiments created equals the number of barcodes present in the barcode file.

Note: A barcode file contains one barcode per line.

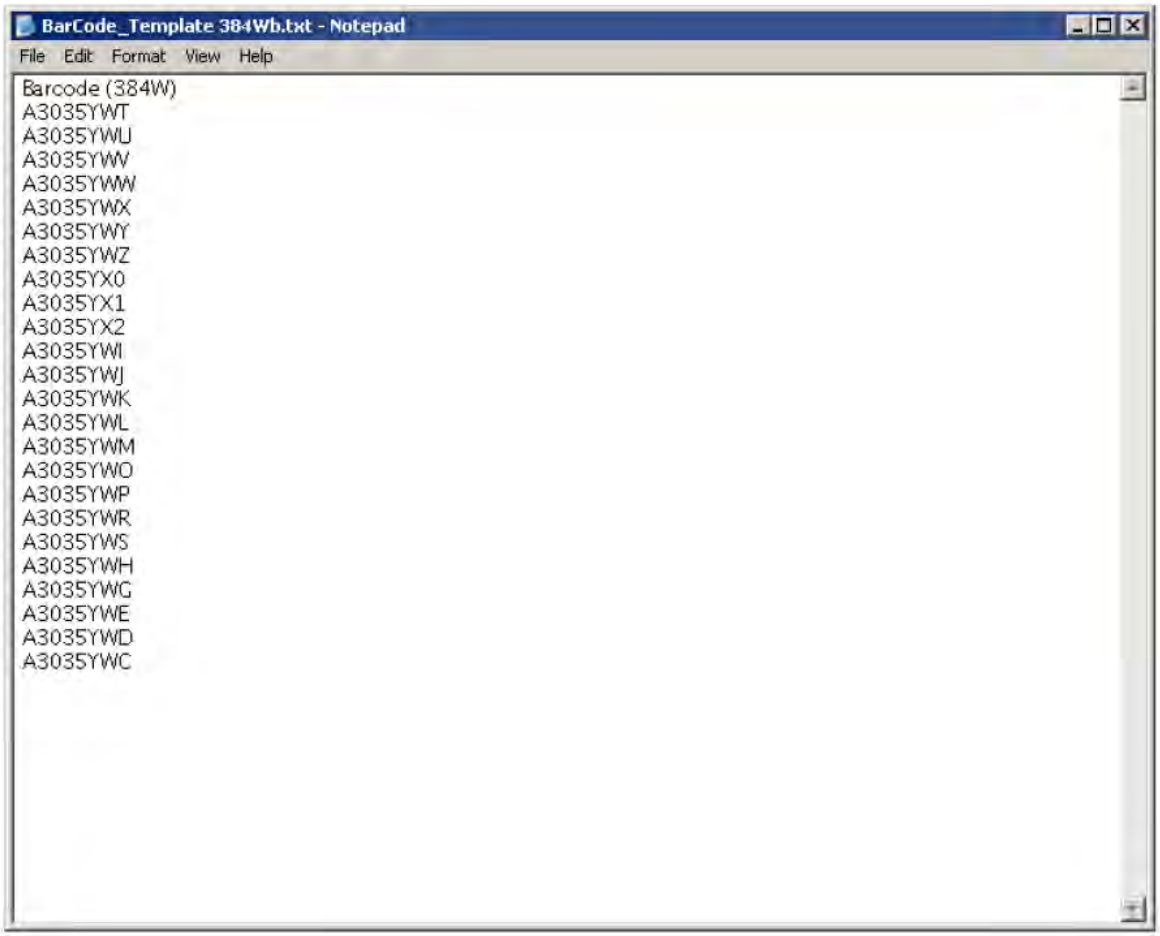


Figure 8 Example barcode file

4. *(Optional)* Edit the file name format. Use the **File Name Preview** to verify your settings.
   - Select the check box to include or exclude the **Custom Name Field_Plate Barcode** attribute from the file name. If included, click the **Custom Name Field**, then enter up to 100 letters and/or numbers to identify the batch of experiments.

     Note: The file name can contain a total of 100 characters, including all file name attributes.
   - Click **Move Up** or **Move Down** to change the order of the selected file name attributes.
5. Select the **Sample Files** folder.
   a. Click **Browse**, then locate and select a folder. Refer to the **Expected Sample File Name** for an example of a file name.

   b. Click **Validate** to visually check that experiment files are matched to sample files. If they do not match, the **Matching Sample File** shows the missing file as **Not found** in red.
6. Select the location for the experiment files to be created:
   a. Click **Browse** in the **Export Setup Files To:** field.

   b. Review the location for the experiment files. Navigate to a new location if you do not want to export the experiment files to that folder, then click **Select**.
7. Click **Create Experiments**.
   A confirmation message is displayed when the batch of experiments has been created.

Create an experiment using the ReadiApp feature

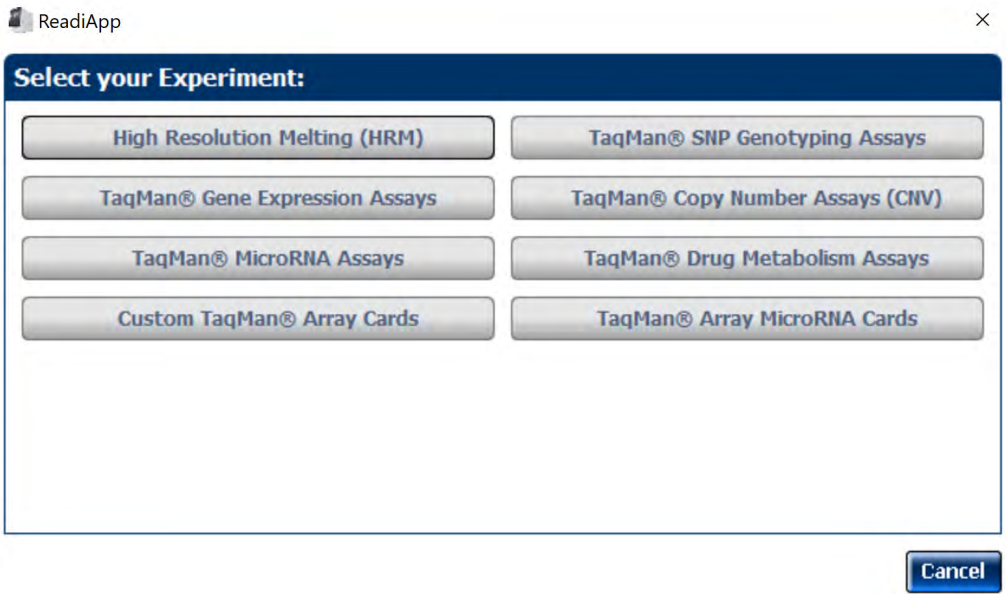
You can use the **ReadiApp** feature to set up an experiment in the QuantStudio™ 12K Flex Software. The **ReadiApp** feature provides a shortcut to create experiments for the assays purchased from Thermo Fisher Scientific.

The default templates available in the QuantStudio™ 12K Flex Software include:

- High Resolution Melting (HRM)
- TaqMan™ Gene Expression Assay
- TaqMan™ MicroRNA Assay
- Custom TaqMan™ Array Card
- TaqMan™ SNP Genotyping Assay
- TaqMan™ Copy Number Assay
- TaqMan™ Drug Metabolism Genotyping Assay
- TaqMan™ MicroRNA Assay (TaqMan™ Array Card)

1. Log in to the QuantStudio™ 12K Flex Software and, from the Tools menu on the Home screen, click **ReadiApp.**
2. Click the assay to use to set up an experiment.

   Note: Click **Cancel** to exit the ReadiApp dialog box.



A new experiment is created using the setup information from the template.

3. *(Optional)* Edit the experiment properties.
4. Proceed to preparing reactions, running the experiment, and analyzing the data.

Part II

Running standard curve experiments

3 About standard curve experiments

IMPORTANT! First-time users of the QuantStudio™ 12K Flex Real–Time PCR System, see the part "Getting started" on page 17 and Appendix A, "Documentation and support". The sections provide information and general instructions that are applicable to all the experiments described in this document.

Note: For more information about any of the topics discussed in this guide, access the Help from within the QuantStudio™ 12K Flex Software by pressing F1, clicking in the toolbar, or selecting **Help ▸ QuantStudio 12K Flex Software Help**.

Before you begin

The Standard Curve method is used for determining absolute target quantity in samples. With the standard curve method, the software measures amplification of the target in samples and in a standard dilution series. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates the absolute quantity of target in the samples.

Assemble required components

- **Sample**—The tissue group that you are testing for a target gene.
- **Standard**—A sample that contains known quantities of the target; used in quantification experiments to generate standard curves.
- **Standard dilution series**—A set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards.
- **Replicates**—The total number of identical reactions containing identical samples, components, and volumes.
- **Negative Controls**—Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

PCR Options

When performing real-time PCR, choose between:

- Singleplex PCR and multiplex PCR (see "Singleplex and multiplex PCR" on page 88)
  and
- 1-step RT-PCR and 2-step RT-PCR (see "1– and 2-step RT-PCR" on page 88)

Singleplex and multiplex PCR

You can perform a PCR reaction using either:

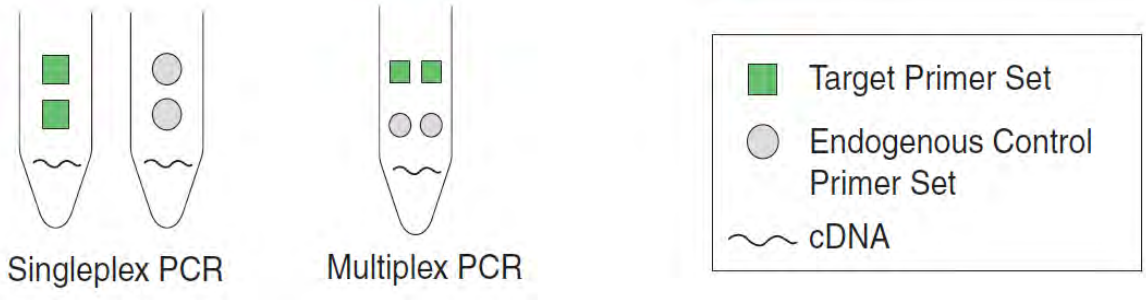
- **Singleplex PCR**—In singleplex PCR a single primer set is present in the reaction tube or well. Only one target or endogenous control can be amplified per reaction.

  or

- **Multiplex PCR**—In multiplex PCR, two or more primer sets are present in the reaction tube or well. Each set amplifies a specific target or endogenous control.

  One example is a probe that is labeled with FAM™ dye detects the target and a probe that is labeled with VIC™ dye detects the endogenous control.

IMPORTANT! SYBR™ Green reagents cannot be used for multiplex PCR.



1– and 2-step RT-PCR

You can perform reverse transcription (RT) and PCR in a single reaction (1-step) or in separate reactions (2-step). The reagent configuration you use depends on whether you are performing 1- or 2-step RT-PCR.

- **1-step RT-PCR**—In 1-step RT-PCR, RT and PCR take place in one buffer system. Using one buffer system provides the convenience of a single-tube preparation for RT and PCR amplification. However, you cannot use Fast PCR master mix or the carryover prevention enzyme, AmpErase™ UNG (uracil-N-glycosylase), to perform 1-step RT-PCR.
- **2-step RT-PCR**—2-step RT-PCR is performed in two separate reactions: First, total RNA is reverse-transcribed into cDNA, then the cDNA is amplified by PCR. This method is useful for detecting multiple transcripts from a single cDNA template or for storing cDNA aliquots for later use. The AmpErase™ UNG enzyme can be used to prevent carryover contamination.

Note: The Standard Curve example experiment is designed for singleplex PCR, where every well contains a primer/probe set for a single target; the reactions are set up for a 2-step RT-PCR.

About the example experiment

To illustrate how to perform Standard Curve experiments, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with the QuantStudio™ 12K Flex Real–Time PCR System.

The objective of the Standard Curve example experiment is to determine the quantity of the RNase P gene in two populations.

In the standard curve example experiment:

- The samples are genomic DNA isolated from two populations.
- The target is the RNase P gene.
- One standard curve is set up for the RNase P gene (target). The standard used for the standard dilution series contains known quantities of the RNase P gene. Because a single target is being studied, only one standard curve is required.

  IMPORTANT! In experiments where multiple targets are being studied, a standard curve is required for each target.

- The Standard Curve is a five-point dilution with 16 technical replicates per point.
- The experiment is designed for singleplex PCR, where every well contains a primer/probe set for a single target.
- Reactions are set up for 2-step RT-PCR.
- Primer/probe sets are from an Applied Biosystems™ RNase P assay.

  Note: The human RNase P FAM™ dye-labeled MGB probe is not available as a TaqMan™ Gene Expression Assay. It can be ordered as a Custom TaqMan™ Gene Expression Assay (Cat. No. 4331348).

4 Design the experiment

This chapter explains how to design the example experiment from the **Experiment Setup** menu.

Note: To automatically export the analyzed data to a specified location, select the **Auto Export** task from the **Export** screen, before running the experiment. For more information about Auto Export, see the part "Getting started" on page 17.

Define the experiment properties

Click **Experiment Setup** ▸ **Experiment Properties** to create a new experiment in the QuantStudio™ 12K Flex Software.

Field or selection	Entry
Experiment Name field	*384-Well Fast Standard Curve Example*
Barcode field	Leave field empty
User Name field	*Example User*
Comments field	*Standard Curve example*
Block	**384-Well**
Type of experiment	**Standard Curve**
Reagents	**TaqMan Reagents**
Properties	**Fast**

Save the experiment.

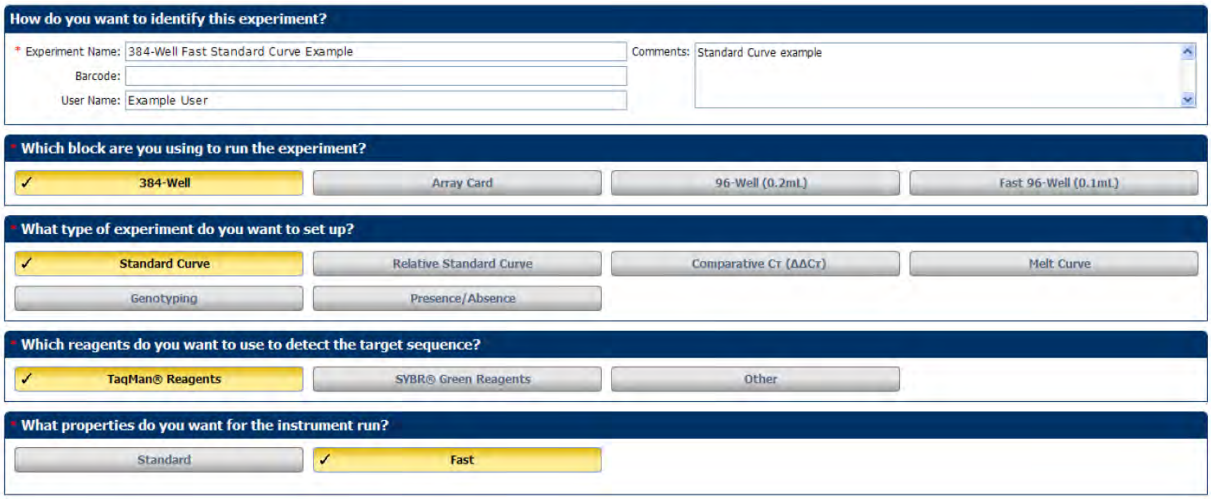


Figure 9 Experiment Properties screen

Define targets, samples, and biological replicates

1. Click **Define** to access the **Define** screen.
2. Enter the following target information.

Target name	Reporter	Quencher	Color
RNaseP	FAM	NFQ-MGB	

3. Enter the following sample information.

Sample name	Color
Pop1	
Pop2	

4. In the **Passive Reference** dropdown list, select **ROX** .

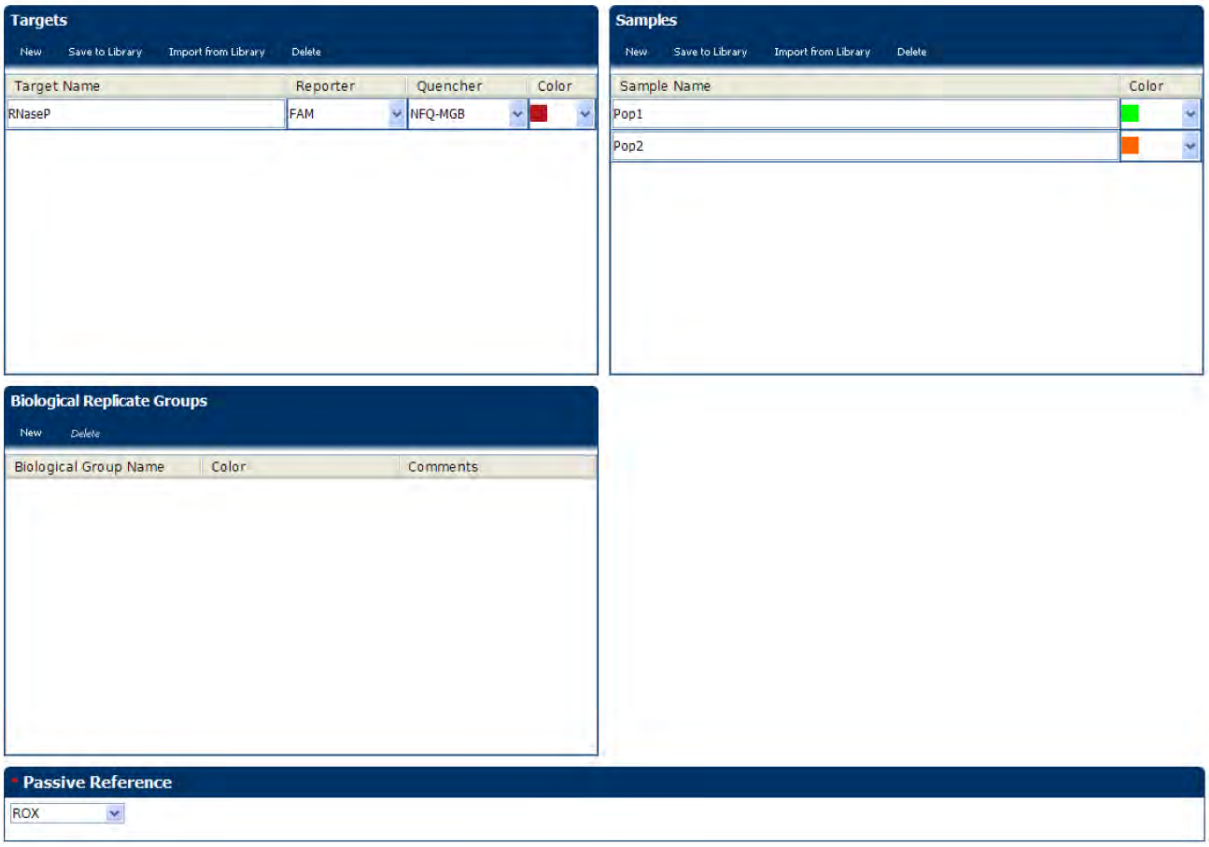


Figure 10 Define screen

Note: This example experiment does not define biological replicate groups. Leave the **Biological Replicate Groups** pane blank.

Assign targets, samples, and biological groups

1. Click **Assign** to access the **Assign** screen.

2. Define and set up standards.
   a. In the **Assign** screen, click **Define and Set Up Standards**.

   b. In the **Define and Set Up Standards** dialog box, in the **Select a target for this standard curve** dropdown list, select **RNaseP**.

   c. Define the standard curve.

Field	Enter
# of Points field	*5*
# of Replicates field	*16*
Starting Quantity field	*1250.0*
Serial Factor field	*2x*

   d. In the **Use Wells** section, select the **Let Me Select Wells** radio button.

   e. Click **Apply**, then click **Close**.

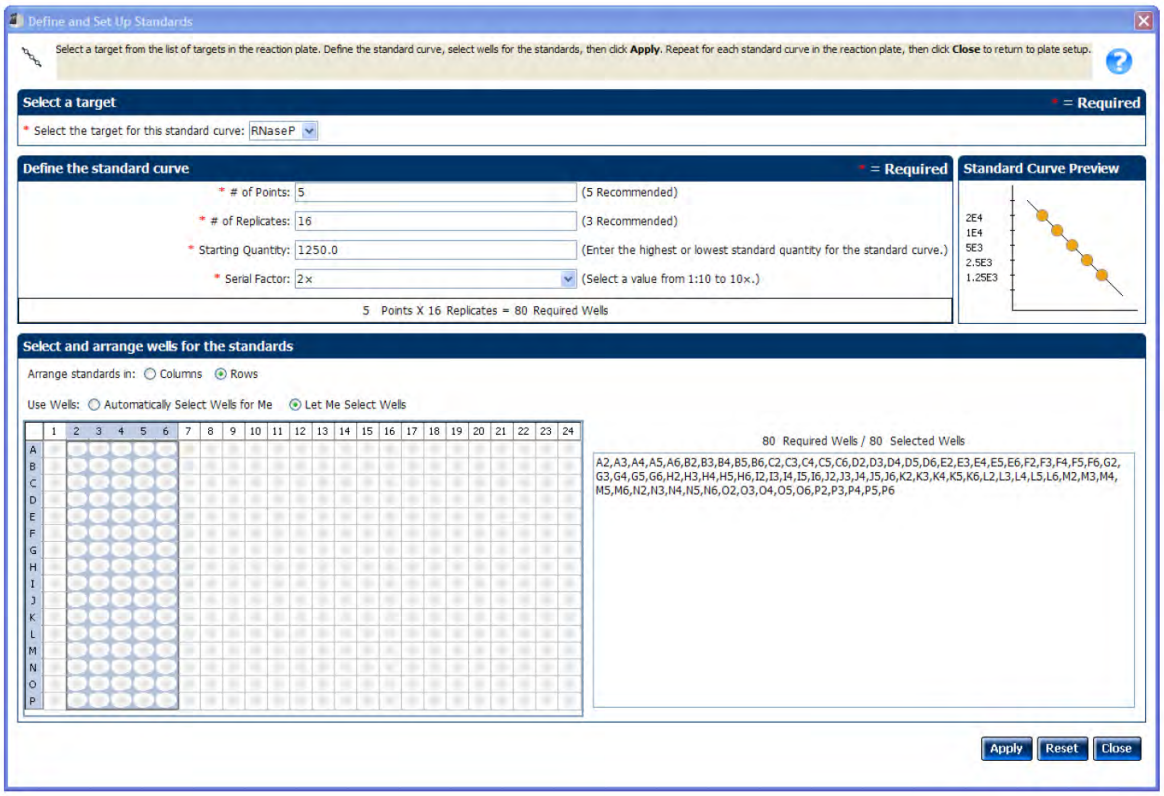


Figure 11 Define and Set Up Standards dialog box

3. Assign targets and samples.

Target name	Well number	Task	Quantity	Sample name
RNaseP	A1 - P1 (column 1)	Negative	None	None
RNaseP	A2 - P2 (column 2)	Standard	1250	None
RNaseP	A3 - P3 (column 3)	Standard	2500	None
RNaseP	A4 - P4 (column 4)	Standard	5000	None
RNaseP	A5 - P5 (column 5)	Standard	10000	None
RNaseP	A6 - P6 (column 6)	Standard	20000	None
RNaseP	A7 - P15 (columns 7 - 15)	Unknown	Determined by run	Pop1
RNaseP	A16 - P24 (columns 16 - 24)	Unknown	Determined by run	Pop2

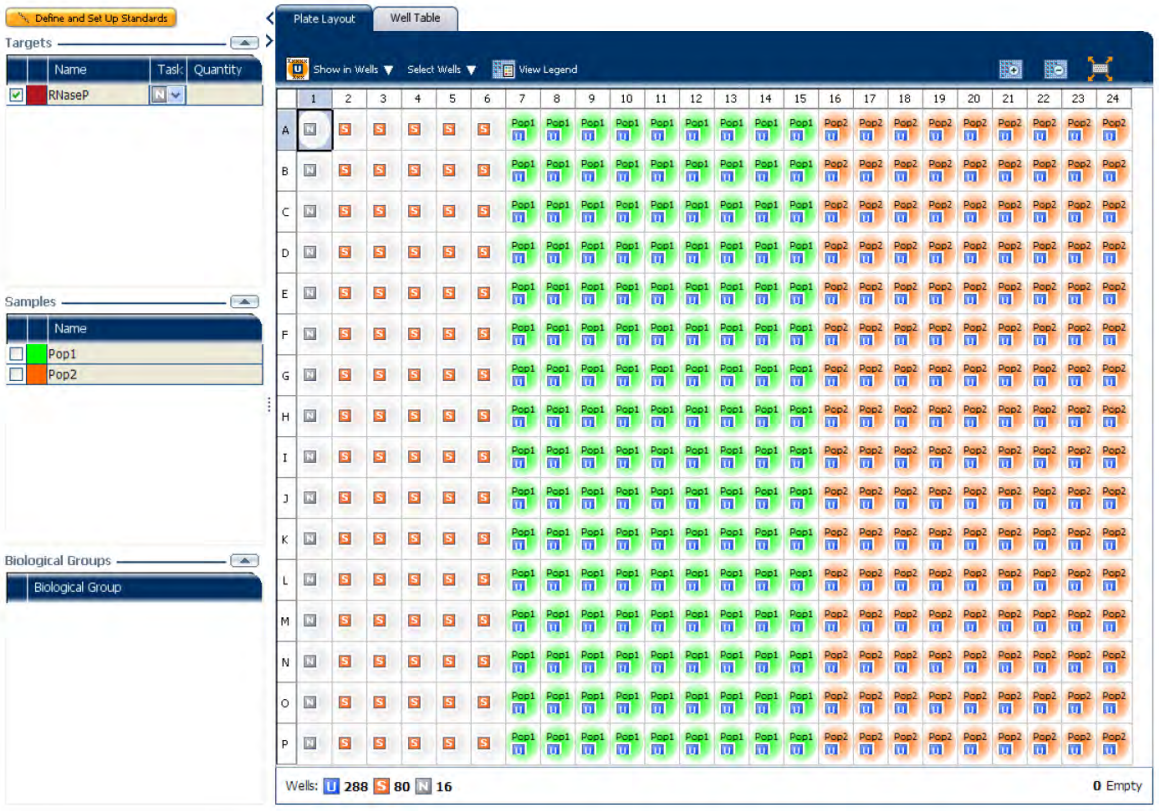


Figure 12 Assign screen

Set up the run method

Click **Run Method** to access the **Run Method** screen. Set the thermal profile under the **Graphical View** tab. Enter the following information:

- Reaction Volume Per Well: ***20 µL***
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Hold Stage	Step 1	1.9°C/s	95°C	20 seconds
PCR Stage Number of Cycles: 40 (default) Enable AutoDelta: Unchecked (default) Starting Cycle: Disabled when Enable AutoDelta is unchecked	Step 1	1.9°C/s	95°C	1 second
	Step 2	1.6°C/s	60°C	20 seconds

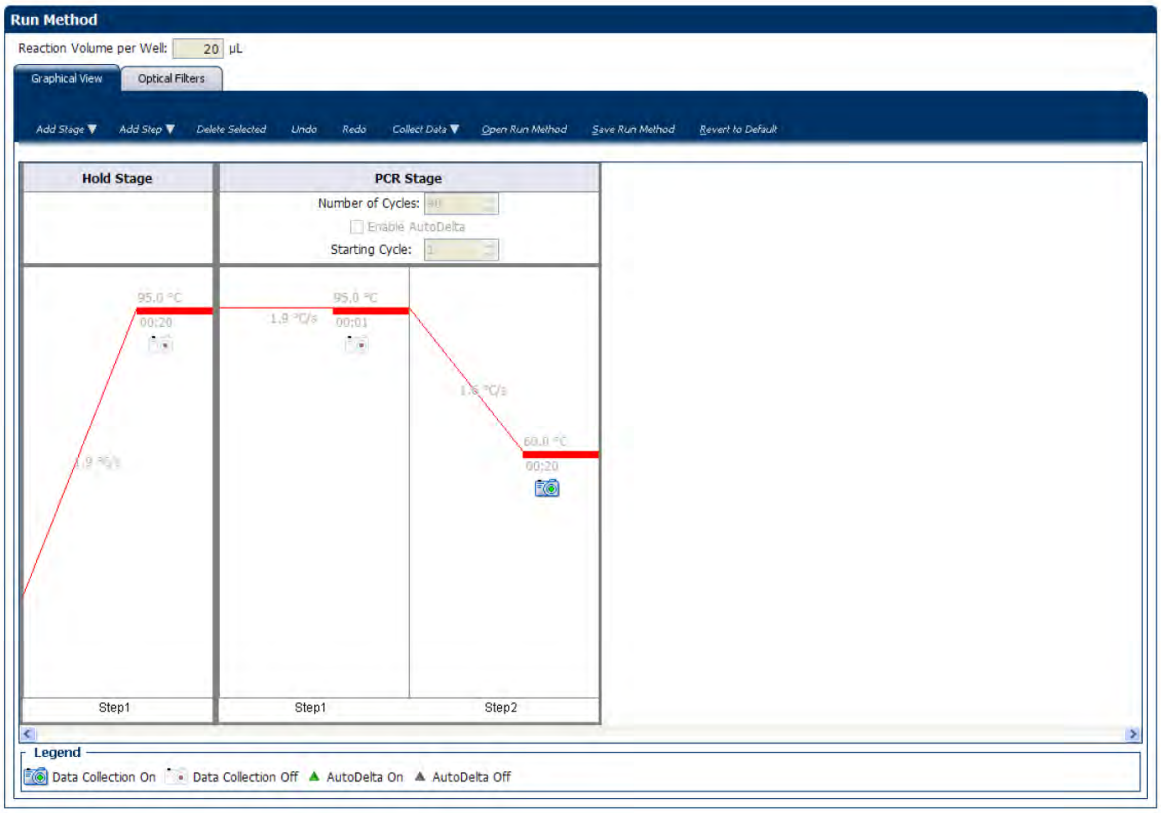


Figure 13 Run Method screen

More information

Information	Reference
Consumables	Chapter 1, “General information and instructions”
Using other quantification methods	• “Running relative standard curve experiments” on page 145 • “Running comparative Ct experiments” on page 198
Using alternative setup	Chapter 2, “Experiment shortcuts”

5 Prepare the reactions

This chapter explains how to prepare the PCR reactions for the Standard Curve example experiment.

Assemble required materials

- Items listed in the part “Getting started” on page 17
- Samples - Human Raji cell line-derived cDNA samples (100 ng/µL)
- Example experiment reaction mix components:
  - TaqMan™ Fast Universal PCR Master Mix
  - TaqMan™ RNase P Detection Reagents Kit (Cat. No. 4316831)

Prepare the sample dilutions

To determine the quantity of the RNase P gene in the example experiment, dilute the samples (as directed below) before adding the samples to the final reaction mix.

The stock concentration of each sample is 100 ng/µL. After dilution, the sample Pop1 has a concentration of 6.6 ng/µL and Pop 2 has a concentration of 3.3 ng/µL. Add 2µL to each reaction.

Use this table for sample dilution volumes for the example experiment.

Sample name	Stock concentration	Sample volume	Diluent volume	Total volume of diluted sample
Pop1	100.0 ng/µL	25 µL	355 µL	380 µL
Pop2	100.0 ng/µL	12.5 µL	367.5µL	380 µL

Note: For your own experiment, adjust the input amounts of the template depending on the template type and target abundance.

1. Label a separate microcentrifuge tube for each diluted sample:
   - Pop 1
   - Pop 2
2. Add the required volume of water (diluent) to each empty tube.

Tube	Sample name	Diluent volume
1	Pop 1	355 µL
2	Pop 2	367.5 µL

3. Add the required volume of sample stock to each tube.

Tube	Sample name	Sample volume
1	Pop 1	25 µL
2	Pop 2	12.5µL

4. Vortex each diluted sample for 3 to 5 seconds, then centrifuge the tubes briefly.

5. Place the diluted samples on ice until you prepare the reaction plate.

Prepare the standard dilution series

The standard concentration in stock is 20,000 copies/µL.

Standard name (labeled tube)	Dilution point	Source	Source volume	Diluent volume	Total volume	Standard concentration
RNase P Std. 1	1 (20,000)	Stock	18 µL	18 µL	36 µL	10,000 copies/µL
RNase P Std. 1	2 (10,000)	Dilution 1	18 µL	18 µL	36 µL	5,000 copies/µL
RNase P Std. 1	3 (5,000)	Dilution 2	18 µL	18 µL	36 µL	2,500 copies/µL
RNase P Std. 1	4 (2500)	Dilution 3	18 µL	18 µL	36 µL	1,250 copies/µL
RNase P Std. 1	5 (1250)	Dilution 4	18 µL	18 µL	36 µL	625 copies/µL

1. Prepare five standard dilutions.

   Note: For dilution 1, first vortex the stock for 3 to 5 seconds, then centrifuge the RNase P Std. 1 tube briefly before pipetting stock into the tube.

   Perform the following steps for each dilution:

   a. Use a new pipette tip to add 18 µL of source to the tube containing the standard.

   b. Vortex the tube for 3 to 5 seconds, then centrifuge the tube briefly.

2. Place the standards on ice until you prepare the reaction plate.

Prepare the reaction mix

For the RNase P assay (Standard Curve example experiment), the following table lists the universal assay conditions (volume and final concentration) for using the TaqMan™ Fast Universal PCR Master Mix.

Reaction component	Volume for 1 reaction	Volume for 384 reactions + 10% excess
TaqMan™ Fast Universal PCR Master Mix	5 µL	2,112 µL
RNase P Assay (20×)	0.5 µL	2,11.2 µL
Water	3.5 µL	1,478.4 µL
Total reaction mix volume	**9 µL**	**3,801.6 µL**

1. Label an appropriately sized tube for the reaction mix: RNase P Reaction Mix.
2. Add the required volumes of each reaction mix component to the tube.

   Note: Do not add the sample or standard at this time.

3. Mix thoroughly by gently pipetting up and down several times, then cap the tube.
4. Centrifuge the tube briefly to remove air bubbles.
5. Place the reaction mix on ice until you prepare the reaction plate.

   Note: You can separately add the sample to the reaction plate, as opposed to preparing individual reaction mixes for each sample.

Prepare the reaction plate

Example experiment reaction plate components

The reaction plate for the Standard Curve example experiment contains:

- A MicroAmp™ Optical 384-Well Reaction Plate
- Reaction volume: 10 µL/well
- 288 Unknown wells (U)
- 80 Standard wells (S)
- 16 Negative Control wells (N)

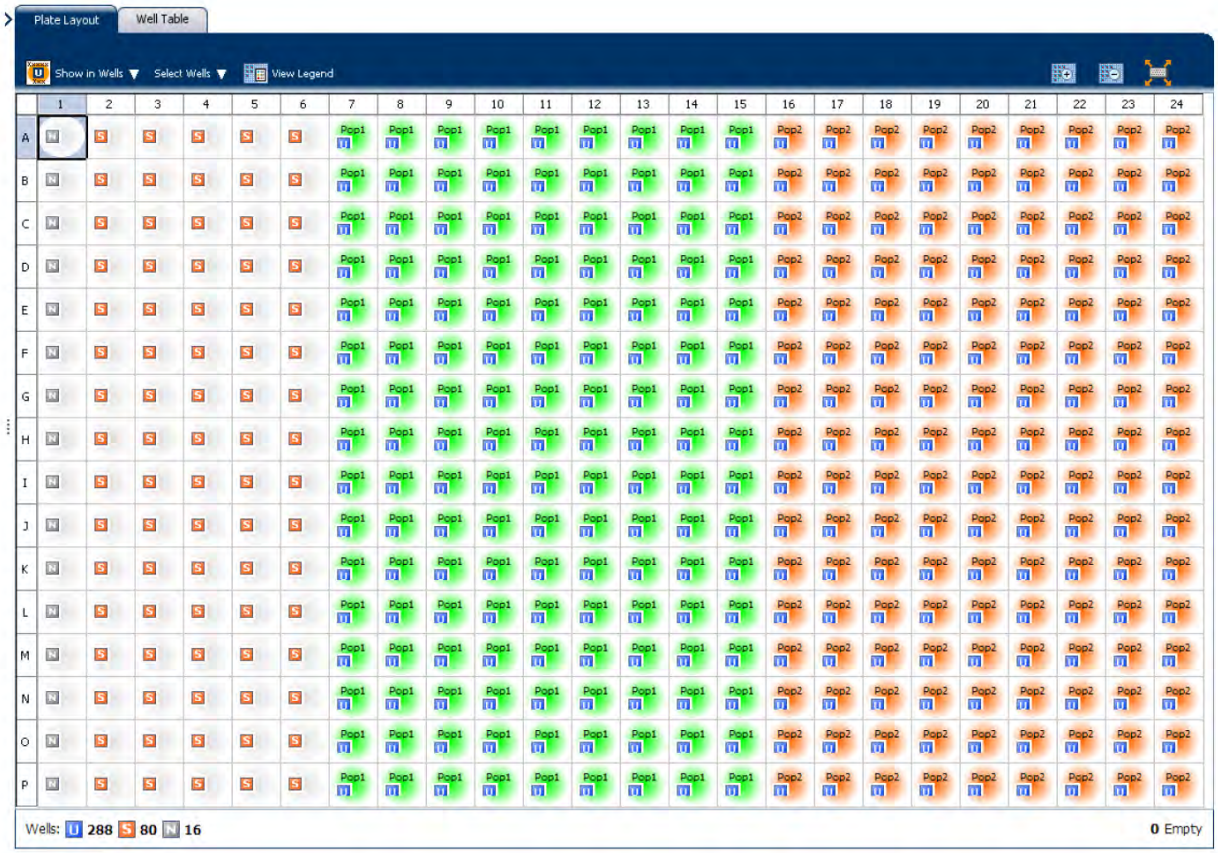


Figure 14 Plate layout

Prepare the reaction plate components

1. Prepare the negative control reactions for the target.
   a. To an appropriately sized tube, add the volumes of reaction mix and water listed below.

Tube	Reaction mix	Reaction mix volume (includes 10% overage)	Water volume (includes 10% overage)
1	RNase P reaction mix	157.5 µL	17.5 µL

   b. Mix the reaction by gently pipetting up and down, then cap the tube.

   c. Centrifuge the tube briefly to remove air bubbles.

   d. Add 10 µL of the negative control reaction to the appropriate wells in the reaction plate.

2. For each replicate group, prepare the standard reactions.
   a. To appropriately sized tubes, add the volumes of reaction mix and standard listed below.

Tube	Standard reaction	Reaction mix	Reaction mix volume (includes 10% overage)	Standard	Standard volume
1	RNase P Std 1	RNase P reaction mix	157.5 µL	RNase P Std 1	17.5 µL
2	RNase P Std 2	RNase P reaction mix	157.5 µL	RNase P Std 2	17.5 µL
3	RNase P Std 3	RNase P reaction mix	157.5 µL	RNase P Std 3	17.5 µL
4	RNase P Std 4	RNase P reaction mix	157.5 µL	RNase P Std 4	17.5 µL
5	RNase P Std 5	RNase P reaction mix	157.5 µL	RNase P Std 5	17.5 µL

   b. Mix the reactions by gently pipetting up and down, then cap the tubes.

   c. Centrifuge the tubes briefly to remove air bubbles.

   d. Add 10 µL of the standard reaction to the appropriate wells in the reaction plate.

3. For each replicate group, prepare the reactions for the unknowns.
   a. To appropriately sized tubes, add the volumes of reaction mix and sample listed below.

Tube	Unknown reaction	Reaction mix	Reaction mix volume (includes 10% excess)	Sample	Sample volume (µL)
1	RNase P pop1	RNase P reaction mix	1,422 µL	pop1	158 µL
2	RNase P pop2	RNase P reaction mix	1,422 µL	pop2	158 µL

   b. Mix the reactions by gently pipetting up and down, then cap the tubes.

   c. Centrifuge the tubes briefly to remove air bubbles.

   d. Add 10 µL of the unknown (sample) reaction to the appropriate wells in the reaction plate.

4. Seal the reaction plate with optical adhesive film.

5. Centrifuge the reaction plate briefly to remove air bubbles.

6. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.

7. Until you are ready to perform the run, place the reaction plate at 4°C, in the dark.

For more information

Information	Reference
Assigning the reaction plate components	Chapter 1, “General information and instructions”
Sealing the reaction plate	Chapter 1, “General information and instructions”

6 Run the experiment

This chapter explains how to run the example experiment on the QuantStudio™ 12K Flex Real-Time PCR Instrument.

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the instrument is in operation.

Start the run

1. Open the Standard Curve example file that you created using instructions in Chapter 4, “Design the experiment”.
2. Load the reaction plate into the instrument.
3. Start the run.

Monitor the run

You can monitor an experiment run in three ways:

- From the **Run** screen of the QuantStudio™ 12K Flex Software, while the experiment is in progress
- From the **Instrument Console** screen of the QuantStudio™ 12K Flex Software, to monitor an experiment started from another computer or from the instrument touchscreen (see “Monitor a run from the software Instrument Console screen” on page 103)
- From the instrument touchscreen (see “Monitor a run from the instrument touchscreen” on page 108)

Monitor a run from the software Instrument Console screen

1. In the **Instrument Console** screen, select the icon of the instrument that you are using to run the experiment.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. In the **Instrument Manager** screen, click **Monitor Running Experiment** to access the **Run** screen.

View the amplification plot

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the QuantStudio™ 12K Flex Software for potential problems.

Click **Amplification Plot** from the **Run Experiment** menu, select the **Plate Layout** tab, then select the wells to view.

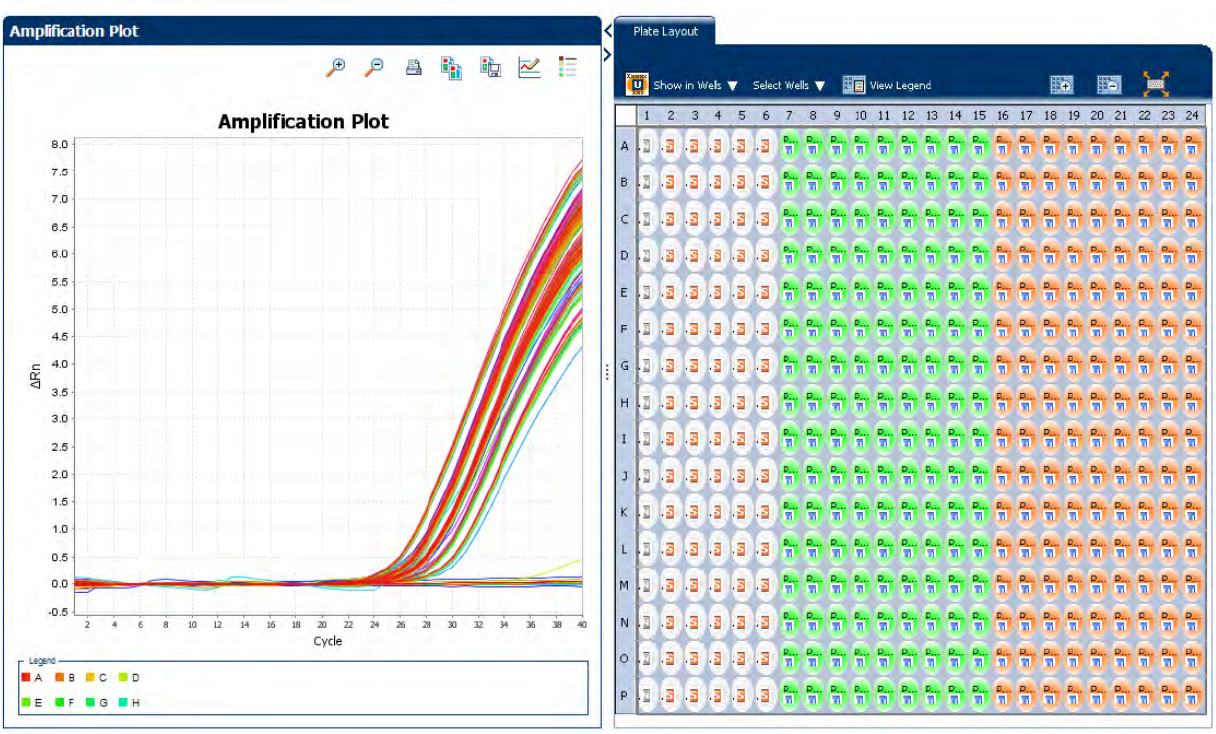


Figure 15 The Amplification Plot screen as it appears at the end of the example experiment

View the temperature plot

Click **Temperature Plot** from the **Run Experiment** menu.

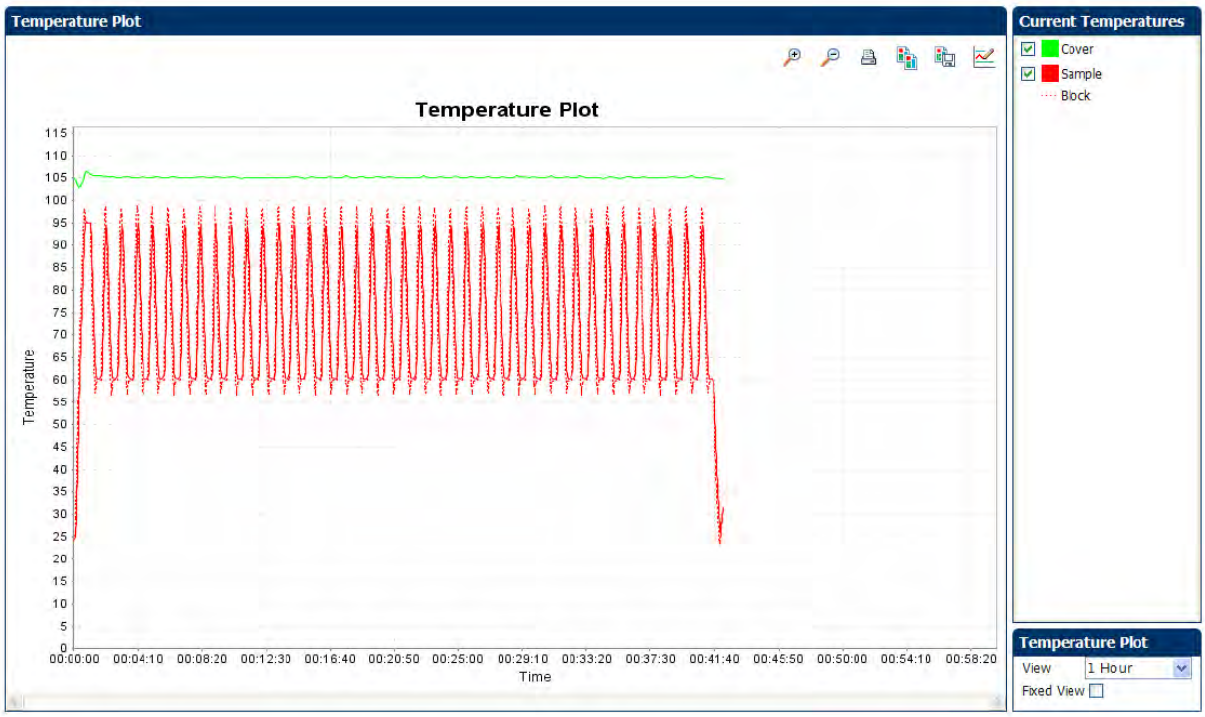


Figure 16 The Temperature Plot screen as it appears during the example experiment

Note: The sample temperature displayed in the **Current Temperatures** group is an estimated value.

View the run method

Click **Run Method** from the **Run Experiment** menu.

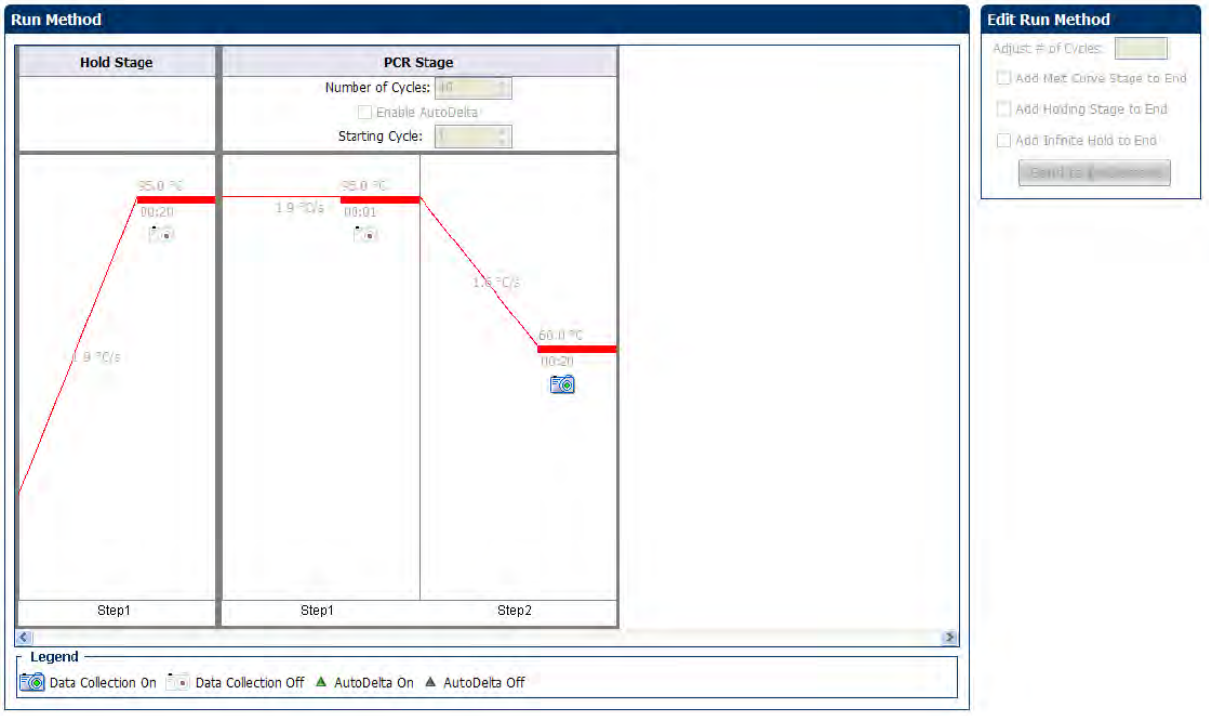


Figure 17 The Run Method screen as it appears in the example experiment

View the run data

Click **View Run Data** from the **Run Experiment** menu.

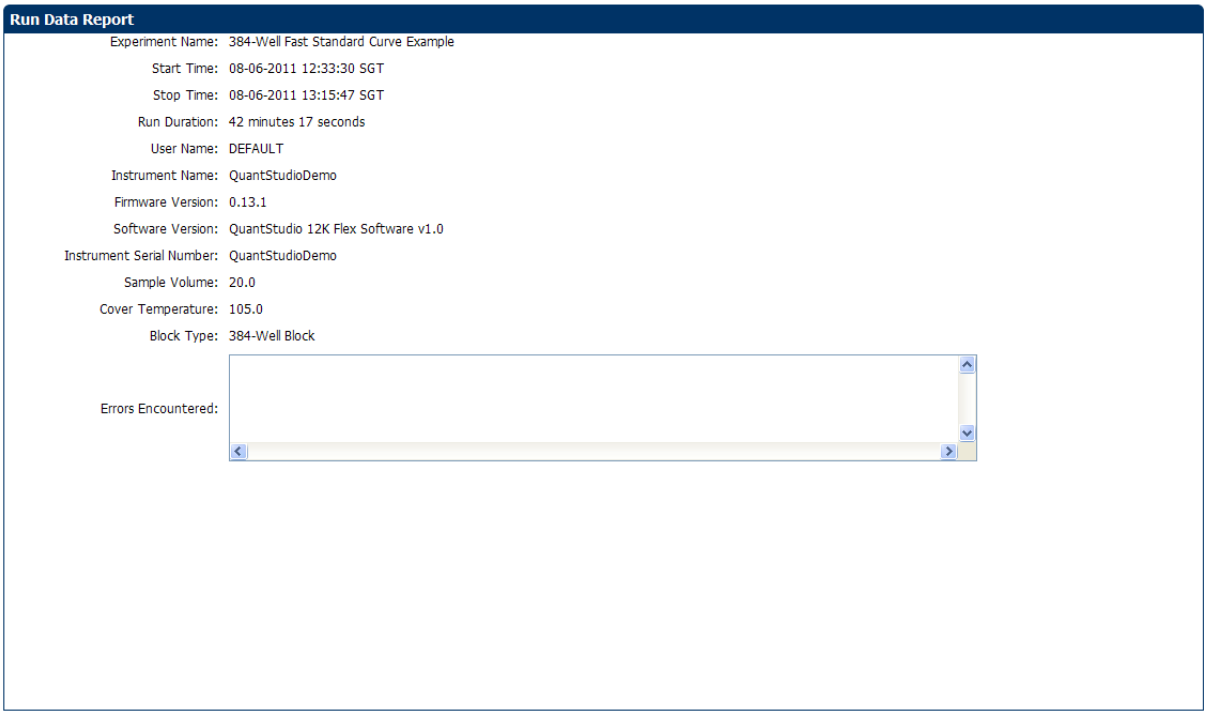


Figure 18 The View Run Data screen as it appears in the example experiment

Monitor a run from the instrument touchscreen

You can view the progress of the run from the instrument touchscreen.

The following figures are for visual representation only. Actual results vary with the experiment.

Figure 19 Experiment View tab

Figure 20 Time View tab

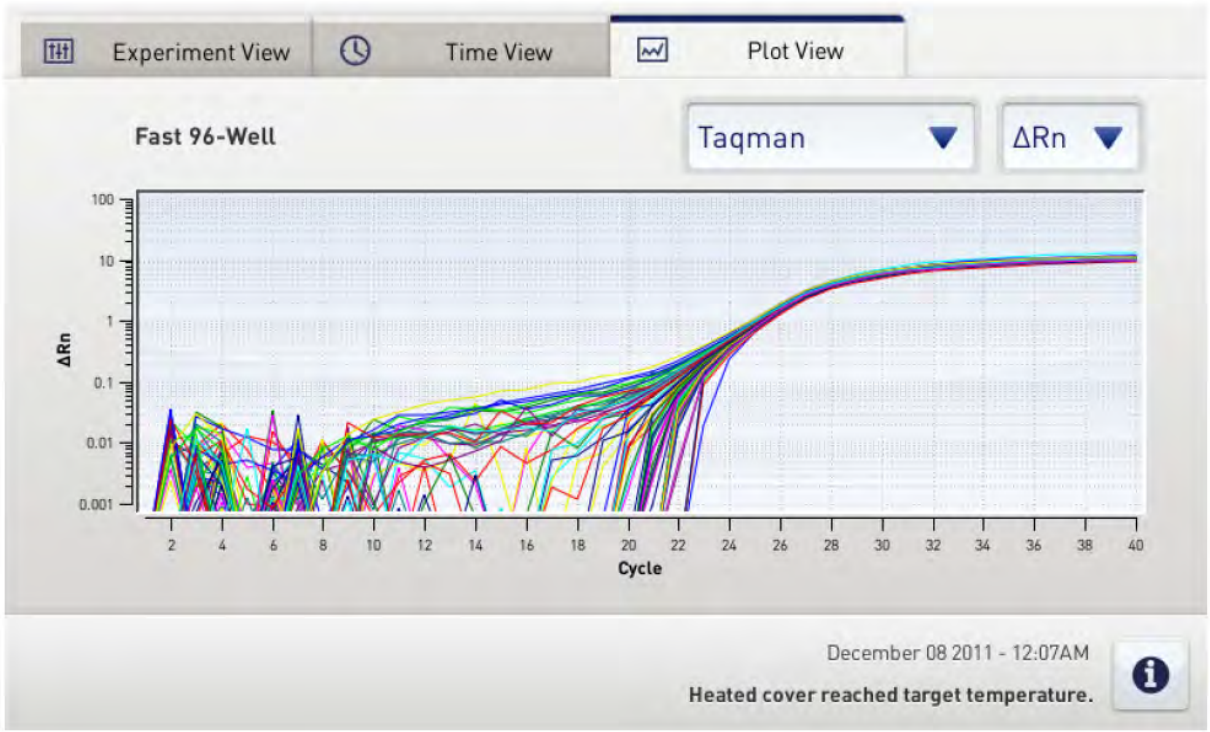


Figure 21 Plot View tab

7 Review results and adjust experiment parameters

The following topics are covered in this chapter:

- Review the analyzed data using several of the analysis screens and publish the data (see “Review results” on page 110)
- Modify experiment parameters to troubleshoot problems with experiment results before rerunning an experiment (see “Adjust parameters for re-analysis of your own experiments” on page 135)

Review results

Analyze the example experiment

1. Open the example experiment file.
   See Chapter 6, “Run the experiment”.
2. Click **Analyze**.

Note: You can also access the experiment to analyze from the **Home** screen.

The software analyzes the data using the default analysis settings.

View the standard curve plot

The Standard Curve Plot screen displays the standard curve for samples designated as standards. The QuantStudio™ 12K Flex Software calculates the quantity of an unknown target from the standard curve.

Purpose

The purpose of viewing the standard curve for the example experiment is to identify the following items:

- Slope and amplification efficiency
- R^2 value (correlation coefficient)
- C_T values

View and assess the standard curve plot

1. From the **Experiment Menu** pane, select **Analysis** ▸ **Standard Curve**.

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

2. Display all 384 wells in the Standard Curve Plot screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.

3. Enter the plot settings.

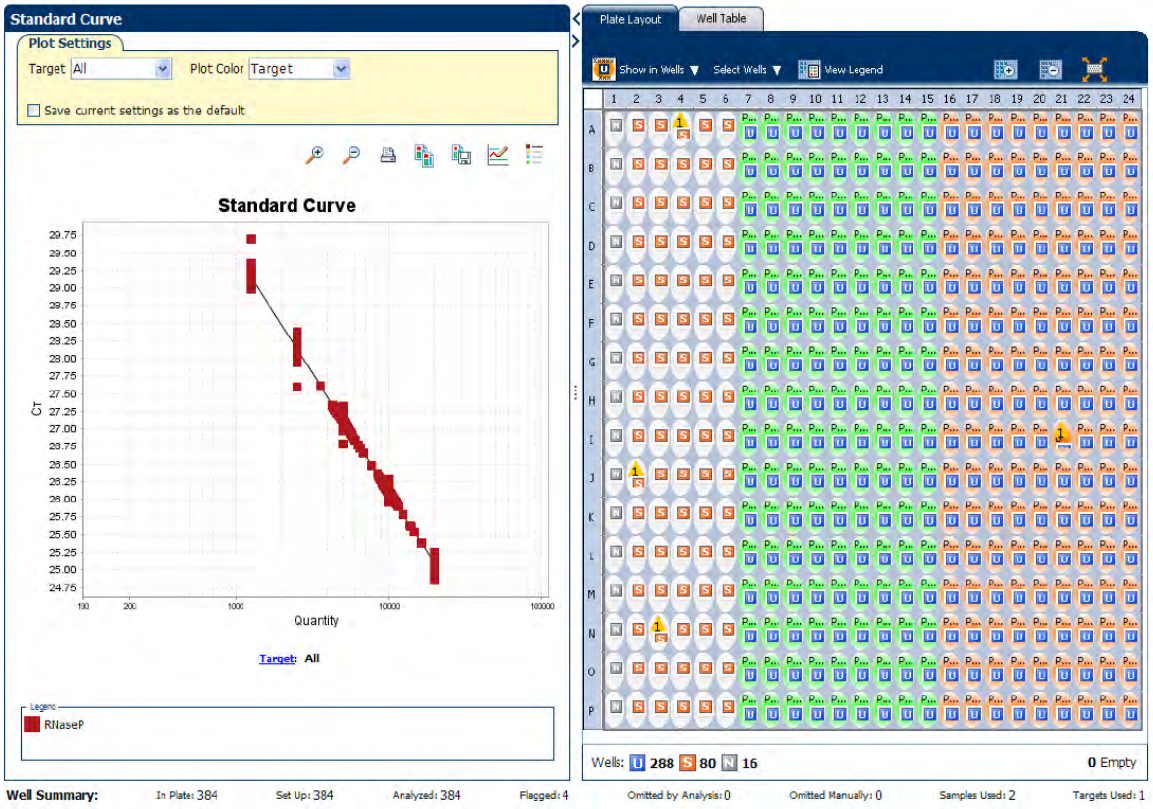
Menu	Selection
Target	All
Plot Color	Target
 (This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**).	Check (default)

4. View the values displayed below the standard curve.

Menu	Selection
Slope	-3.372
R2	0.994
Amplification efficiency	97.944%
Error	0.03

5. Check that all samples are within the standard curve.

   In the example experiment, as shown below, all samples (blue dots) are within the standard curve (red dots).



6. Check the C_T values.
   a. Click the **Well Table** tab.
   b. From the **Group By** menu, select **Replicate**.

c. Look at the values in the **C_T** column.

   In the example experiment, the C_T values fall within the expected range (>8 and < 35).

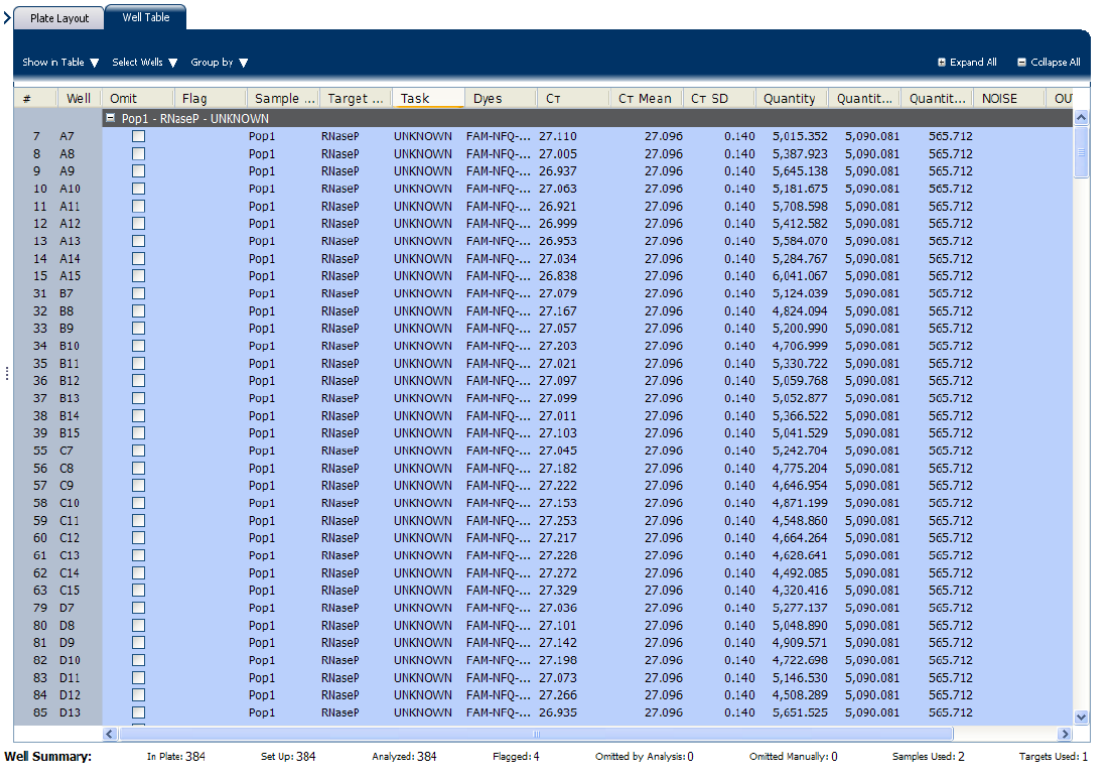


Plate Layout | Well Table

Show in Table | Select Wells | Group by | Expand All | Collapse All

#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	CT	CT Mean	CT SD	Quantity	Quantit...	Quantit...	NOISE	OU
		Pop1 - RNaseP - UNKNOWN													
7	A7			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.110	27.096	0.140	5,015.352	5,090.081	565.712		
8	A8			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.005	27.096	0.140	5,387.923	5,090.081	565.712		
9	A9			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.937	27.096	0.140	5,645.138	5,090.081	565.712		
10	A10			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.063	27.096	0.140	5,181.675	5,090.081	565.712		
11	A11			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.921	27.096	0.140	5,708.598	5,090.081	565.712		
12	A12			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.999	27.096	0.140	5,412.582	5,090.081	565.712		
13	A13			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.953	27.096	0.140	5,584.070	5,090.081	565.712		
14	A14			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.034	27.096	0.140	5,284.767	5,090.081	565.712		
15	A15			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.838	27.096	0.140	6,041.067	5,090.081	565.712		
31	B7			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.079	27.096	0.140	5,124.039	5,090.081	565.712		
32	B8			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.167	27.096	0.140	4,824.094	5,090.081	565.712		
33	B9			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.057	27.096	0.140	5,200.990	5,090.081	565.712		
34	B10			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.203	27.096	0.140	4,706.999	5,090.081	565.712		
35	B11			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.021	27.096	0.140	5,330.722	5,090.081	565.712		
36	B12			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.097	27.096	0.140	5,059.768	5,090.081	565.712		
37	B13			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.099	27.096	0.140	5,052.877	5,090.081	565.712		
38	B14			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.011	27.096	0.140	5,366.522	5,090.081	565.712		
39	B15			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.103	27.096	0.140	5,041.529	5,090.081	565.712		
55	C7			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.045	27.096	0.140	5,242.704	5,090.081	565.712		
56	C8			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.182	27.096	0.140	4,775.204	5,090.081	565.712		
57	C9			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.222	27.096	0.140	4,646.954	5,090.081	565.712		
58	C10			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.153	27.096	0.140	4,871.199	5,090.081	565.712		
59	C11			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.253	27.096	0.140	4,548.860	5,090.081	565.712		
60	C12			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.217	27.096	0.140	4,664.264	5,090.081	565.712		
61	C13			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.228	27.096	0.140	4,628.641	5,090.081	565.712		
62	C14			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.272	27.096	0.140	4,492.085	5,090.081	565.712		
63	C15			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.329	27.096	0.140	4,320.416	5,090.081	565.712		
79	D7			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.036	27.096	0.140	5,277.137	5,090.081	565.712		
80	D8			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.101	27.096	0.140	5,048.890	5,090.081	565.712		
81	D9			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.142	27.096	0.140	4,909.571	5,090.081	565.712		
82	D10			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.198	27.096	0.140	4,722.698	5,090.081	565.712		
83	D11			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.073	27.096	0.140	5,146.530	5,090.081	565.712		
84	D12			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.266	27.096	0.140	4,508.289	5,090.081	565.712		
85	D13			Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.935	27.096	0.140	5,651.525	5,090.081	565.712		

Well Summary: In Plate: 384 | Set Up: 384 | Analyzed: 384 | Flagged: 4 | Omitted by Analysis: 0 | Omitted Manually: 0 | Samples Used: 2 | Targets Used: 1

Tips for analyzing your own experiments

When you analyze your own standard curve experiment, look for the following items:

- **Slope and amplification efficiency values**—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. The following factors affect amplification efficiency:
  - Range of standard quantities—For accurate and precise efficiency measurements, use a broad range of standard quantities, 5 to 6 logs (10^5 to 10^6 fold).
  - Number of standard replicates—For accurate efficiency measurements, include replicates to decrease the effects of pipetting inaccuracies.
  - PCR inhibitors—PCR inhibitors in the reaction can reduce amplification efficiency.
- **R^2 values (correlation coefficient)**—The R^2 value is a measure of the closeness of fit between the regression line and the individual C_T data points of the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points. An R^2 value >0.99 is desirable.
- **C_T values**—The threshold cycle (C_T) is the PCR cycle number at which the fluorescence level meets the threshold.
  - A C_T value >8 and <35 is desirable.
  - A C_T value <8 indicates that there is too much template in the reaction.
  - A C_T value >35 indicates a low amount of target in the reaction. For C_T values >35, expect a higher standard deviation.

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

- Omit wells (see “Omit wells to improve CT precision” on page 140).
  Or
- Rerun the experiment.

Assess amplification results using the amplification plot

Amplification plots available for viewing

The **Amplification Plot** screen displays amplification of all samples in the selected wells. There are three plots available:

- **ΔRn vs Cycle**—ΔRn is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. This plot displays ΔRn as a function of cycle number. Use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle**—Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays Rn as a function of cycle number. Use this plot to identify and examine irregular amplification.
- **C_T vs Well**—C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. Use this plot to locate outlying amplification (outliers).

Each plot can be viewed as a linear graph or a log10 graph.

Purpose

The purpose of viewing the amplification plot for the example experiment is to identify:

- Correct baseline and threshold values
- Outliers

View the amplification plot

1. In the **Experiment Menu** pane, click **Analysis** ▸ **Amplification Plot**.

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

2. Display the RNase P wells in the **Amplification Plot** screen. Click the **Plate Layout** tab. Enter the Plot Settings.
   a. Select the **Plate Layout** tab.

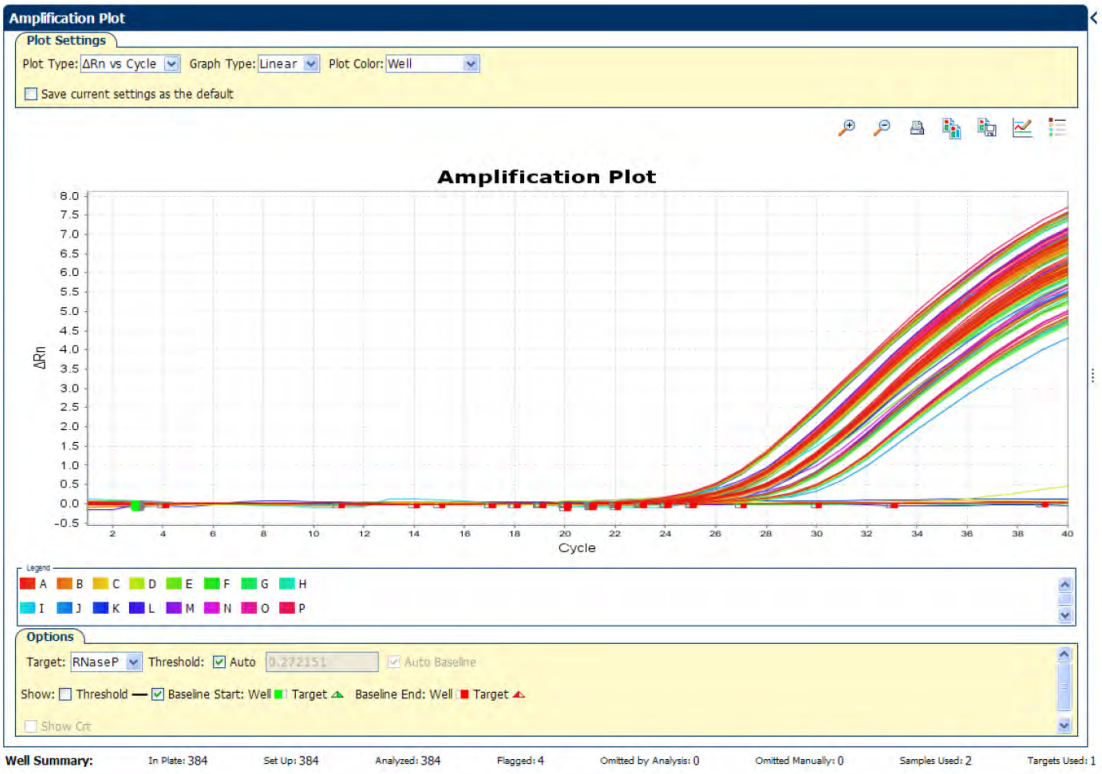
b. In the **Select Wells** dropdown list, select **Target ▸ RNaseP**.

3. In the Amplification Plot screen, select the following.

Item	Select
Plot Type dropdown list	**ΔRn vs Cycle**
Plot Color dropdown list	**Well** (default)
 (This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**).	Check (default)

4. View the baseline values.
   a. From the **Graph Type** dropdown list, select **Linear**.
   b. Select the **Baseline** checkbox to show the start cycle and end cycle.

c. Confirm that the baseline is set correctly. The end cycle should be set a few cycles before the cycle number where significant fluorescent signal is detected. In the example experiment, the baseline is set correctly.

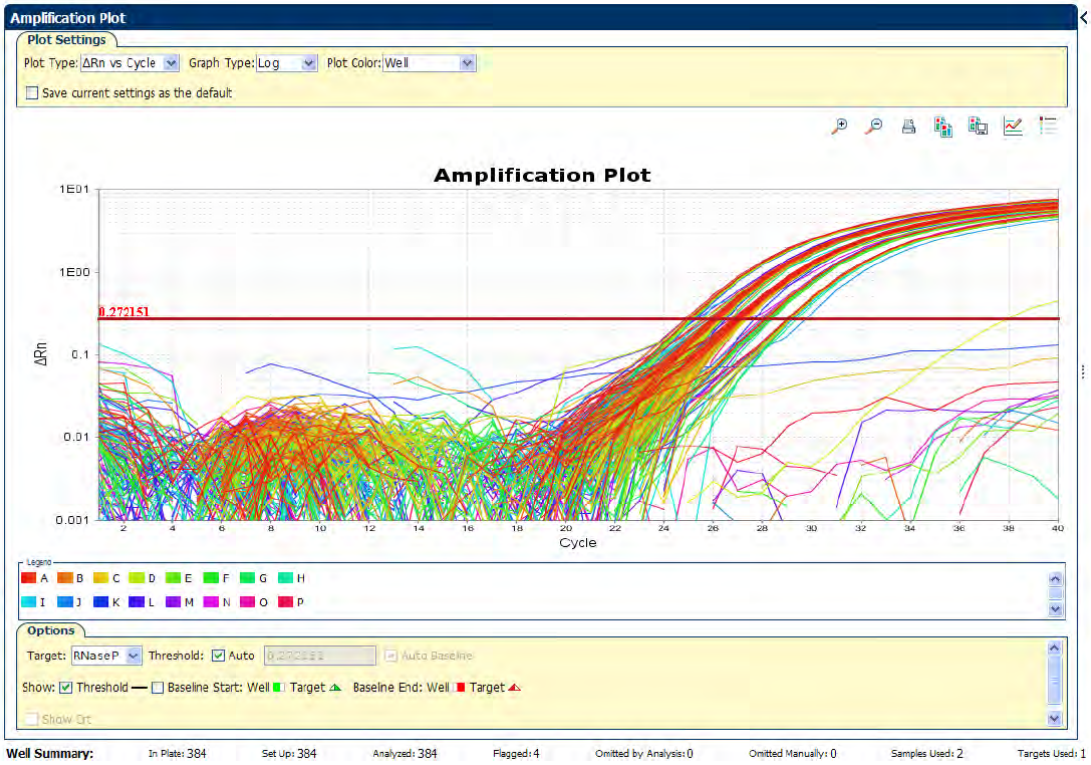


5. View the threshold values.

Item	Select
Graph Type dropdown list	**Log**
Target dropdown list	**RNaseP**

a. Select the **Threshold** check box to show the threshold.

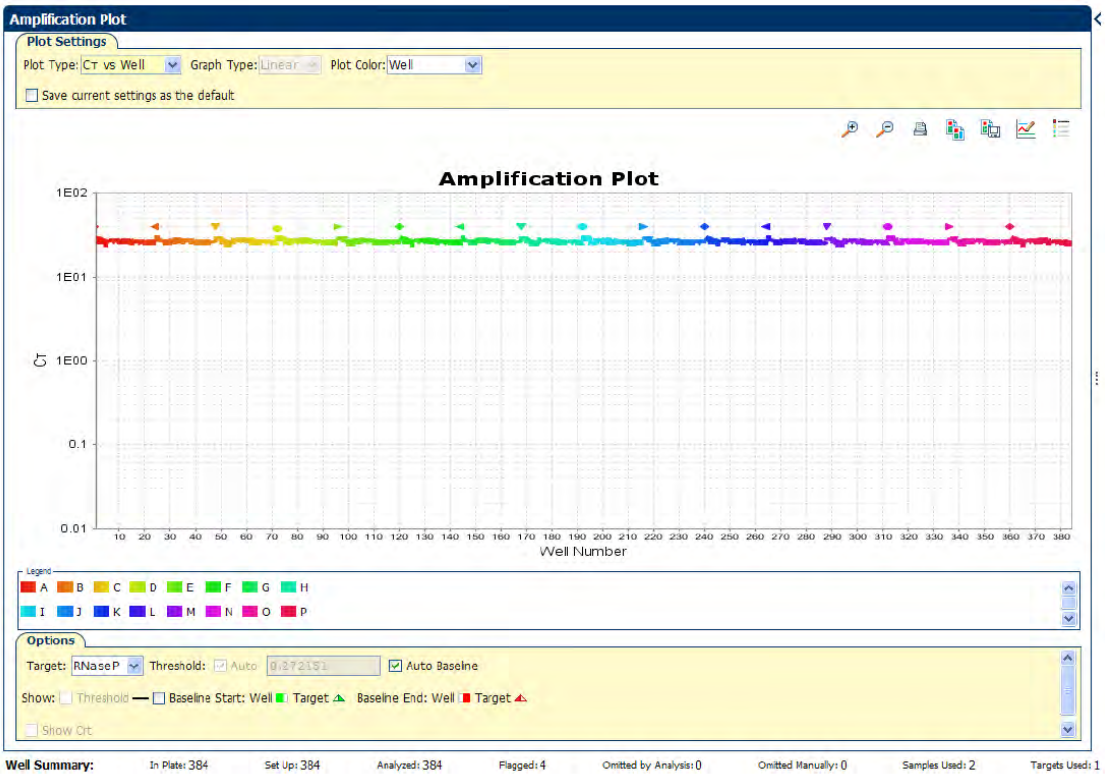
b. Confirm that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.



6. Locate outliers.

a. In the **Plot Type** dropdown list, select **C_T vs. Well**.

b. Look for outliers from the amplification plot. In the example experiment, there are no outliers for RNase P.



Tips for analyzing your own experiments

When you analyze your own standard curve experiment, look for the following items:

- **Outliers**
- **A typical amplification plot**—The QuantStudio™ 12K Flex Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear phase
  - Exponential (geometric phase)
  - Baseline

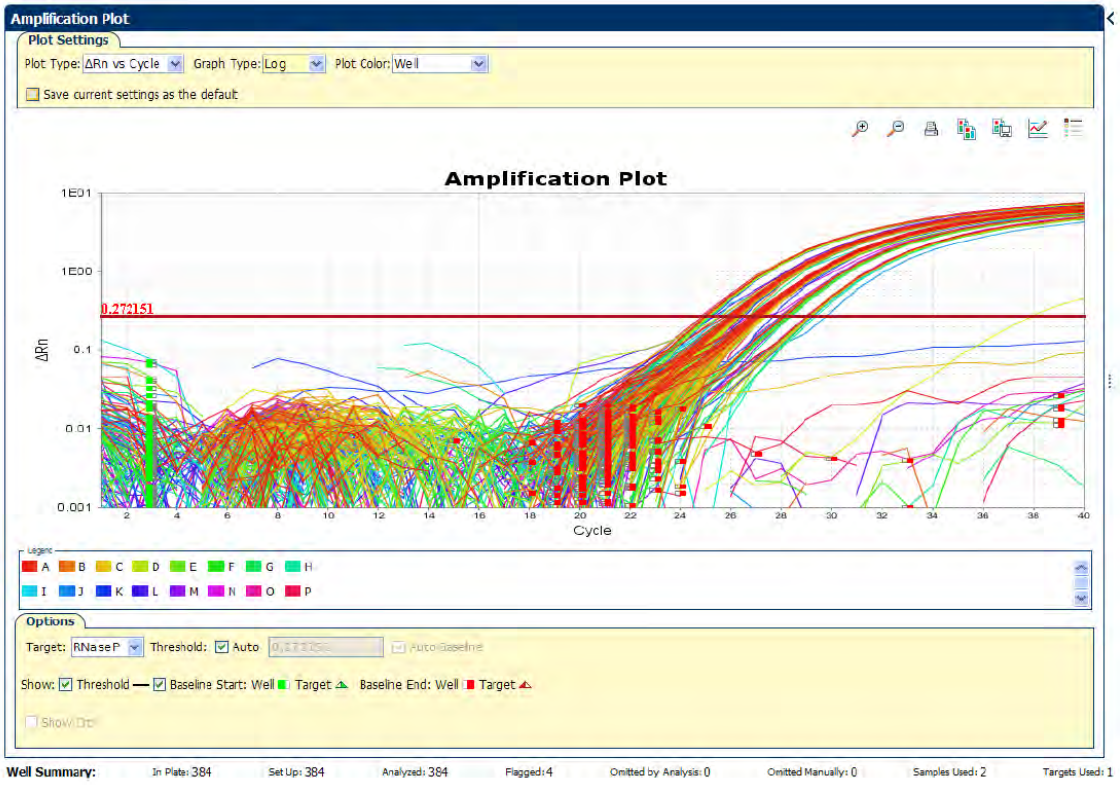
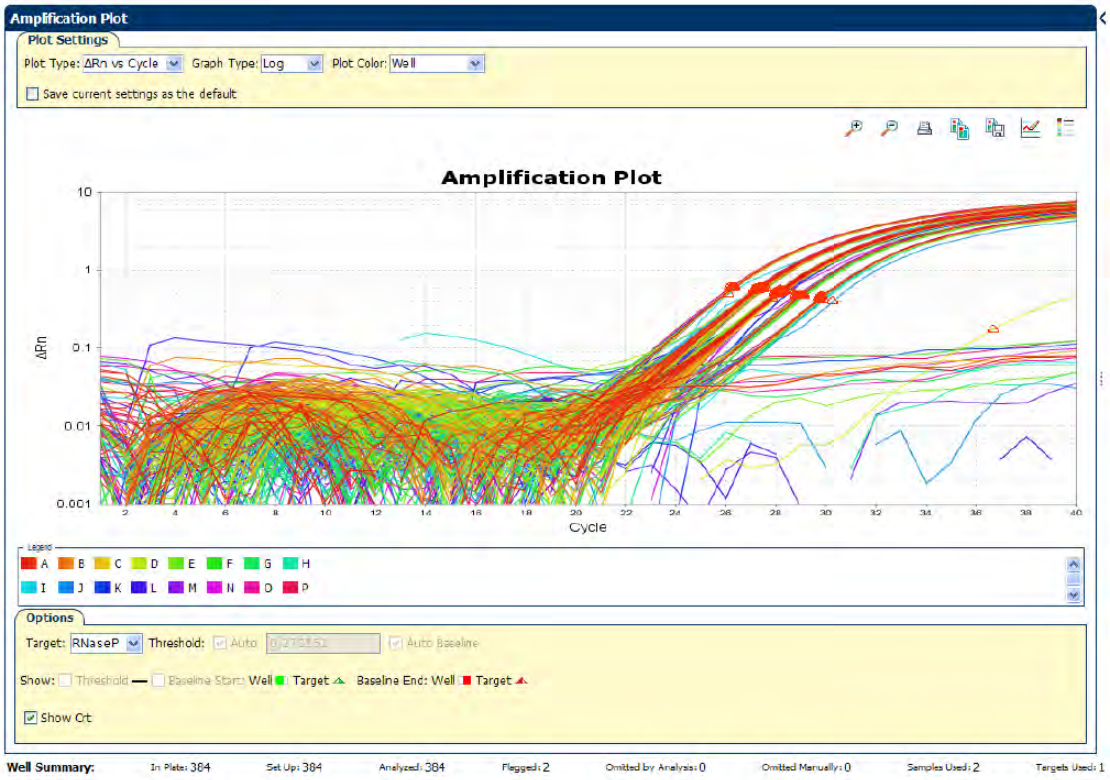


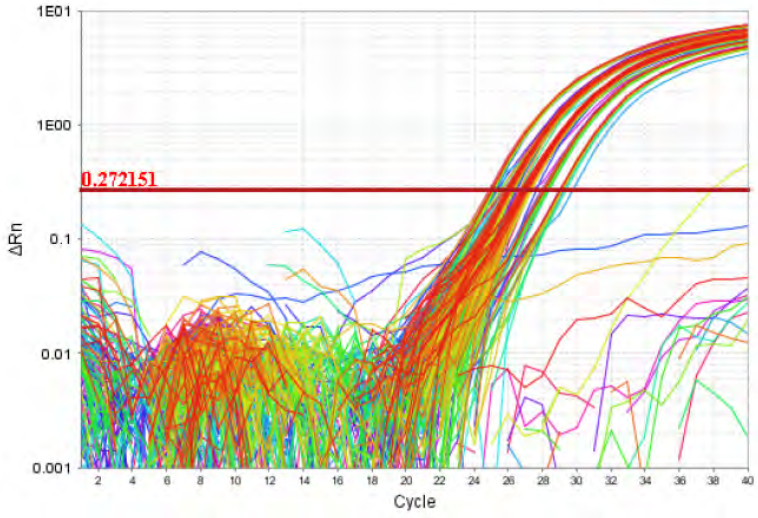
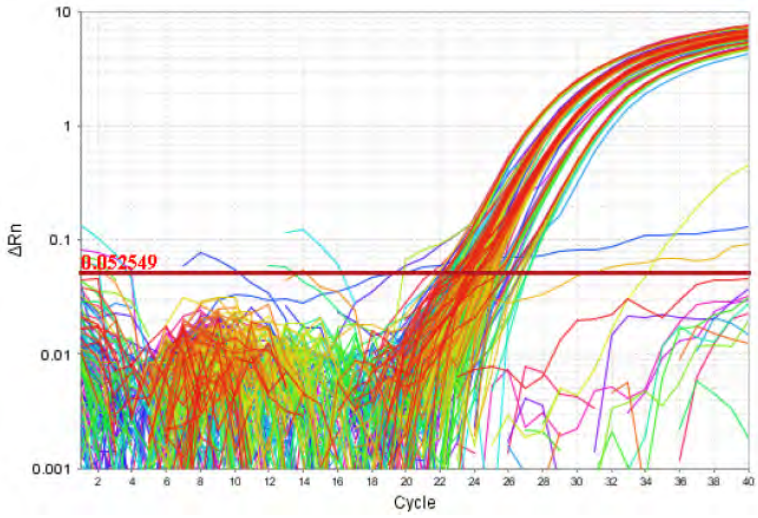
Figure 22 Typical amplification plot

IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudio™ 12K Flex Software. We recommend that you examine the Amplification Plot screen and review the assigned baseline and threshold values for each well after analysis.

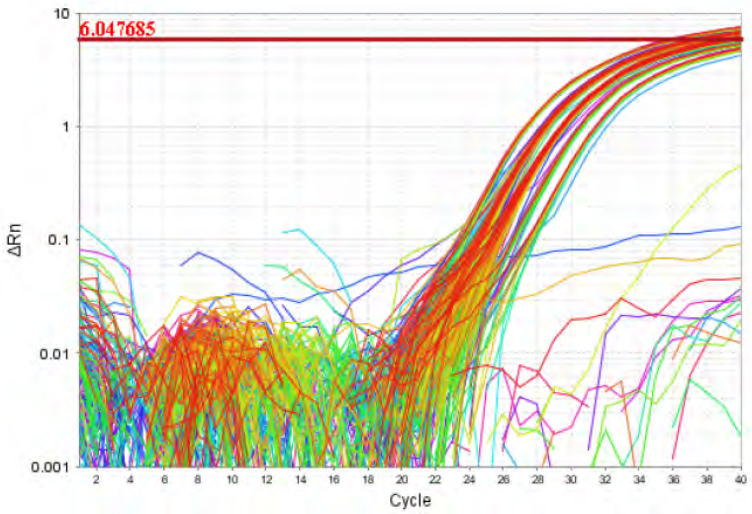
Note: If you use the Relative Threshold algorithm to analyze an experiment that includes amplification, select to view the analysis results using the ΔRn vs Cycle, Rn vs Cycle, or C_{RT} vs Well plot type and Linear or Log graph type. Select the **Show Crt** checkbox to view the derived fractional cycle on the amplification plot.


Amplification Plot
Plot Settings
Plot Type: ΔRn vs Cycle
Graph Type: Log
Plot Color: Well
Save current settings as the default
Amplification Plot
ΔRn
Cycle
Options
Target: RNaseP
Threshold:
Auto
Auto Baseline
Show:
Threshold
Baseline Start: Well
Target
Baseline End: Well
Target
Show Ct
Well Summary:
In Plate: 384
Set Up: 384
Analyzed: 384
Flagged: 2
Omitted by Analysis: 0
Omitted Manually: 0
Samples Used: 2
Targets Used: 1

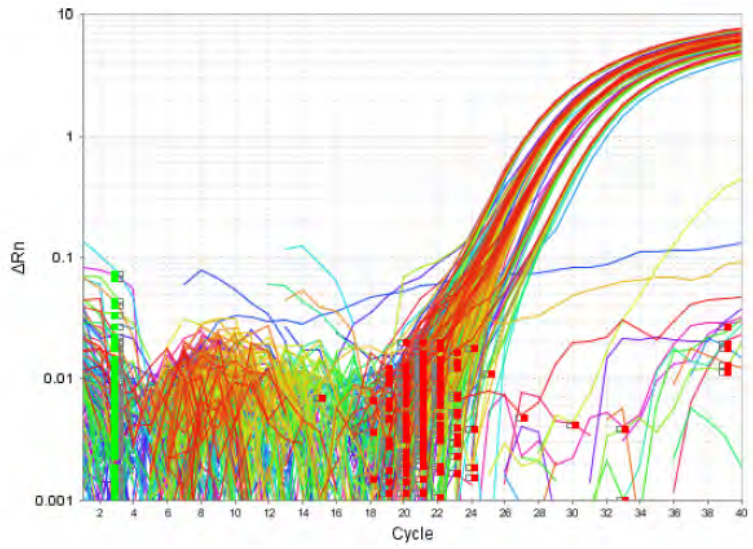
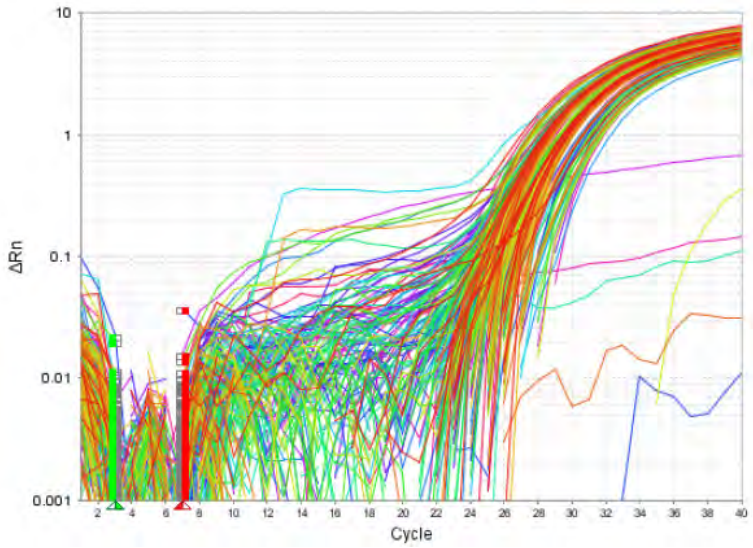
- **Correct threshold values:**

Condition	Example
Threshold Set Correctly The threshold is set in the exponential phase of the amplification curve. Threshold settings above or below the optimum increase the standard deviation of the replicate groups.	
Threshold Set Too Low The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the exponential phase of the curve.	

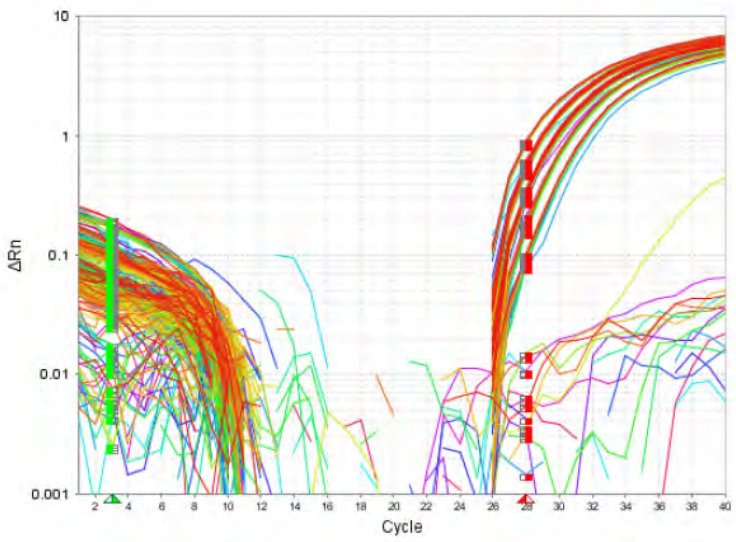
(continued)

Condition	Example
Threshold Set Too High The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar down into the exponential phase of the curve.	

- **Correct baseline values:**

Condition	Example
Baseline Set Correctly The amplification curve begins after the maximum baseline.	
Baseline Set Too Low The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.	

(continued)

Condition	Example
Baseline Set Too High The amplification curve begins before the maximum baseline. Decrease the End Cycle value.	

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

- Omit wells (see “Omit wells to improve CT precision” on page 140).

  Or
- Manually adjust the baseline and/or threshold (see “Adjust analysis settings” on page 135).

Identify well problems using the well table

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

- The sample name, target name, task, and dyes
- The calculated threshold cycle (C_T), normalized fluorescence (Rn), and quantity values
- Comments
- Flags

Purpose

The purpose of viewing the well table is to identify the following items:

- Quantity values
- Flags
- C_T values (including C_T standard deviation)

View the well table

1. From the **Experiment Menu** pane, select **Analysis**, then select the **Well Table** tab.

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

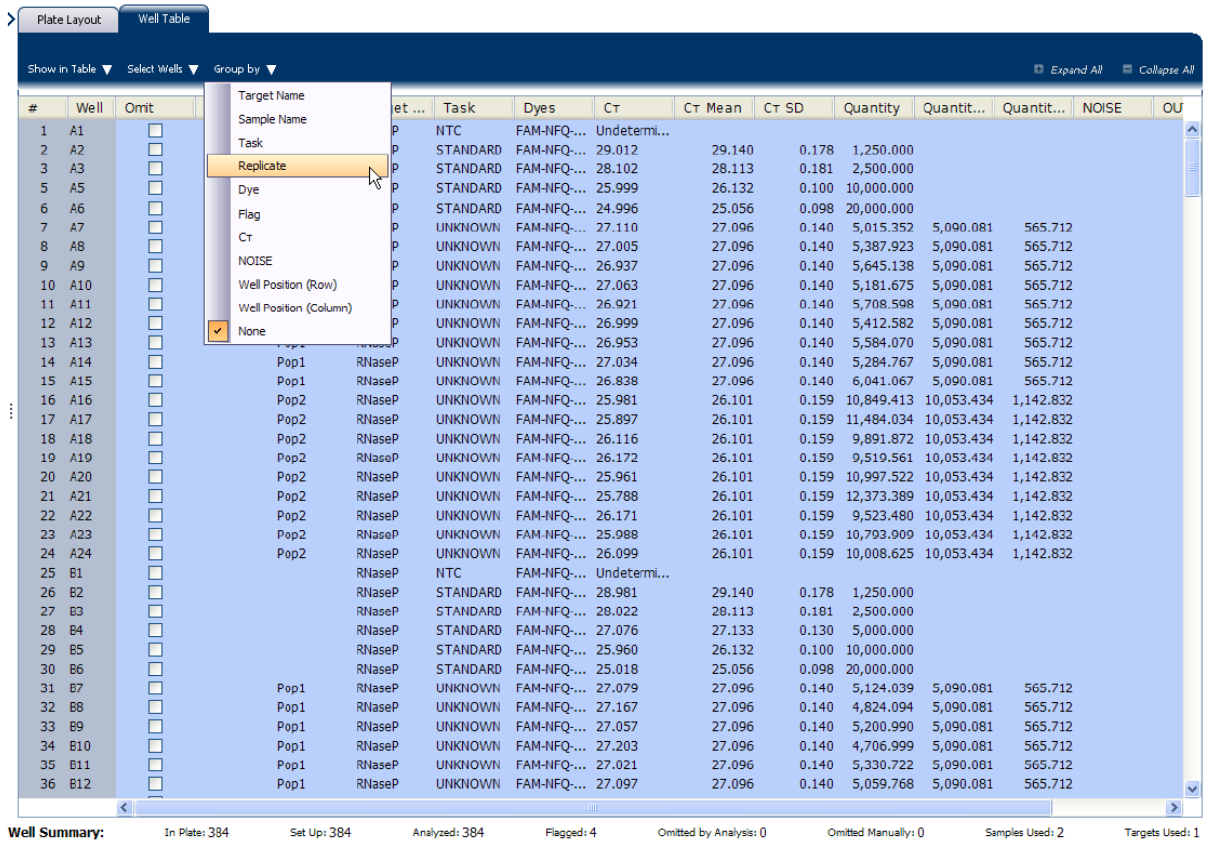
2. Use the **Group By** dropdown list to group wells by a specific category. For the example experiment, group the wells by replicate, flag, or C_T value.

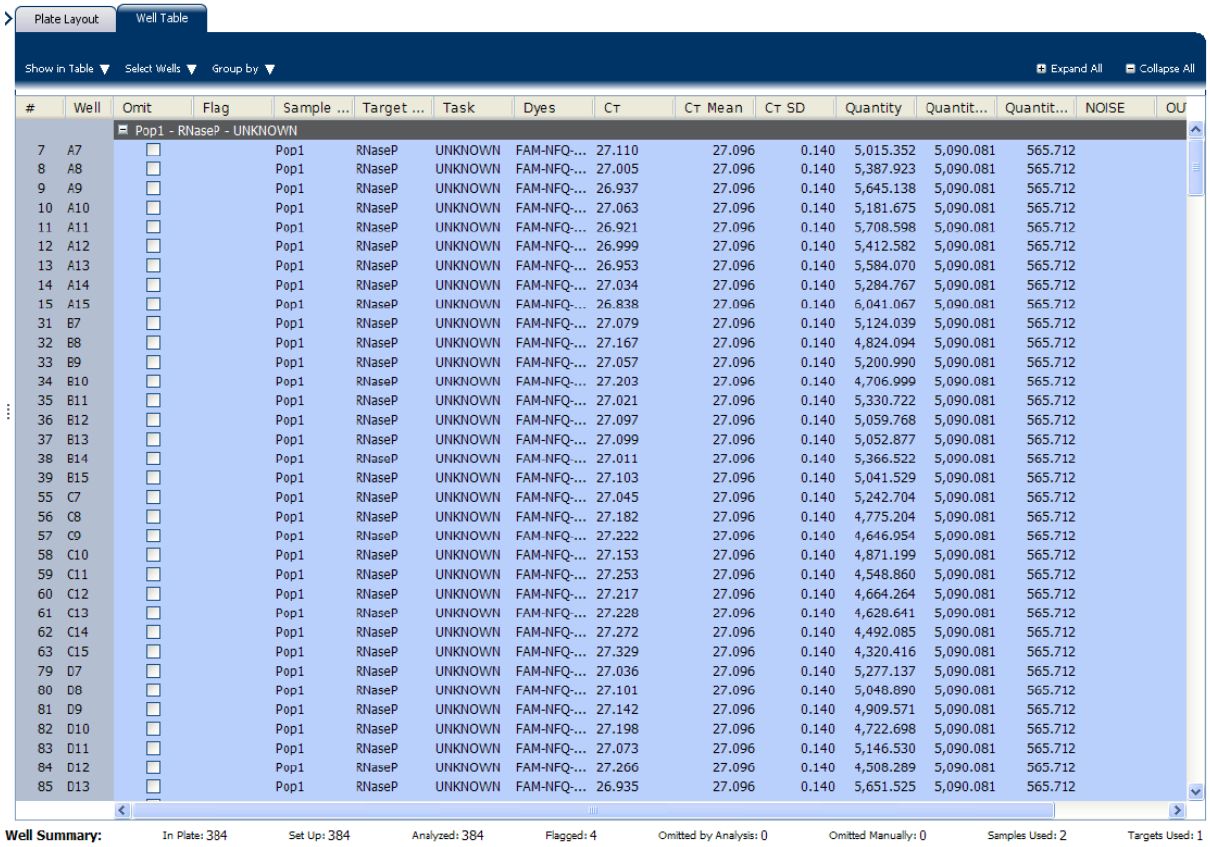
   Note: You can select only one category at a time.

Group by replicate

From the **Group By** dropdown list, select **Replicate**. The software groups the replicate wells: negative controls, standards, and samples. In the example experiment, note that the quantity values within each replicate group are similar.

Note: In the example experiment, the **Quantity** column, **Quantity Mean** column, and **Quantity SD** column have been moved from their default locations to the beginning of the Well Table. To move a column, click and drag on the column heading.



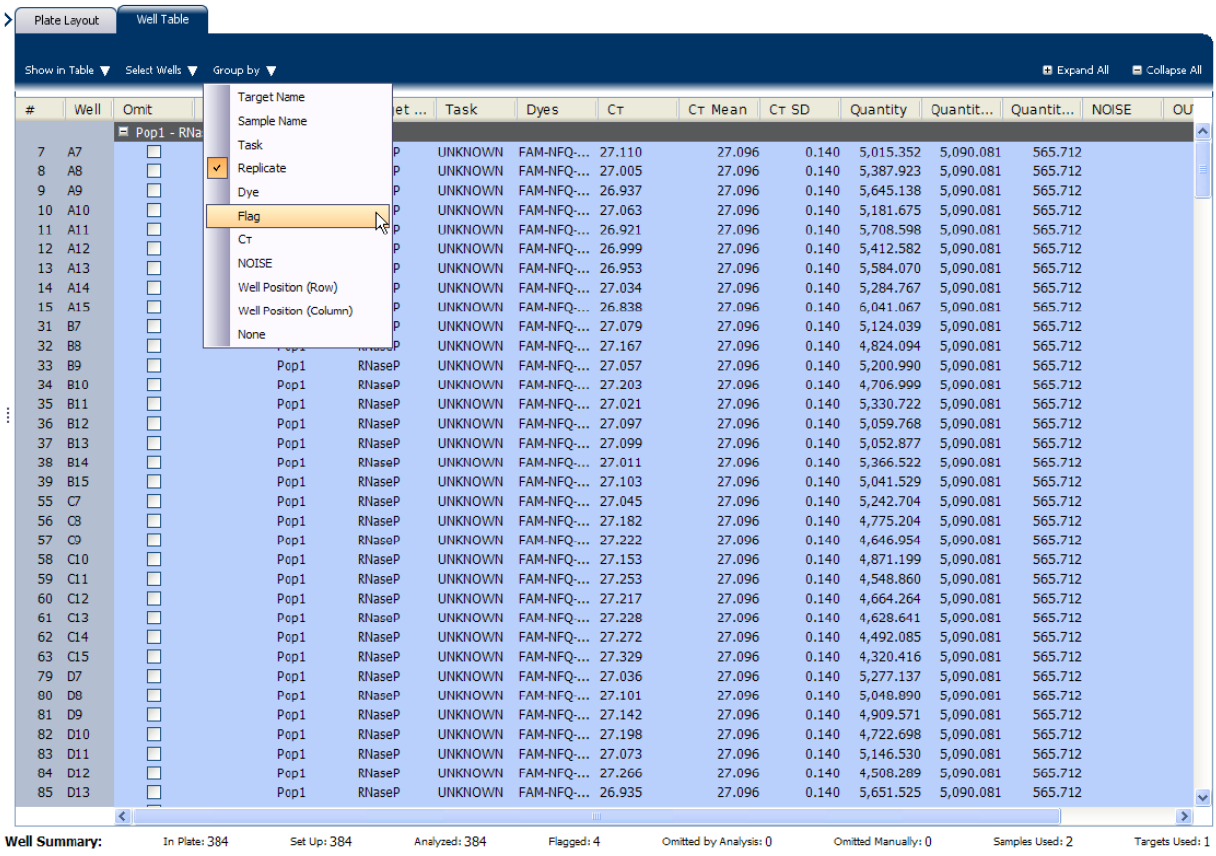


#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	Cт	Cт Mean	Cт SD	Quantity	Quantit...	Quantit...	NOISE	OU
7	A7	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.110	27.096	0.140	5,015.352	5,090.081	565.712		
8	A8	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.005	27.096	0.140	5,387.923	5,090.081	565.712		
9	A9	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.937	27.096	0.140	5,645.138	5,090.081	565.712		
10	A10	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.063	27.096	0.140	5,181.675	5,090.081	565.712		
11	A11	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.921	27.096	0.140	5,708.598	5,090.081	565.712		
12	A12	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.999	27.096	0.140	5,412.582	5,090.081	565.712		
13	A13	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.953	27.096	0.140	5,584.070	5,090.081	565.712		
14	A14	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.034	27.096	0.140	5,284.767	5,090.081	565.712		
15	A15	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.838	27.096	0.140	6,041.067	5,090.081	565.712		
31	B7	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.079	27.096	0.140	5,124.039	5,090.081	565.712		
32	B8	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.167	27.096	0.140	4,824.094	5,090.081	565.712		
33	B9	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.057	27.096	0.140	5,200.990	5,090.081	565.712		
34	B10	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.203	27.096	0.140	4,706.999	5,090.081	565.712		
35	B11	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.021	27.096	0.140	5,330.722	5,090.081	565.712		
36	B12	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.097	27.096	0.140	5,059.768	5,090.081	565.712		
37	B13	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.099	27.096	0.140	5,052.877	5,090.081	565.712		
38	B14	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.011	27.096	0.140	5,366.522	5,090.081	565.712		
39	B15	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.103	27.096	0.140	5,041.529	5,090.081	565.712		
55	C7	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.045	27.096	0.140	5,242.704	5,090.081	565.712		
56	C8	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.182	27.096	0.140	4,775.204	5,090.081	565.712		
57	C9	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.222	27.096	0.140	4,646.954	5,090.081	565.712		
58	C10	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.153	27.096	0.140	4,871.199	5,090.081	565.712		
59	C11	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.253	27.096	0.140	4,548.860	5,090.081	565.712		
60	C12	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.217	27.096	0.140	4,664.264	5,090.081	565.712		
61	C13	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.228	27.096	0.140	4,628.641	5,090.081	565.712		
62	C14	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.272	27.096	0.140	4,492.085	5,090.081	565.712		
63	C15	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.329	27.096	0.140	4,320.416	5,090.081	565.712		
79	D7	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.036	27.096	0.140	5,277.137	5,090.081	565.712		
80	D8	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.101	27.096	0.140	5,048.890	5,090.081	565.712		
81	D9	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.142	27.096	0.140	4,909.571	5,090.081	565.712		
82	D10	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.198	27.096	0.140	4,722.698	5,090.081	565.712		
83	D11	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.073	27.096	0.140	5,146.530	5,090.081	565.712		
84	D12	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	27.266	27.096	0.140	4,508.289	5,090.081	565.712		
85	D13	☐		Pop1	RNaseP	UNKNOWN	FAM-NFQ-...	26.935	27.096	0.140	5,651.525	5,090.081	565.712		

Figure 23 Well table

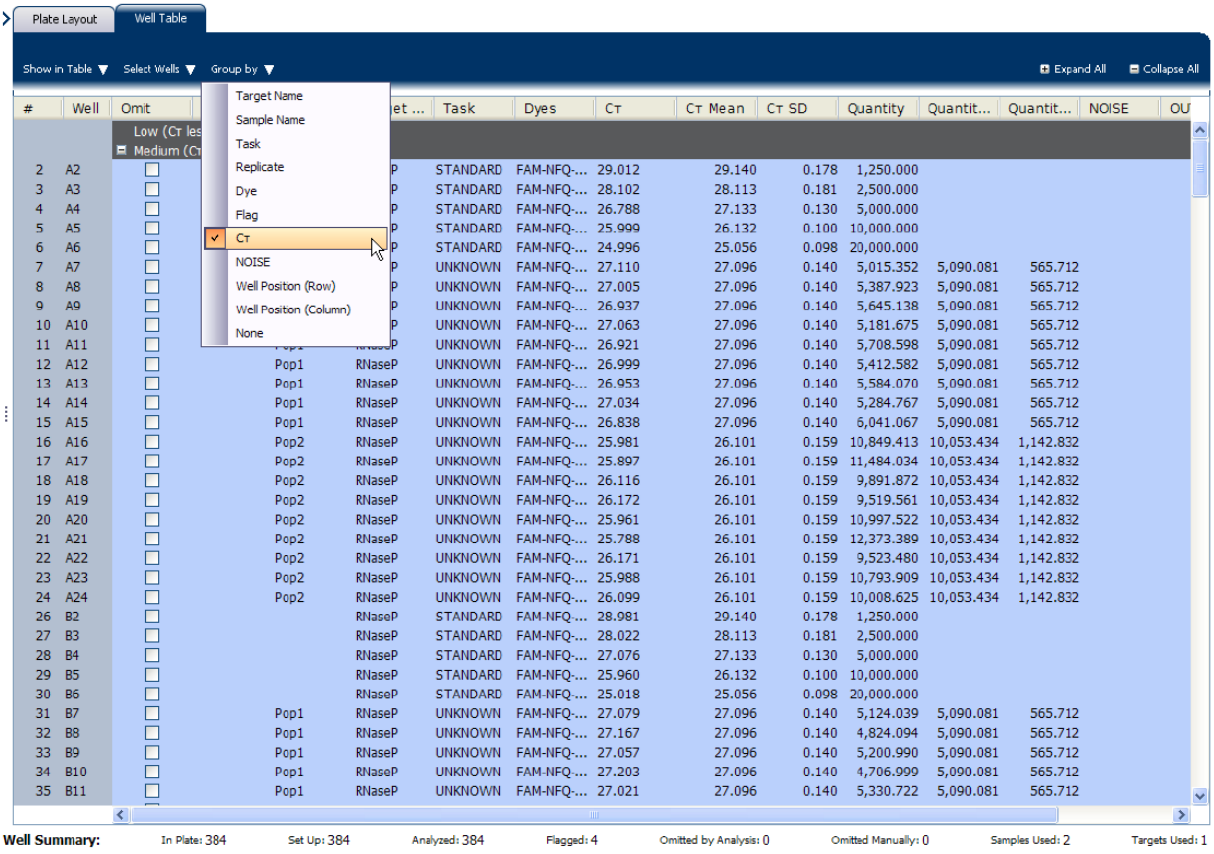
Group by flag

From the **Group By** dropdown list, select **Flag**. The software groups the flagged and unflagged wells. In the example experiment, there are four flagged wells.



Group by C_T value

From the **Group By** dropdown list, select **C_T**. The software groups the wells by C_T value: low, medium, high, and undetermined. In the example experiment, the C_T values are within the expected range (greater than 8 and less than 35).



Tips for analyzing your own experiments

- **Replicate**—The software groups the wells by replicate: negative controls, standards, and samples. Look in the quantity columns to make sure the quantity values for each replicate group are similar indicating tight C_T precision.
- **Flag**—The software groups the flagged and unflagged wells. A flag indicates that the software has found a potential error in the flagged well. For a description of the flags, see “Flag settings” on page 137.
- **C_T**—The threshold cycle (C_T) is the PCR cycle number at which the fluorescence level meets the threshold. A C_T value greater than 8 and less than 35 is desirable. A C_T value less than 8 indicates that there is too much template in the reaction. A C_T value greater than 35 indicates a low amount of target in the reaction. For C_T values greater than 35, expect a higher standard deviation.

Confirm accurate dye signal using the multicomponent plot

The **Multicomponent Plot** screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the standard curve example experiment, you review the Multicomponent Plot for the following items:

- ROX™ dye (passive reference)
- FAM™ dye (reporter)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

View the multicomponent plot

1. From the **Experiment Menu** pane, select **Analysis** ▸ **Multicomponent Plot**.

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

2. Display the unknown and standard wells one at a time in the **Multicomponent Plot** screen.
   a. Click the **Plate Layout** tab.
   b. Select one well in the plate layout. The well is displayed in the Multicomponent Plot.

      Note: If you select multiple wells, the Multicomponent Plot displays the data for all selected wells simultaneously.

3. From the **Plot Color** dropdown list, select **Dye**.

4. Click **Show a legend for the plot** (default).

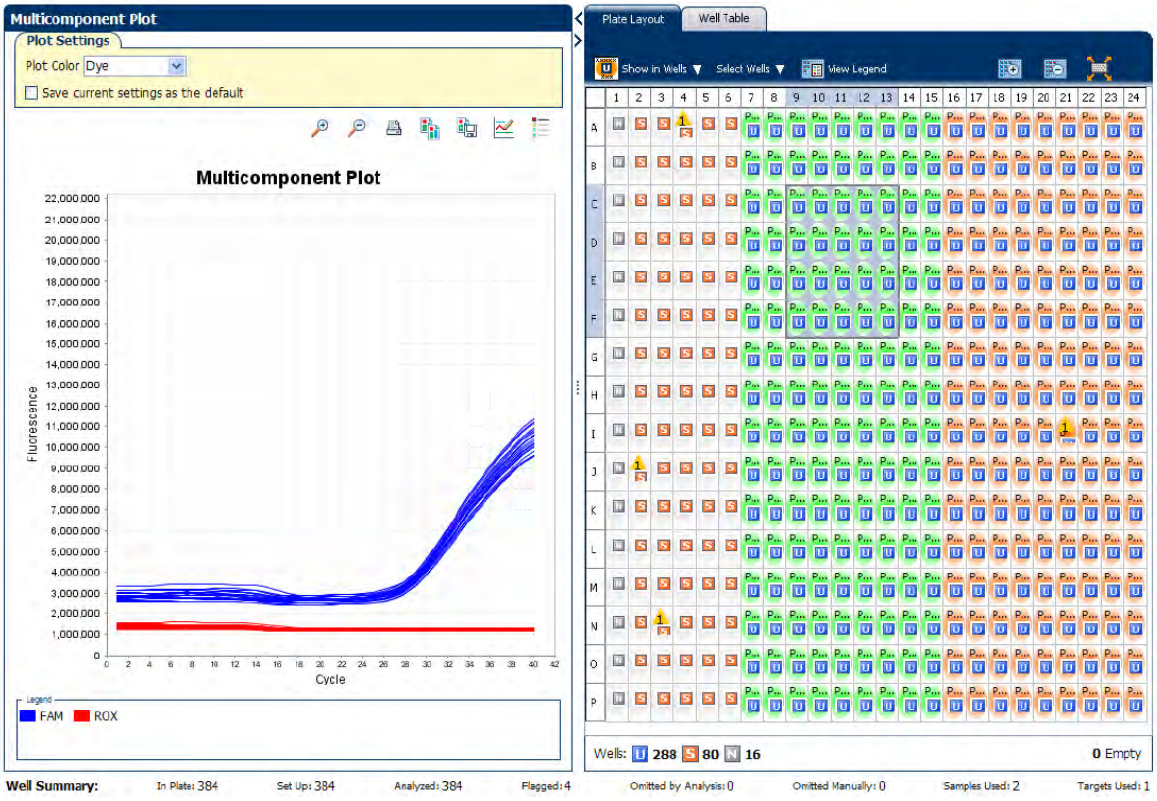
   Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

5. Check the ROX™ dye signal.

   In the example experiment, the ROX™ dye signal remains constant throughout the PCR process. Aa constant ROX™ dye signal indicates typical data.

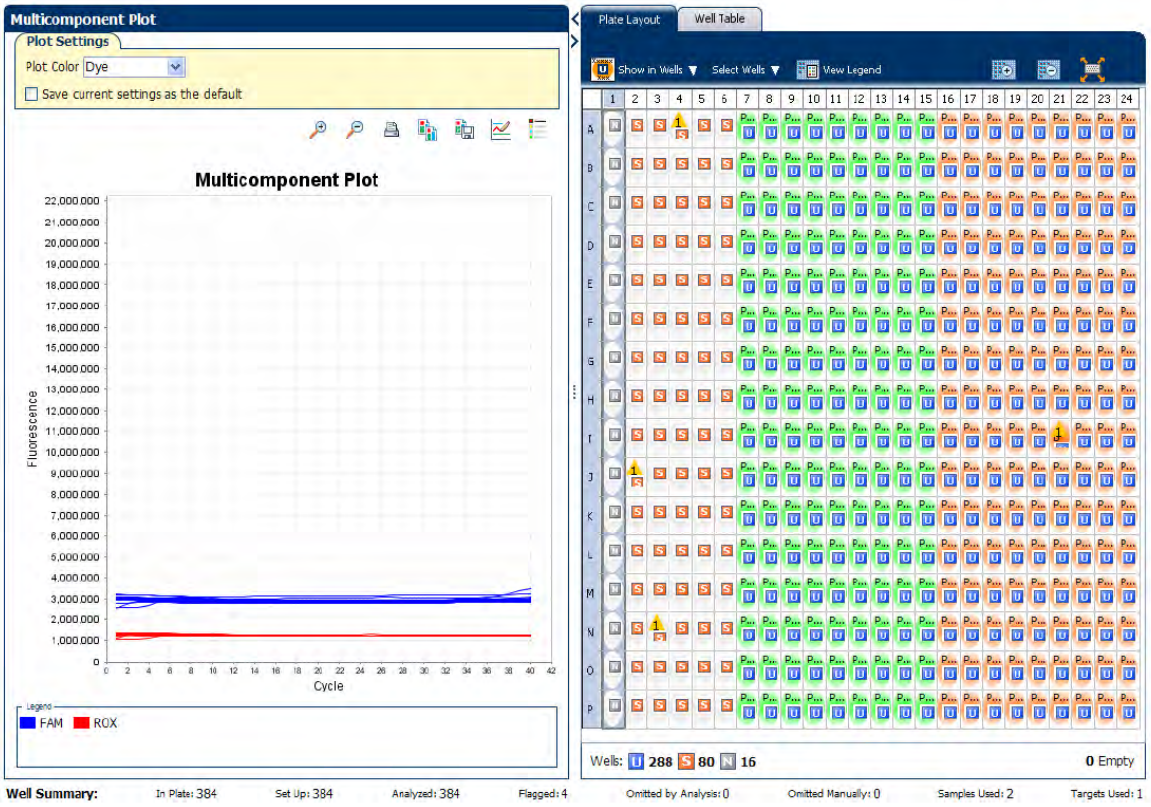
6. Check the FAM™ dye signal.

   In the example experiment, the FAM™ dye signal increases throughout the PCR process; increase in FAM™ dye signal indicates normal amplification.



7. Select the negative control wells one at time and check for amplification.

   In the example experiment, there is no amplification in the negative control wells.



Tips for confirming dye accuracy in your own experiment

When you analyze your own standard curve experiment, look for the following items:

- **Passive reference**—The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye**—The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- **Irregularities in the signal**—There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative Control wells**—There should not be any amplification in the negative control wells.

Determine signal accuracy using the raw data plot

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

Purpose

In the standard curve example experiment, you review the Raw Data Plot for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the raw data plot

1. From the **Experiment Menu** pane, select **Analysis** ▸ **Raw Data Plot**.

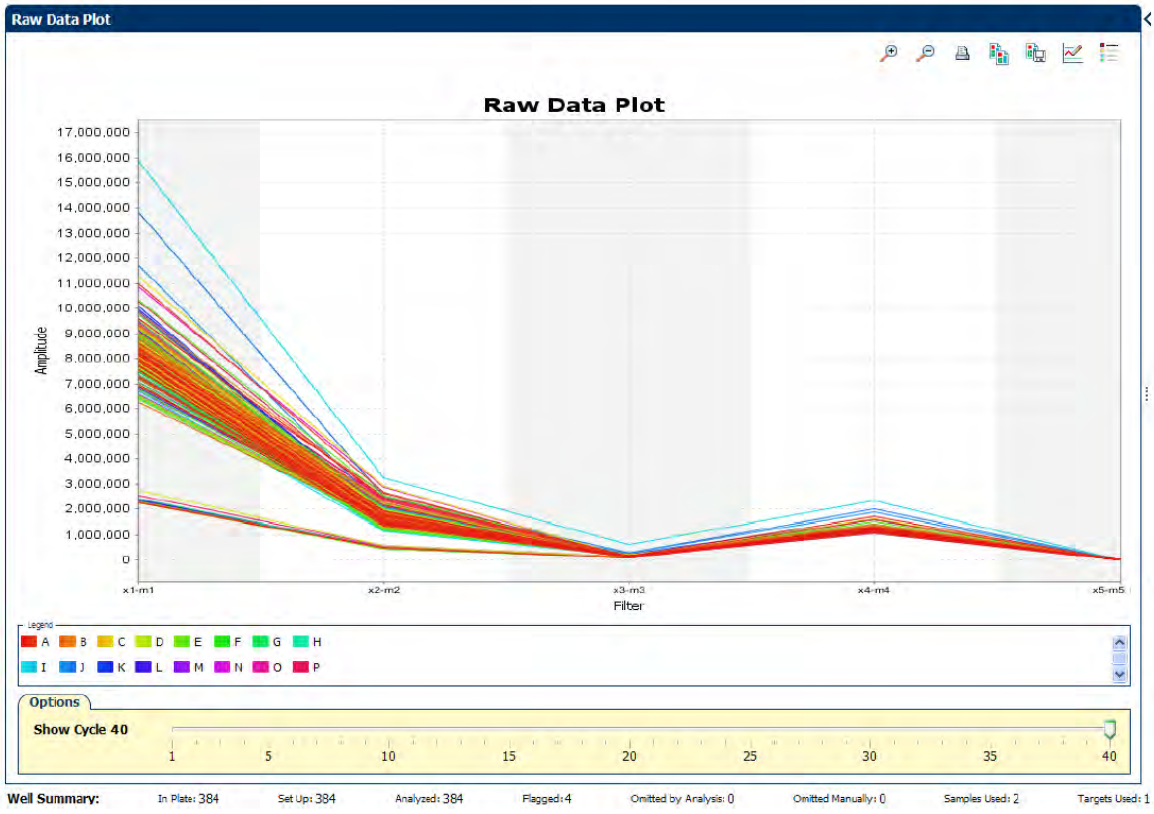
   Note: If no data are displayed, click **Analyze**.

2. Display all 384 wells in the **Raw Data Plot** screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.

3. Click **Show a legend for the plot** (default).

   The legend displays the color code for each row of the reaction plate (see the legend in the Raw Data Plot shown below).

4. Click and drag the **Show Cycle** pointer from cycle 1 to cycle 40. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.



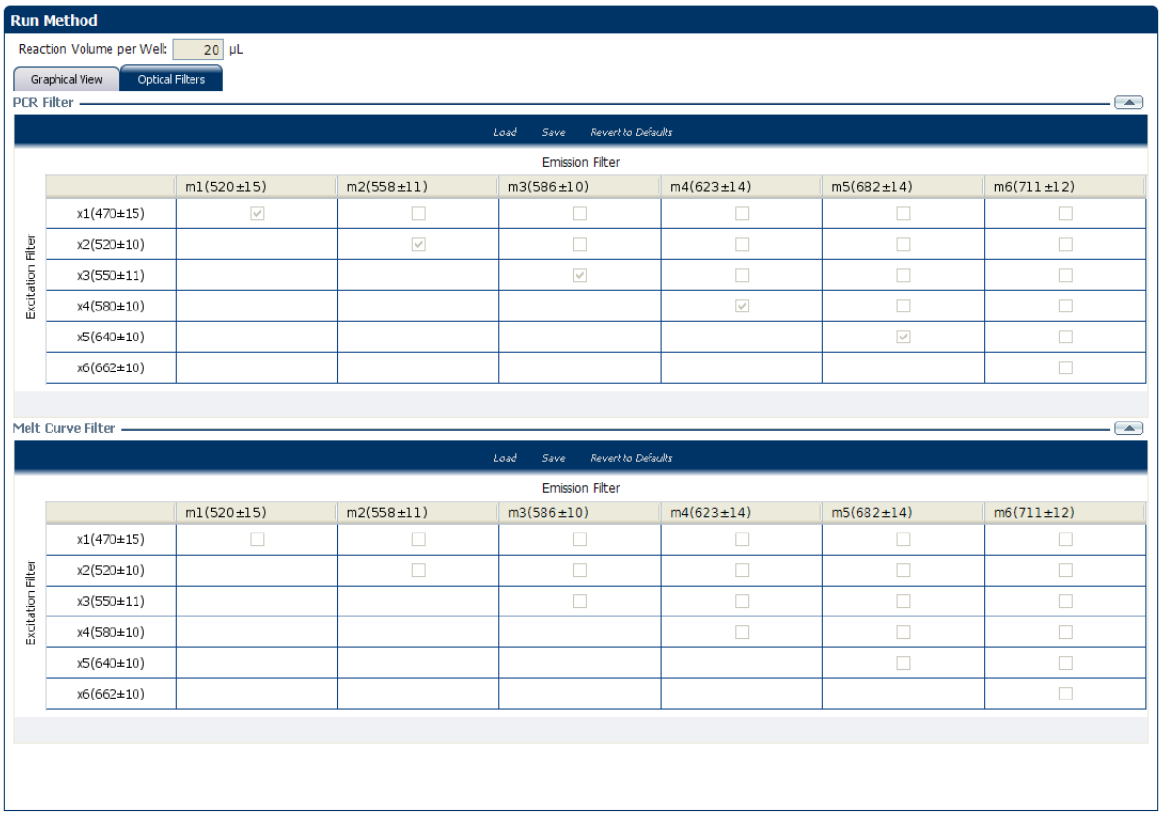


Figure 24 filters used for the example experiment

Tips for determining signal accuracy in your own experiments

When you analyze your own standard curve experiment, look for the following items in each filter:

- Characteristic signal growth
- No abrupt changes or dips

Review the flags in the QC summary

The **QC Summary** screen displays a list of the QuantStudio™ 12K Flex Software flags, including the flag frequency and location for the open experiment.

1. From the **Experiment Menu** pane, select **Analysis ▸ QC Summary**.

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

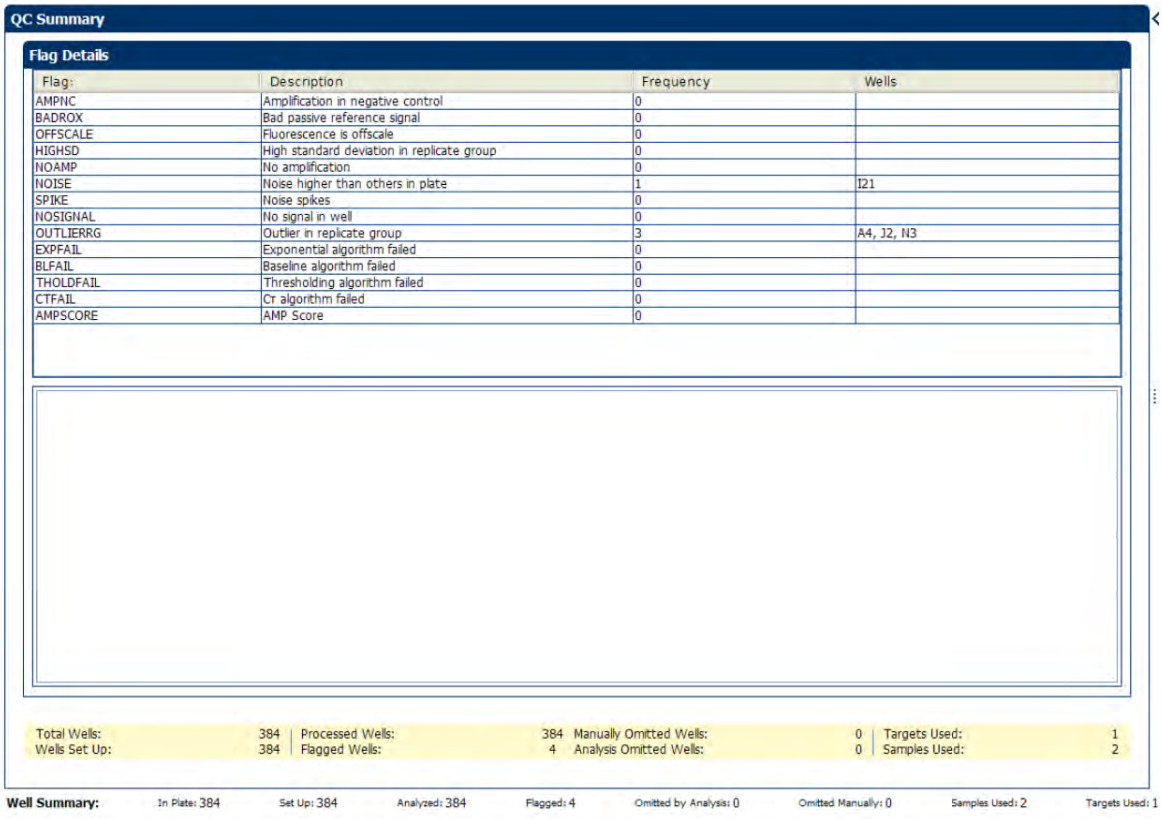
2. Review the Flags Summary.

   Note: A 0 displayed in the **Frequency** column indicates that the flag does not appear in the experiment. If the frequency is greater than 0, the flag appears somewhere in the experiment. The well position is listed in the **Wells** column.

   In the example experiment, there are four flagged wells.

3. In the **Flag Details** table, click each flag with a frequency greater than 0 to display detailed information about the flag. In the example experiment, the **Frequency** column displays **0** for all flags.

4. *(Optional)* For those flags with frequency greater than 0, click the troubleshooting link to view information on correcting the flag.



Possible flags

The flags listed below might be triggered by the experiment data.

Note: To change the flag settings, see “Flag settings” on page 137.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate

(continued)

Flag	Description
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
AMPSCORE	Amplification in the linear region is below a certain threshold, corresponding to the score set in the analysis settings
Secondary analysis flags	
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the **Frequency** column for each flag).

For more information

Information	Reference
Publishing data	Chapter 1, “General information and instructions”

Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

The **Analysis Settings** dialog box displays the analysis settings for the threshold cycle (C_T), flags, and advanced options.

If the default analysis settings in the QuantStudio™ 12K Flex Software are not suitable for your own experiment, you can change the settings in the **Analysis Settings** dialog box, then reanalyze your experiment.

View the analysis settings

1. From the **Experiment Menu** pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the **Analysis Settings** dialog box.
   In the example experiment, the default analysis settings are used for each tab.
   - C_T Settings
   - Flag Settings
   - Advanced Settings
   - Standard Curve Settings

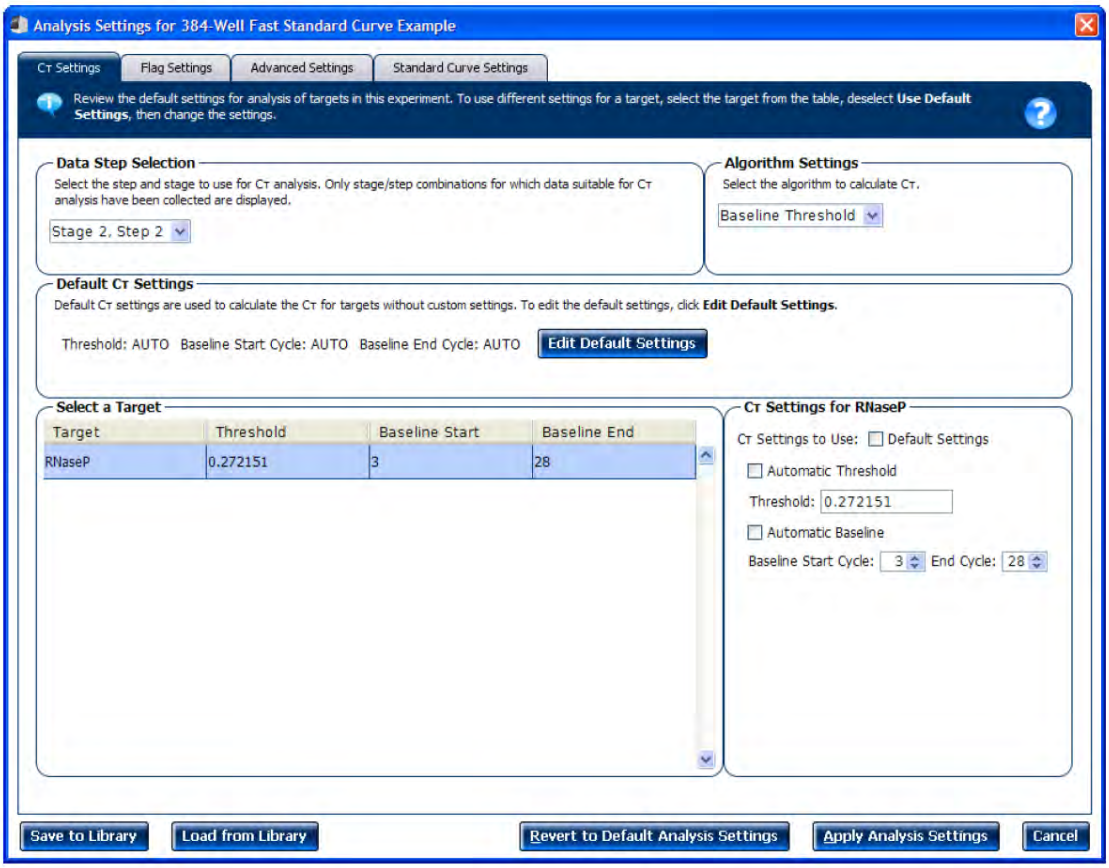


Figure 25 Analysis Settings dialog box for a Standard Curve experiment

3. View and, if necessary, change the analysis settings (see “Adjust analysis settings” on page 137).

Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see “About the analysis settings library” on page 62.

4. Click **Apply Analysis Settings** to apply the current analysis settings.

Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

C_T settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method.

- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

  The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for quantification.

  The Relative Threshold algorithm is a well-based analysis based on the PCR reaction efficiency and fitted to the Amplification curve. This setting is ideal for a single sample across genes with no dependence on targets, thereby reducing variability. It is not necessary to set either a baseline or a threshold when you use the Relative Threshold algorithm, so any settings for baseline or threshold will not affect the analysis.

- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the targets that do not have custom settings. To edit the default settings, click **Edit Default Settings**.

- **C_T Settings for Target**

  When you manually set the threshold and baseline, we recommend the following settings:

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

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag settings

Use the **Flag Settings** tab to perform the following tasks:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the QuantStudio™ 12K Flex Software.

1. In the **Use** column, select the checkboxes for flags to apply during analysis.

2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

3. In the **Reject Well** column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations might change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.

4. Click **Apply Analysis Settings** in the **Analysis Settings** dialog box.

   If the run status is complete, the data are reanalyzed.

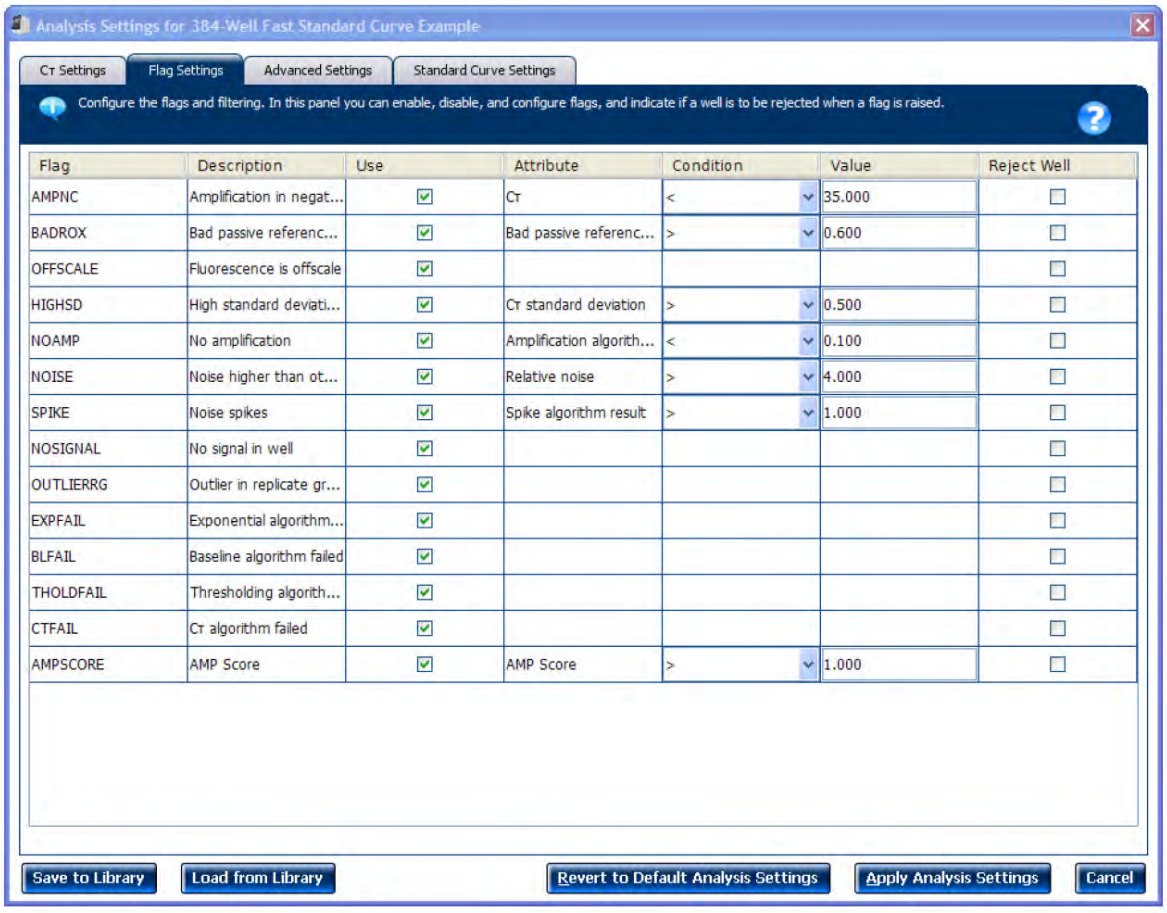


Figure 26 Flag Settings tab

Advanced settings

Use the **Advanced Settings** tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold algorithm.

The following steps describe the use of custom baseline settings for a well-target combination.

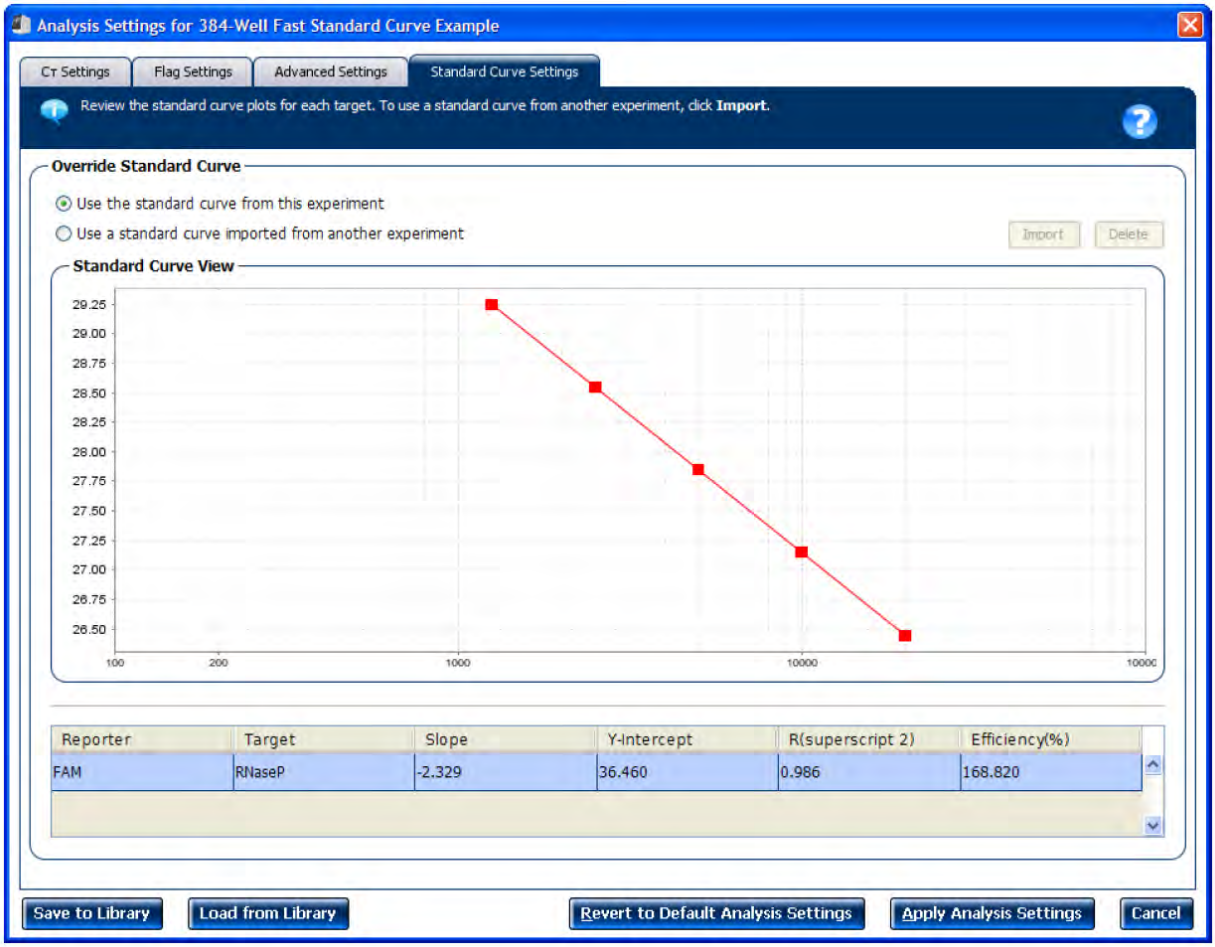
1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** checkbox.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** checkbox.
   - To define the baseline, deselect the **Automatic Baseline** checkbox, then enter the baseline start cycle and baseline end cycle.

Standard curve settings

Use this tab to review the settings of the current standard curve experiment or to import the standard curve from an external experiment (with the same samples and targets) and apply it to the current experiment.

Note: The run method must be the same. We recommend using the standard curve from the current experiment.

For the example experiment, the settings from the current experiment have been used.



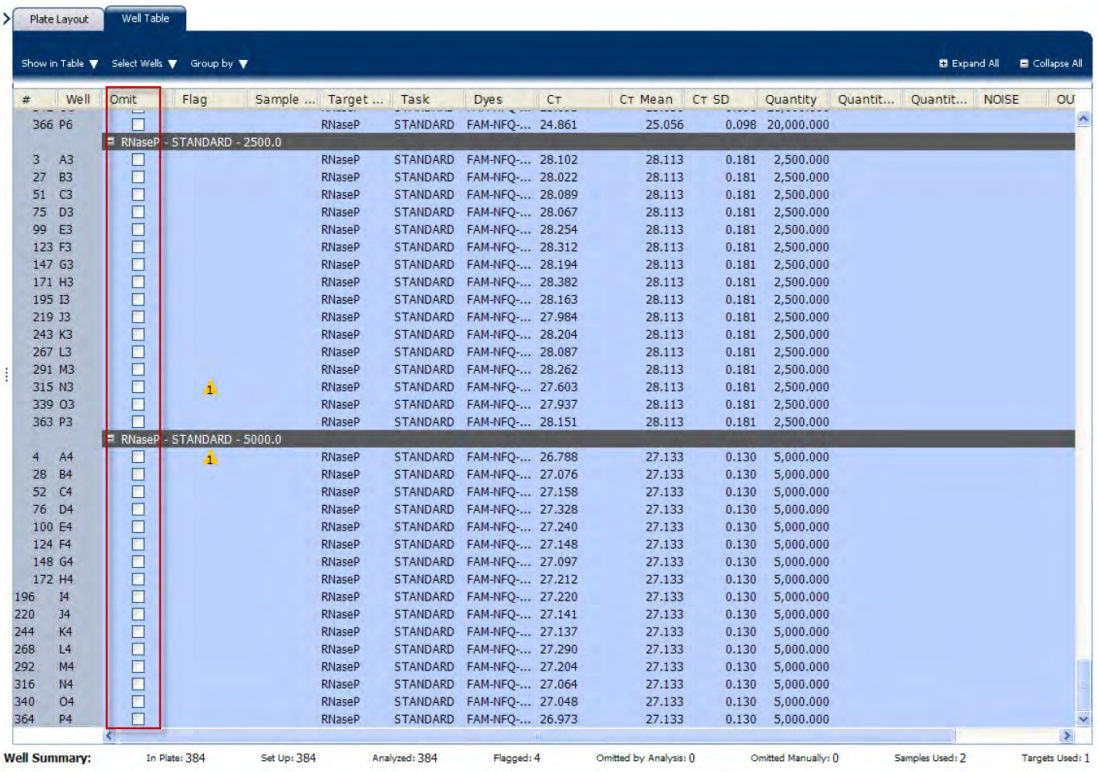
Omit wells to improve C_T precision

Experimental error might cause some wells to be amplified insufficiently or not at all. These wells typically produce C_T values that differ significantly from the average for the associated replicate wells. If included in the calculations, these outliers can result in erroneous measurements. To support C_T precision, omit the outliers from the analysis.

1. From the **Experiment Menu** pane, select **Analysis** ▸ **Amplification Plot**.

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

2. In the **Amplification Plot** screen, select **C_T vs Well** from the **Plot Type** dropdown list.
3. Select the **Well Table** tab.
4. In the Well Table, view outliers.
   a. From the **Group By** dropdown list, select **Replicate**.
   b. Look for any outliers in the replicate group (make sure they are flagged).
   c. Select the **Omit** checkbox next to outlying well or outlying wells, as shown below.



5. Click **Analyze** to reanalyze the experiment data with the outlying well or outlying wells removed from the analysis.

Note: You can also omit undesirable wells in an experiment from the **Plate Layout** screen. To omit a well from the **Plate Layout** screen, right-click the well, then click **Omit**.

---

8 Export analysis results

1. Open a Standard Curve example experiment file that has been analyzed.
   For information about analysis, see Chapter 7, “Review results and adjust experiment parameters”.

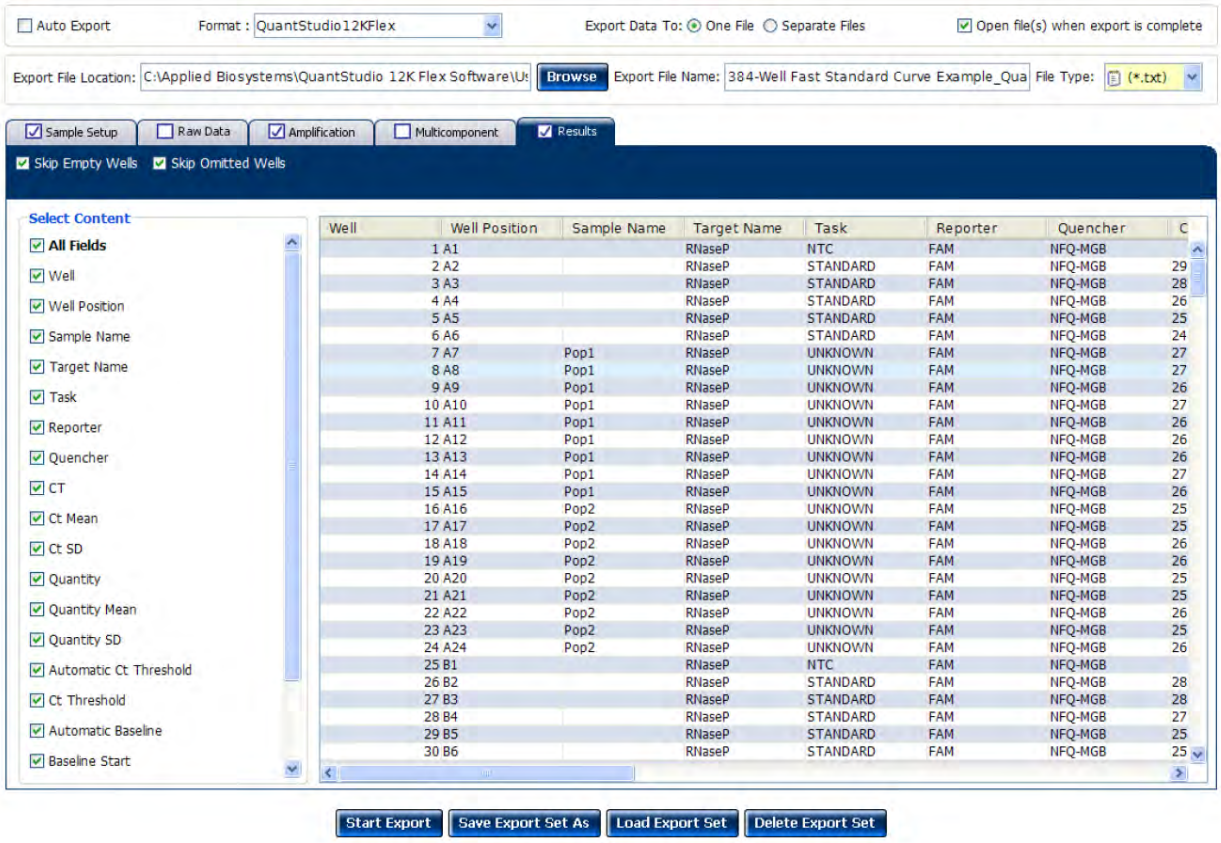
2. In the **Experiment Menu** pane, click **Export**.

   Note: To export data automatically after analysis, select the **Auto Export** checkbox during experiment setup or before running the experiment. The **Auto Export** checkbox is unchecked for the example experiment.

3. In the **Format** dropdown list, select **QuantStudio 12K Flex format**.

4. Complete the **Export** dialog box as shown below.

Field or Selection	Entry
Select Data to export/Select Content	Results
Export Data To options	**One File** radio button
Export File Name field	Enter a file name or use the default file name
File Type dropdown list	***.txt**
Export File Location field	Use the default file location or click **Browse** to select a different location



Well	Well Position	Sample Name	Target Name	Task	Reporter	Quencher	C
1	A1		RNaseP	NTC	FAM	NFQ-MGB	
2	A2		RNaseP	STANDARD	FAM	NFQ-MGB	29
3	A3		RNaseP	STANDARD	FAM	NFQ-MGB	28
4	A4		RNaseP	STANDARD	FAM	NFQ-MGB	26
5	A5		RNaseP	STANDARD	FAM	NFQ-MGB	25
6	A6		RNaseP	STANDARD	FAM	NFQ-MGB	24
7	A7	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	27
8	A8	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	27
9	A9	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	26
10	A10	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	27
11	A11	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	26
12	A12	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	26
13	A13	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	26
14	A14	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	27
15	A15	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	26
16	A16	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	25
17	A17	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	25
18	A18	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	26
19	A19	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	26
20	A20	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	25
21	A21	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	25
22	A22	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	26
23	A23	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	25
24	A24	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	26
25	B1		RNaseP	NTC	FAM	NFQ-MGB	
26	B2		RNaseP	STANDARD	FAM	NFQ-MGB	28
27	B3		RNaseP	STANDARD	FAM	NFQ-MGB	28
28	B4		RNaseP	STANDARD	FAM	NFQ-MGB	27
29	B5		RNaseP	STANDARD	FAM	NFQ-MGB	25
30	B6		RNaseP	STANDARD	FAM	NFQ-MGB	25

Figure 27 Export screen

384-Well Fast Standard Curve Example_QuantStudio_export.txt - Notepad

File Edit Format View Help

```
* Barcode = NA
* Block Type = 384-Well Block
* Calibration Background is expired  = No
* Calibration Background performed on  = 2011-08-05 01:46:18 AM SGT
* Calibration FAM is expired  = No
* Calibration FAM performed on  = 2011-08-05 02:04:58 AM SGT
* Calibration ROI is expired  = No
* Calibration ROI performed on  = 2011-08-04 11:42:31 AM SGT
* Calibration ROX is expired  = No
* Calibration ROX performed on  = 2011-08-05 02:23:30 AM SGT
* Calibration Uniformity is expired  = No
* Calibration Uniformity performed on  = 2011-08-05 01:56:21 AM SGT
* Calibration VIC is expired  = No
* Calibration VIC performed on  = 2011-08-05 02:15:08 AM SGT
* Chemistry = TAQMAN
* Comment = NA
* Date Created = 1970-01-01 07:30:00 AM SGT
* Experiment File Name = C:\Program Files\Applied Biosystems\QuantStudio12KFlex\examples\Standard Curve\384-Well Fast Standard Curve
Example.eds
* Experiment Name = 384-Well Fast Standard Curve Example
* Experiment Run End Time = 2011-08-06 13:15:47 PM SGT
* Experiment Type = Standard Curve
* Instrument Name = QuantStudioDemo
* Instrument Serial Number = QuantStudioDemo
* Instrument Type = QuantStudio 12K Flex
* Passive Reference = ROX
* Quantification Cycle Method = Ct
* Signal Smoothing On = true
* Stage/ Cycle where Analysis is performed = Stage 2, Step 2
* User Name = NA

[Sample Setup]
Well    Well Position   Sample Name     Sample Color    Biogroup Name   Biogroup Color  Target Name     Target Color    Task    Reporter
Quencher        Quantity        Comments
1       A1                                      RNaseP  "RGB(176,23,31)"        NTC     FAM     NFQ-MGB
2       A2                                      RNaseP  "RGB(176,23,31)"        STANDARD        FAM     NFQ-MGB "1,250.000"
3       A3                                      RNaseP  "RGB(176,23,31)"        STANDARD        FAM     NFQ-MGB "2,500.000"
4       A4                                      RNaseP  "RGB(176,23,31)"        STANDARD        FAM     NFQ-MGB "5,000.000"
5       A5                                      RNaseP  "RGB(176,23,31)"        STANDARD        FAM     NFQ-MGB "10,000.000"
6       A6                                      RNaseP  "RGB(176,23,31)"        STANDARD        FAM     NFQ-MGB "20,000.000"
7       A7      Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
8       A8      Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
9       A9      Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
10      A10     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
11      A11     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
12      A12     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
13      A13     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
14      A14     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
15      A15     Pop1    "RGB(0,255,0)"                  RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
16      A16     Pop2    "RGB(255,102,0)"                        RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
17      A17     Pop2    "RGB(255,102,0)"                        RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
18      A18     Pop2    "RGB(255,102,0)"                        RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
19      A19     Pop2    "RGB(255,102,0)"                        RNaseP  "RGB(176,23,31)"        UNKNOWN FAM     NFQ-MGB
```

Figure 28 Exported file

Part III

Running relative standard curve experiments

9 About relative standard curve experiments

IMPORTANT! First-time users of the QuantStudio™ 12K Flex Real–Time PCR System, see the part "Getting started" on page 17 and Appendix A, "Documentation and support". The sections provide information and general instructions that are applicable to all the experiments described in this document.

Note: For more information about any of the topics discussed in this guide, access the Help from within the QuantStudio™ 12K Flex Software by pressing F1, clicking in the toolbar, or selecting **Help ▸ QuantStudio 12K Flex Software Help**.

About relative standard curve experiments

The Relative Standard Curve method is used to determine relative target quantity in samples. The QuantStudio™ 12K Flex Software measures amplification of the target and of the endogenous control in samples, in a reference sample, and in a standard dilution series. Measurements are normalized using the endogenous control. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates target quantity and endogenous control quantity in the samples and the reference sample. For each sample and reference sample, the target quantity is normalized by endogenous control quantity (quantity of target/quantity of endogenous control). The normalized quotient from samples is divided by the quotient from the reference sample to get relative quantification (fold change). The software determines the relative quantity of target in each sample by comparing target quantity in each sample to target quantity in the reference sample.

Relative Standard Curve experiments are commonly used to perform the following tasks:

- Compare expression levels of a gene in different tissues
- Compare expression levels of a gene in a treated sample and an untreated sample
- Compare expression levels of wild-type alleles and mutated alleles
- Analyze the gene expression changes over time under specific treatment conditions

Assemble the required components

- **Sample**—The tissue group that you are testing for a target gene.
- **Reference sample (also called a calibrator)**—The sample used as the basis for relative quantification results. For example, in a study of drug effects on gene expression, an untreated control is an appropriate reference sample.
- **Standard**—A sample that contains known quantities of the target; used in quantification experiments to generate standard curves.
- **Standard dilution series**—A set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards.
- **Endogenous control**—A gene that is used to normalize template input differences, and sample-to-sample or run-to-run variation.
- **Replicates**—The total number of identical reactions containing identical components and identical volumes.
- **Negative Controls**—Wells that contain water or buffer instead of sample template. No amplification of the target should occur in the negative control wells.

PCR options

When performing real-time PCR, choose between:

- Singleplex PCR and multiplex PCR (see “Singleplex and multiplex PCR” on page 147)

  and
- 1-step RT-PCR and 2-step RT-PCR (see “1- and 2-step RT-PCR” on page 148)

Singleplex and multiplex PCR

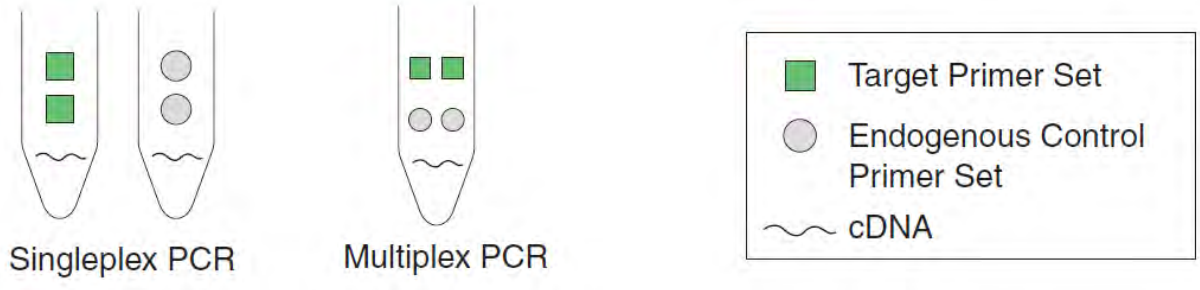
You can perform a PCR reaction using either:

- **Singleplex PCR**—In singleplex PCR a single primer and probe set is present in the reaction tube or well. Only one target or endogenous control can be amplified per reaction.

  Or
- **Multiplex PCR**—In multiplex PCR, two or more primer and probe sets are present in the reaction tube or well. Each set amplifies a specific target or endogenous control.

  One example is a probe that is labeled with FAM™ dye detects the target and a probe that is labeled with VIC™ dye detects the endogenous control.

IMPORTANT! SYBR™ Green reagents cannot be used for multiplex PCR.



1- and 2-step RT-PCR

You can perform reverse transcription (RT) and PCR in a single reaction (1-step) or in separate reactions (2-step). The reagent configuration you use depends on whether you are performing 1- or 2-step RT-PCR.

- **1-step PCR**—In 1-step RT-PCR, RT and PCR take place in one buffer system. Using one buffer system provides the convenience of a single-tube preparation for RT and PCR amplification. However, you cannot use Fast PCR Master Mix or the carryover prevention enzyme, AmpErase™ UNG (uracil-N-glycosylase), to perform 1-step RT-PCR.
- **2-step PCR**—2-step RT-PCR is performed in two separate reactions. First, total RNA is reverse-transcribed into cDNA, then the cDNA is amplified by PCR. This method is useful for detecting multiple transcripts from a single cDNA template or for storing cDNA aliquots for later use. The AmpErase™ UNG enzyme can be used to prevent carryover contamination.

About the example experiment

To illustrate how to perform a Relative Standard Curve, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with the QuantStudio™ 12K Flex Real–Time PCR System.

The objective of the Relative Standard Curve example experiment is to compare the expression of the HPRT transcriptional factor (an oncoprotein that activates the transcription of growth-associated genes) in Human cDNA tissues.

In the Relative Standard Curve example experiment:

- The samples are 1, 10, 100, 1000, 10000, Heart, and Kidney.
- The target is HPRT.
- The endogenous control is FAS.
- The reference sample is Kidney.
- One standard curve is set up for HPRT. The standard used for the standard dilution series is a Human cDNA sample of known total concentration.
- One standard curve is set up for FAS (endogenous control). The standard used for the standard dilution series is a Human Male Raji cDNA sample of known total concentration.
- Reactions are set up for 2-step RT-PCR. The SuperScript™ VILO™ cDNA Synthesis Kit is used for reverse transcription. The TaqMan™ Gene Expression Master Mix is used for PCR.
- Select primer and probe sets from the TaqMan™ Gene Expression Assay product line:
  - For the target assay (HPRT), select assay ID Hs99999909_m1.
  - For the endogenous control assay (FAS), select assay ID Hs00907759_m1.

10 Design the experiment

This chapter explains how to design the example experiment from the **Setup** menu in the **Experiment Menu** pane.

Note: To automatically export the analyzed data to a specified location, select the **Auto Export** checkbox in the **Export** screen, before running the experiment. For more information on Auto Export, the part "Getting started" on page 17.

Define the experiment properties

In the **Experiment Menu** pane, click **Setup** ▸ **Experiment Properties**.

Enter or select the following information.

Field or Selection	Entry
Experiment Name field	Enter ***96-Well Relative Std Curve Example***.
Barcode field	Leave the **Barcode** field empty.
User Name field	Enter ***Example User*** or enter a user name.
Comments field	Enter ***Relative Standard Curve example***.
Block	Select **96-Well (0.2 mL)**.
Experiment Type	Select **Relative Standard Curve**.
Reagents	Select **TaqMan Reagents**.
Properties	Select **Standard**.

Save the experiment.

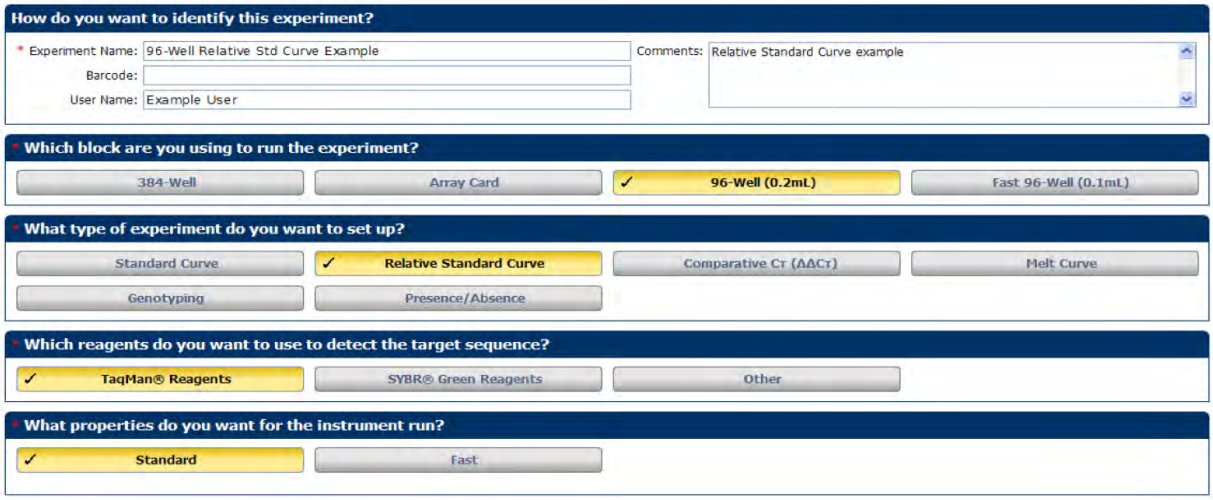


Figure 29 Experiment Properties screen

Define targets, samples, and biological replicates

1. Click **Define** to access the **Define** screen.
2. Enter the following target information.

Target name	Reporter	Quencher	Color
FAS	FAM	NFQ-MGB	
HPRT	FAM	NFQ-MGB	

3. Enter the following sample information.

Sample name	Color
1	
10	
100	

(continued)

Sample name	Color
1000	
10000	
Heart	
Kidney	

4. In the **Passive Reference** dropdown list, select **ROX** .

5. Enter the following analysis settings.

Field	Select
Reference Sample field	**Kidney**
Endogenous Control field	**FAS**

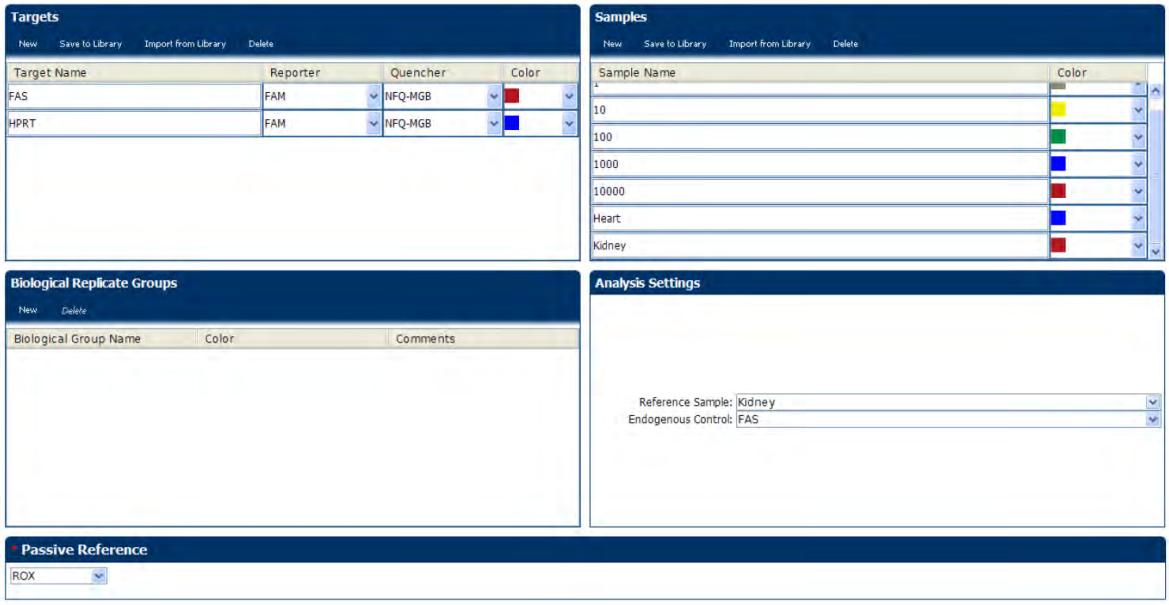


Figure 30 Define screen

Note: This example experiment does not define biological replicate groups. Leave the **Biological Replicate Groups** pane blank.

Assign targets, samples, and biological groups

Note: To automatically set up and assign standards, click Define and Set Up Standards to open the **Define and Set Up Standards** dialog box.

1. Click **Assign** to access the **Assign** screen.
2. For the first standard for the FAS target:
   a. Click-drag to select wells A1-A3.
   b. In the **Targets** list, select the checkbox for the row that is labeled ***FAS***.
   c. In the **Task** dropdown list, select **S**.
   d. In the **Quantity** field, enter ***10,000***.
   e. Repeat steps a on page 152 to c on page 152 for each of the standards for the FAS target, as displayed in Table 2 on page 152.
3. Repeat step 2 on page 152 for each standard for the HPRT target.

Table 2 Targets

Target name	Well number	Task	Quantity	Sample name
FAS	A12	Negative	None	None
	B4 - B6	Unknown	Determined by run	Kidney
	B7 - B9	Unknown	Determined by run	Heart
	A1 - A3	Standard	10,000	10,000
	A4 - A6	Standard	1,000	1,000
	A7 - A9	Standard	100	100
	A10 - A12	Standard	10	10
	B1 - B3	Standard	1	1
HPRT	D12	Negative	None	None
	D4 - D6	Unknown	Determined by run	Kidney
	D7 - D9	Unknown	Determined by run	Heart
	C1 - C3	Standard	10,000	10,000
	C4 - C6	Standard	1,000	1,000
	C7 - C9	Standard	100	100
	C10 - C12	Standard	10	10
	D1 - D3	Standard	1	1

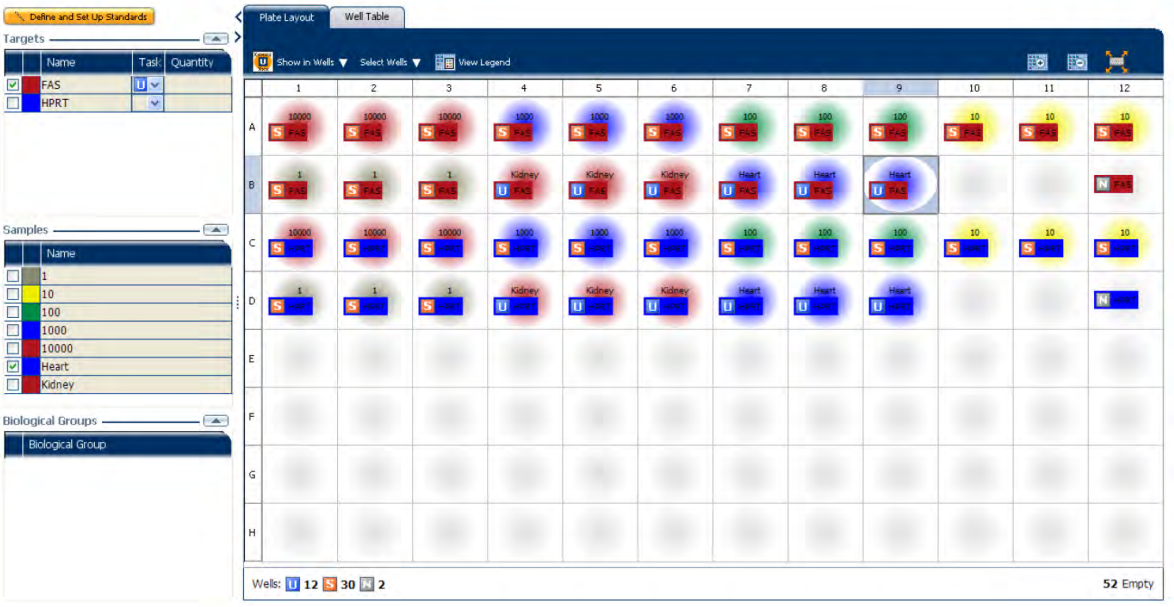


Figure 31 Assign screen

Set up the run method

Click **Run Method** to access the **Run Method** screen. Set the thermal profile under the **Graphical View** tab. Enter the following information:

- Reaction Volume Per Well: ***20 µL***
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Hold Stage	Step 1	1.6°C/s	50°C	2 minutes
	Step 2	1.6°C/s	95°C	10 minutes
PCR Stage Number of Cycles: 40 Enable AutoDelta: Unchecked (default) Starting Cycle: Disabled when Enable AutoDelta is unchecked	Step 1	1.6°C/s	95°C	15 seconds
	Step 2	1.6°C/s	60°C	1 minute

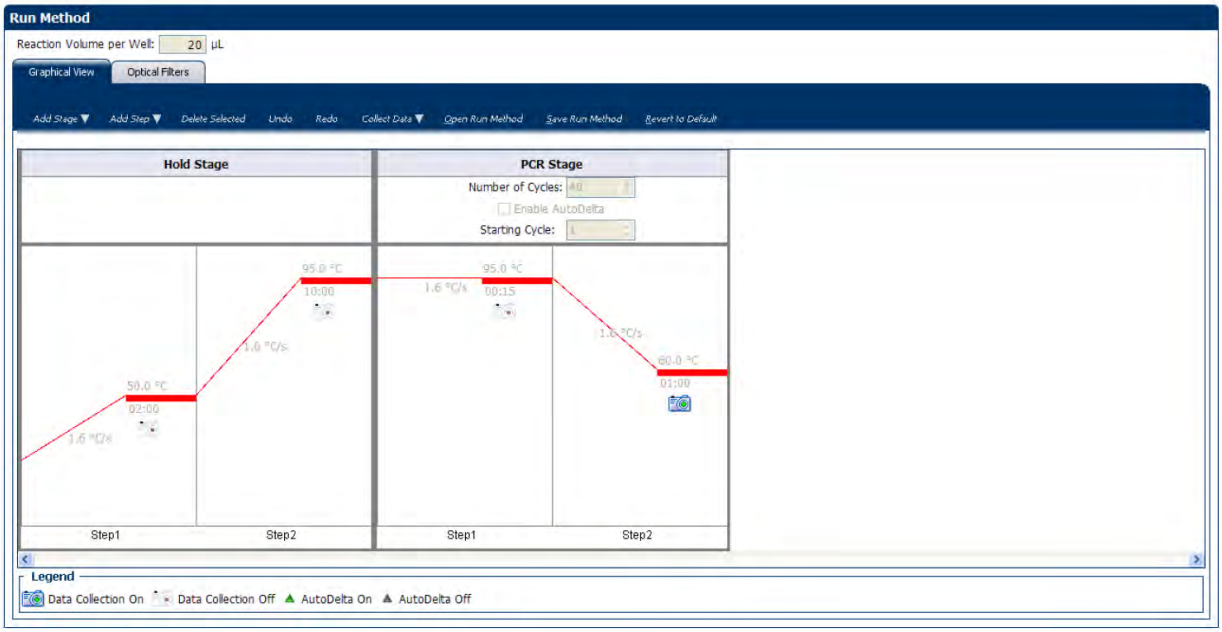


Figure 32 Run Method screen

Tips for designing your own experiment

We recommend the following items for your own experiments:

- Set up a standard curve for each target assay in the reaction plate.
- Identify each target assay with a unique name and color. You can enter up to 100 characters in the **Target Name** field.
- Identify each sample using a unique name and color. You can enter up to 100 characters in the **Sample Name** field.
- Select an endogenous control for each sample. The endogenous control is a target that is present in all samples under investigation. It should be expressed equally in all sample types, regardless of treatment or tissue origin (examples of endogenous controls are ß-actin, GAPDH, and 18S ribosomal RNA [18S rRNA]). The endogenous control is used to normalize the PCR results. The endogenous control corrects for variable sample mass, nucleic acid extraction efficiency, reverse transcription efficiency, and pipette calibration errors.

  Note: Each sample type (for example, each tissue in a study comparing multiple tissues) requires an endogenous control.

- Enter at least five dilution points for each standard curve in the reaction plate.
- Enter at least three replicates (identical reactions) for each point in the standard curve and for each sample reaction.
- Enter at least three negative control reactions for each target assay.

- Carefully consider the appropriate range of standard quantities for your assay because the range of standard quantities affects the amplification efficiency calculations.
  - For more accurate measurements of amplification efficiency, use a broad range of standard quantities, spanning between 5 and 6 logs. If you do so, use a PCR product or a highly concentrated template, such as a cDNA clone.
  - If you have a limited amount of cDNA template and/or if the target is a low-copy number transcript, or known to fall within a given range, a narrow range of standard quantities may be necessary.
- Minimally run a five-point curve of 1:10 dilutions to minimize the effects of small pipetting errors.
- Select a reference sample from your previously defined samples. Amplification results from the samples and from the reference sample are compared to determine relative expression.
- Select an endogenous control from your previously defined target assays. Amplification results from the endogenous control are used to normalize the amplification results from the target for differences in the amount of template added to each reaction.

More information

Information	Reference
Consumables	Chapter 1, “General information and instructions”
Using other quantification methods	• “Running standard curve experiments” on page 86 • “Running comparative Ct experiments” on page 198
Using alternative setup	Chapter 2, “Experiment shortcuts”

11 Prepare the reactions

This chapter explains how to prepare the PCR reactions for the Relative Standard Curve example experiment.

Assemble required materials

- Items listed in the part "Getting started" on page 17
- Samples—Total RNA isolated from kidney, heart, liver, and brain tissues
- Example experiment reaction mix components:
  - TaqMan™ Gene Expression Master Mix (2×)
  - FAS Assay Mix (20×)
  - HPRT Assay Mix (20×)

Prepare the template

Prepare the template for the PCR reactions (both samples and standards) using a cDNA synthesis kit. One option is SuperScript™ VILO™ cDNA Synthesis Kit (Cat. No. 11754250).

Example experiment settings

For the Relative Standard Curve example experiment, the template for the PCR reactions is cDNA reverse-transcribed from total RNA samples.

Prepare the template

Use the SuperScript™ VILO™ cDNA Synthesis Kit (Cat. No. 11754250). Follow the procedures for the kit.

1. Isolate total RNA from cells a sample preparation kit appropriate to the tissue or cell type.
2. Quantify and perform quality control on the RNA.
3. Convert the RNA to cDNA by reverse transcription.

Prepare the sample dilutions

For the Relative Standard Curve example experiment, no more than 10% of your reaction should consist of the undiluted cDNA.

1. Label a separate microcentrifuge tube for each diluted sample.
   - Kidney
   - Heart
2. Add the required volume of water (diluent) to each empty tube.

Tube	Sample name	Diluent volume
1	Kidney	76 µL
2	Heart	76 µL

3. Add the required volume of sample stock (100 ng/µL) to each empty tube:

Tube	Sample name	Diluent volume
1	Kidney	4 µL
2	Heart	4 µL

4. Vortex each diluted sample for 3 to 5 seconds, then centrifuge the tubes briefly.
5. Place the diluted samples on ice until you prepare the reaction plate.

Prepare the standard dilution series for FAS and HPRT assays

The same standard materials are used to prepare the exact same dilutions for both the target genes. The prepared standards are then used to generate the two standard curves.

- The stock concentration for cDNA is 100 ng/µL.
- The volumes calculated for both the FAS and HPRT assays are:

Standard name (labeled tube)	Dilution point	Source	Source volume	Diluent volume	Total volume	Standard concentration
Std. 1	1	Stock	20 µL	20 µL	40 µL	100 ng/µL
Std. 2	2	Dilution 1	20 µL	20 µL	40 µL	50 ng/µL
Std. 3	3	Dilution	20 µL	20 µL	40 µL	25 ng/µL
Std. 4	4	Dilution 3	20 µL	20 µL	40 µL	12.5 ng/µL
Std. 5	5	Dilution 4	20 µL	20 µL	40 µL	6.25 ng/µL

1. Label ten separate microcentrifuge tubes for each diluted standard:
   - FAS (FAS Std. 1 - FAS Std. 5)
   - HPRT (HPRT Std. 1 - HPRT Std. 5)
2. Prepare five standard dilutions each for FAS and HPRT:

   Note: For dilution 1, first vortex the stock for 3 to 5 seconds, then centrifuge both the Std. 1 tubes briefly before pipetting 10 µL stock into each Std. 1 tube.
3. For each subsequent dilution, add source to the standard:
   a. Use a new pipette tip to add 10 µL of source to the FAS and HPRT tubes containing the standard.
   b. Vortex the tubes for 3 to 5 seconds, then centrifuge the tubes briefly.
4. Place the standards on ice until you prepare the reaction plate.

Prepare the reaction mix

1. Label an appropriately sized tube for each reaction mix:
   - FAS Reaction Mix
   - HPRT Reaction Mix
2. For the FAS assay, add the required volumes of each component to the FAS Reaction Mix tube:

   Note: Extra volume is already factored in for pipetting error

Component	Volume for 1 reaction	Volume for 30 reactions
TaqMan™ Gene Expression Master Mix (2×)	10 µL	300 µL
FAS Assay Mix (20×)	1 µL	30 µL
Water	8 µL	240 µL
Total Reaction Mix Volume	**19 µL**	**570 µL**

3. For the HPRT assay, add the required volumes of each component to the HPRT Reaction Mix tube:

Component	Volume for 1 reaction	Volume for 30 reactions
TaqMan™ Gene Expression Master Mix (2×)	10 µL	300 µL
HPRT Assay Mix (20×	1 µL	30 µL
Water	8 µL	240 µL
Total Reaction Mix Volume	**19 µL**	**570 µL**

4. Mix the reaction in each tube by gently pipetting up and down, then cap each tube.
5. Centrifuge the tubes briefly to remove air bubbles.

6. Place the reaction mixes on ice until you prepare the reaction plate.

Note: Do not add the sample or standard at this time.

Prepare the reaction plate

Example experiment reaction plate components

The reaction plate for the Relative Standard Curve example experiment contains:

- A MicroAmp™ Optical 96-Well Reaction Plate
- Reaction volume: 20 µL/well
- 12 Unknown wells U
- 30 Standard wells S
- 2 Negative Control wells N
- 52 Empty wells

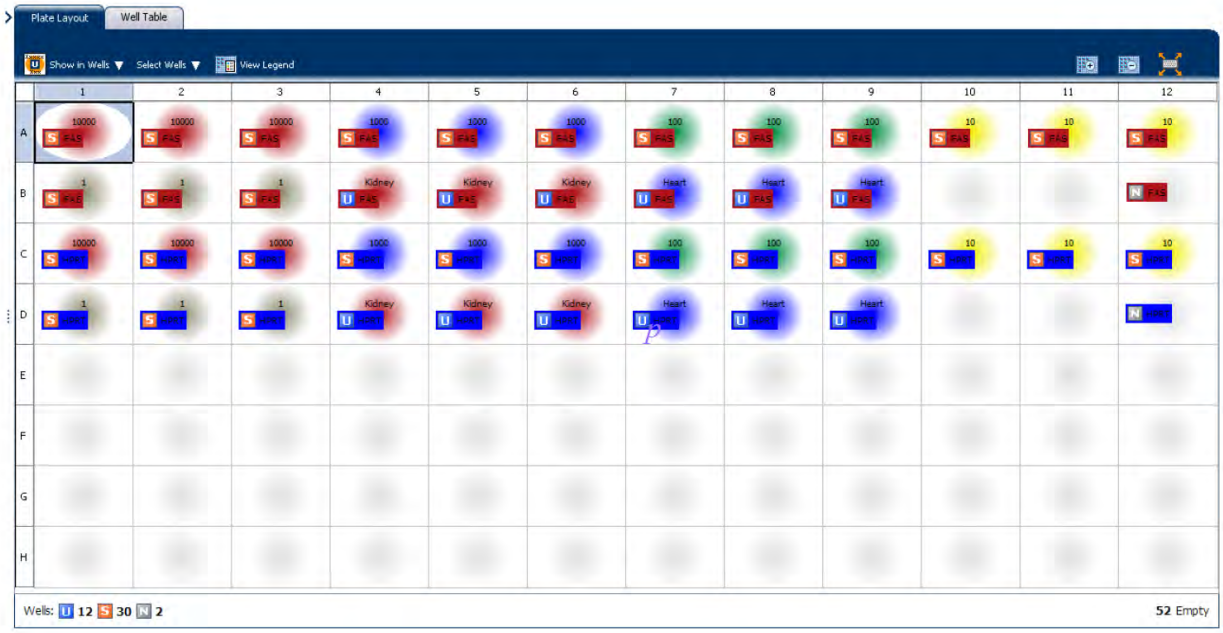


Figure 33 Plate layout for example experiment

To prepare the reaction plate components

1. For each target, prepare the negative control reactions:
   a. To an appropriately sized tube, add the volumes of reaction mix and water listed below.

Tube	Reaction mix	Reaction mix volume	Water volume
1	FAS Reaction Mix	19 µL	1 µL
2	HPRT Reaction Mix	19 µL	1 µL

b. Mix the reaction by gently pipetting up and down, then cap the tube.

c. Centrifuge the tube briefly to remove air bubbles.

d. Add 20 µL of the negative control reaction to the appropriate wells in the reaction plate.

2. For each replicate group, prepare the standard reactions:

a. To appropriately sized tubes, add the volumes of reaction mix and standard listed below.

Tube	Standard rection	Reaction mix	Reaction mix volume	Standard	Standard volume
1	FAS Std. 1	FAS Reaction Mix	76 µL	FAS Std. 1	4 µL
2	FAS Std. 2	FAS Reaction Mix	76 µL	FAS Std. 2	4 µL
3	FAS Std. 3	FAS Reaction Mix	76 µL	FAS Std. 3	4 µL
4	FAS Std. 4	FAS Reaction Mix	76 µL	FAS Std. 4	4 µL
5	FAS Std. 5	FAS Reaction Mix	76 µL	FAS Std. 5	4 µL
6	HPRT Std. 1	HPRT Reaction Mix	76 µL	HPRT Std. 1	4 µL
7	HPRT Std. 2	HPRT Reaction Mix	76 µL	HPRT Std. 2	4 µL
8	HPRT Std. 3	HPRT Reaction Mix	76 µL	HPRT Std. 3	4 µL
9	HPRT Std. 4	HPRT Reaction Mix	76 µL	HPRT Std. 4	4 µL
10	HPRT Std. 5	HPRT Reaction Mix	76 µL	HPRT Std. 5	4 µL

b. Mix the reactions by gently pipetting up and down, then cap the tubes.

c. Centrifuge the tubes briefly to remove air bubbles.

d. Add 20 µL of the standard reaction to the appropriate wells in the reaction plate.

3. For each replicate group, prepare the reactions for the unknowns:

a. To appropriately sized tubes, add the volumes of reaction mix and sample listed below:

Tube	Unknown reaction	Reaction mix	Reaction mix volume	Sample	Sample volume
1	FAS Kidney	FAS Reaction Mix	76 µL	Kidney	4 µL
2	FAS Heart	FAS Reaction Mix	76 µL	Heart	4 µL
3	HPRT Kidney	HPRT Reaction Mix	76 µL	Kidney	4 µL
4	HPRT Heart	HPRT Reaction Mix	76 µL	Heart	4 µL

b. Mix the reactions by gently pipetting up and down, then cap the tubes.

c. Centrifuge the tubes briefly to remove air bubbles.

d. Add 20 µL of the unknown (sample) reaction to the appropriate wells in the reaction plate.

4. Seal the reaction plate with optical adhesive film.

5. Centrifuge the reaction plate briefly to remove air bubbles.
6. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.
7. Until you are ready to perform the run, place the reaction plate at 4°C, in the dark.

Tips for preparing reactions for your own experiments

Tips for preparing templates

When you prepare your own Relative Standard Curve experiment, we recommend the following templates:

- **Complementary DNA (cDNA)**—cDNA reverse-transcribed from total RNA samples.
- **Genomic DNA (gDNA)**—Purified gDNA already extracted from tissue or sample

Tips for preparing sample dilutions

When you prepare your own Relative Standard Curve experiment, for optimal performance of the TaqMan™ Gene Expression Assay or the Custom TaqMan™ Gene Expression Assay, use 10–100 ng of cDNA template per 10–µL reaction.

Tips for preparing the reaction mix

If your experiment includes more than one target assay, prepare the reaction mix for each target assay separately.

Tips for preparing the reaction plate

When you prepare your own Relative Standard Curve experiment, make sure the arrangement of the PCR reactions matches the plate layout displayed in the QuantStudio™ 12K Flex Software.

More information

Information	Reference
Assigning the reaction plate components	Chapter 1, “General information and instructions”
Sealing the reaction plate	Chapter 1, “General information and instructions”

12 Run the experiment

This chapter explains how to run the example experiment on the QuantStudio™ 12K Flex Real-Time PCR Instrument.

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the instrument is in operation.

Start the run

1. Open the Relative Standard Curve example file that you created.
   See Chapter 10, “Design the experiment”.
2. Load the reaction plate into the instrument.
3. Start the run.

Monitor the run

You can monitor an experiment run in three ways:

- From the **Run** screen of the QuantStudio™ 12K Flex Software, while the experiment is in progress
- From the **Instrument Console** screen of the QuantStudio™ 12K Flex Software, to monitor an experiment started from another computer or from the instrument touchscreen (see “Monitor a run from the software Instrument Console screen” on page 162)
- From the instrument touchscreen (see “Monitor a run from the instrument touchscreen” on page 165)

Monitor a run from the software Instrument Console screen

1. In the **Instrument Console** screen, select the icon of the instrument that you are using to run the experiment.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. In the **Instrument Manager** screen, click **Monitor Running Experiment** to access the **Run** screen.

View the amplification plot

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the QuantStudio™ 12K Flex Software for potential problems.

Click **Amplification Plot** from the **Run Experiment** menu, select the **Plate Layout** tab, then select the wells to view.

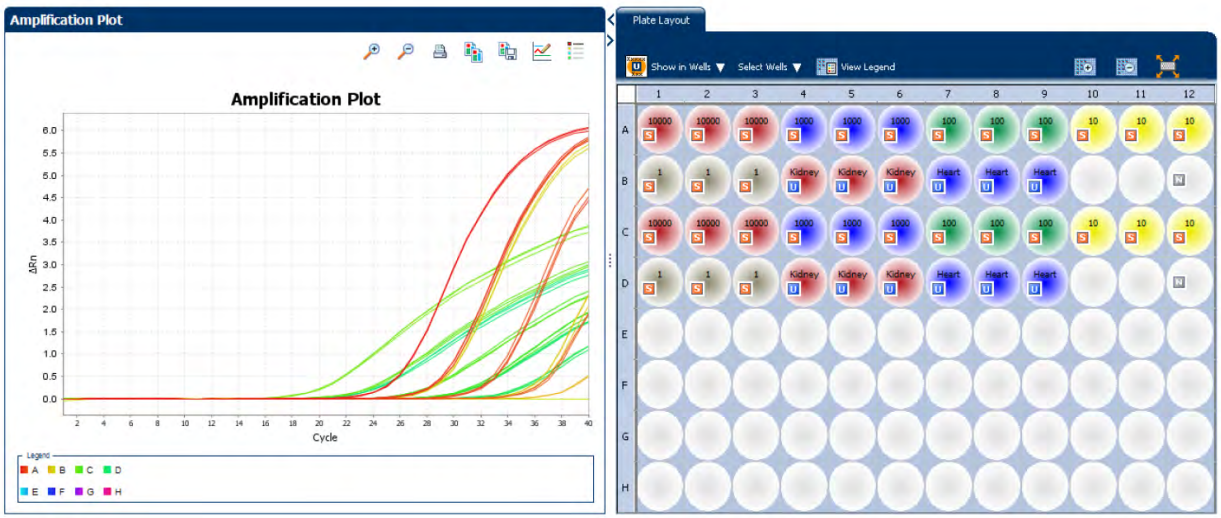


Figure 34 The Amplification Plot screen as it appears at the end of the example experiment

View the temperature plot

Click **Temperature Plot** from the **Run Experiment** menu.

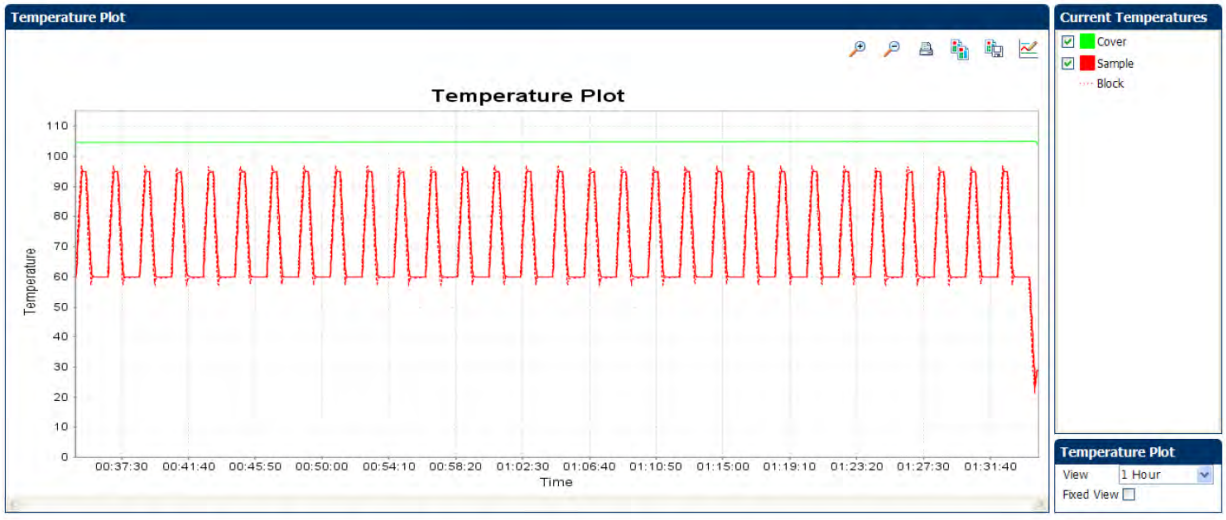


Figure 35 The Temperature Plot screen as it appears during the example experiment

Note: The sample temperature displayed in the **Current Temperatures** group is an estimated value.

View the run method

Click **Run Method** from the **Run Experiment** menu.

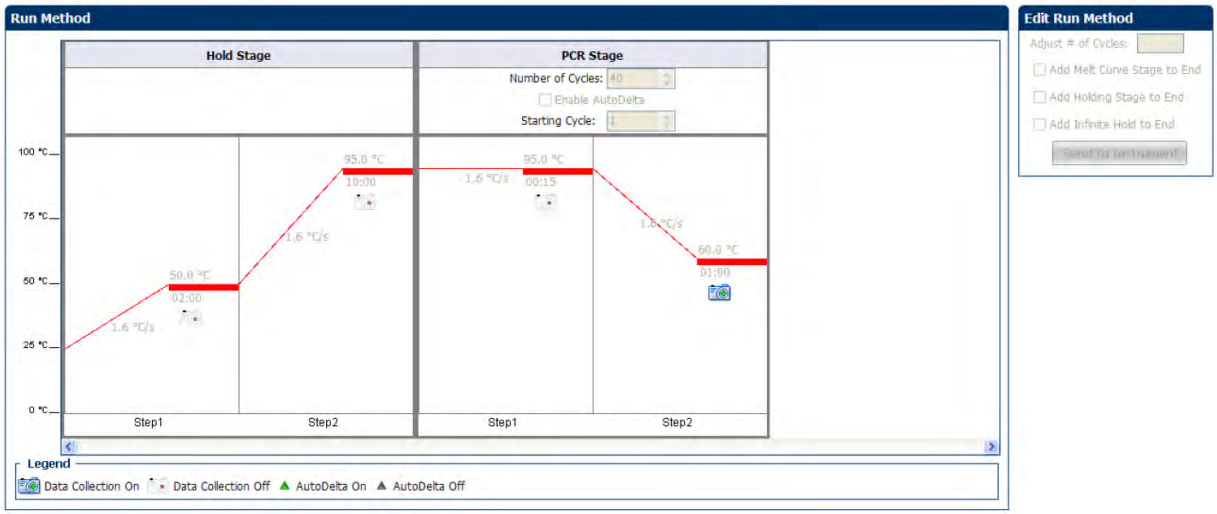


Figure 36 The Run Method screen as it appears in the example experiment

View run data

Click **View Run Data** from the **Run Experiment** menu.

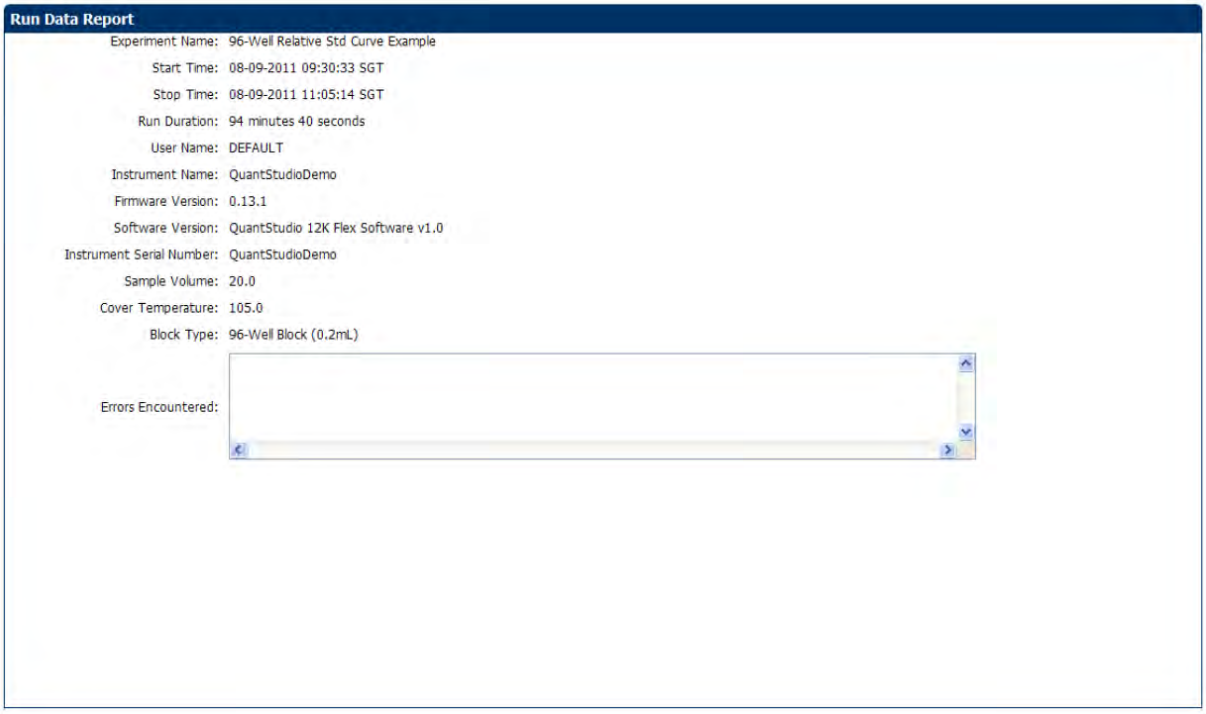


Figure 37 The View Run Data screen as it appears in the example experiment

Monitor a run from the instrument touchscreen

You can view the progress of the run from the instrument touchscreen.

The following figures are for visual representation only. Actual results vary with the experiment.

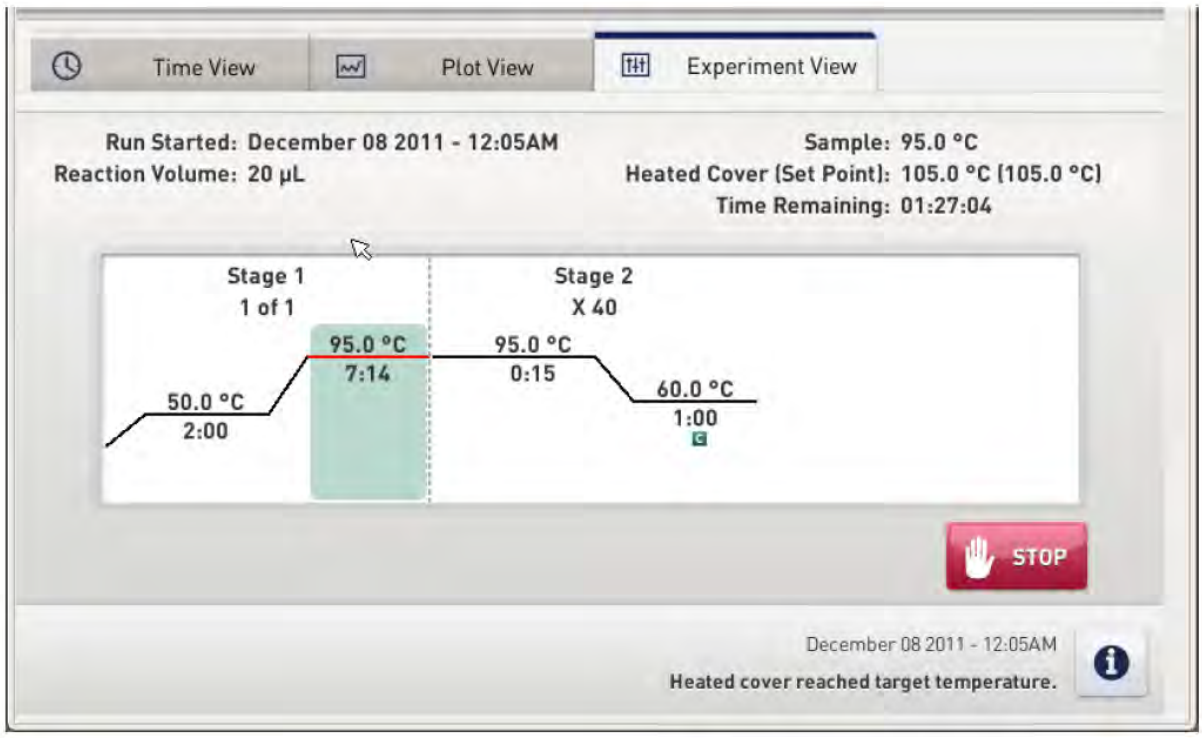


Figure 38 Experiment View tab

Figure 39 Time View tab

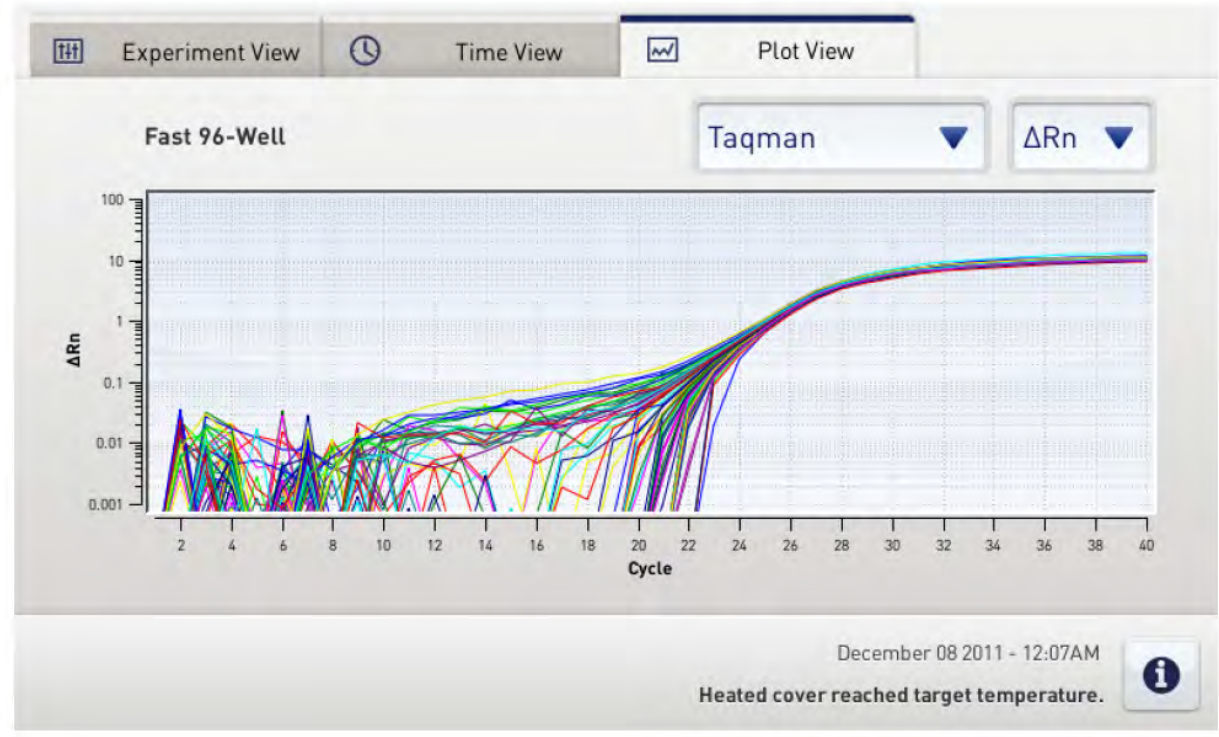


Figure 40 Plot View tab

13 Review results and adjust experiment parameters

The following topics are covered in this chapter:

- Review the analyzed data using several of the analysis screens and publish the data (see “Review results” on page 167)
- Modify experiment parameters to troubleshoot problems with experiment results before rerunning an experiment (see “Adjust parameters for re-analysis of your own experiments” on page 189)

Review results

Analyze the example experiment

1. Open the example experiment file.
   See Chapter 12, “Run the experiment”.
2. Click **Analyze**.

Note: You can also access the experiment to analyze from the **Home** screen.

The software analyzes the data using the default analysis settings.

View the standard curve plot

The Standard Curve Plot screen displays the standard curve for samples designated as standards. The QuantStudio™ 12K Flex Software calculates the quantity of an unknown target from the standard curve.

Example experiment standard curve values

The purpose of viewing the standard curve for the example experiment is to identify the following items:

- Slope and amplification efficiency
- R^2 value (correlation coefficient)
- C_T values

View and assess the standard curve plot

1. From the **Experiment Menu** pane, select **Analysis** ▸ **Standard Curve**.

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

2. Display all 96 wells in the Standard Curve Plot screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.

3. Enter the plot settings.

Menu	Selection
Target	All
Plot Color	Default
 (This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.)	Check (default)

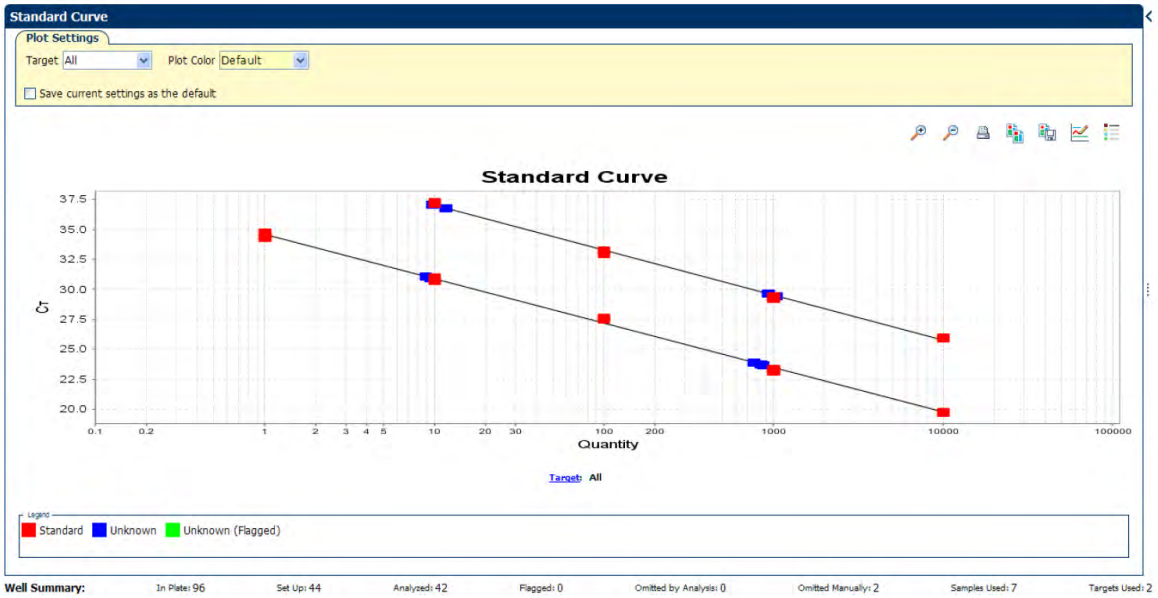
4. View the values displayed below the standard curve.

   In the example experiment, the values for each target fall within the acceptable ranges.

Target	Slope	R2 Value	Amplification efficiency (Eff%)
FAS	-3.38	0.998	97.612
HPRT	-3.652	0.983	87.858

5. Check that all samples are within the standard curve.

   In the example experiment, all samples (blue dots) are within the standard curve (red dots).



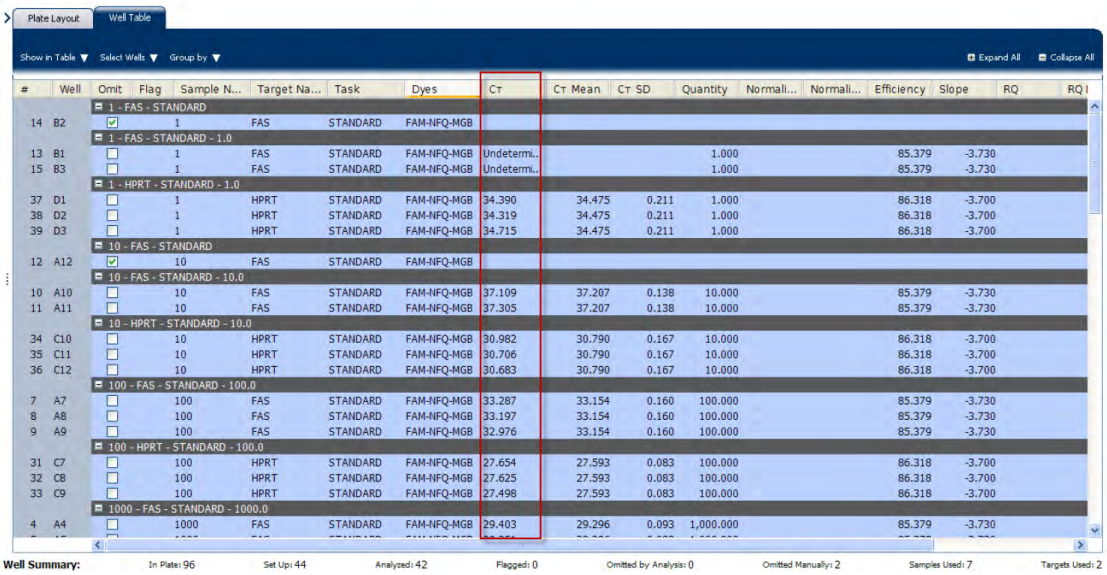
6. Check the C_T values.

   a. Click the **Well Table** tab.

   b. From the **Group By** menu, select **Replicate**.

c. Look at the values in the **C_T** column.

In the example experiment, the C_T values fall within the expected range (>8 and <35).



#	Well	Omit	Flag	Sample N...	Target Na...	Task	Dyes	CT	CT Mean	CT SD	Quantity	Normali...	Normali...	Efficiency	Slope	RQ	RQ I
				1 - FAS - STANDARD													
14	B2	☑		1	FAS	STANDARD	FAM-NFQ-MGB										
				1 - FAS - STANDARD - 1.0													
13	B1	☐		1	FAS	STANDARD	FAM-NFQ-MGB	Undetermi...			1.000			85.379	-3.730		
15	B3	☐		1	FAS	STANDARD	FAM-NFQ-MGB	Undetermi...			1.000			85.379	-3.730		
				1 - HPRT - STANDARD - 1.0													
37	D1	☐		1	HPRT	STANDARD	FAM-NFQ-MGB	34.390	34.475	0.211	1.000			86.318	-3.700		
38	D2	☐		1	HPRT	STANDARD	FAM-NFQ-MGB	34.319	34.475	0.211	1.000			86.318	-3.700		
39	D3	☐		1	HPRT	STANDARD	FAM-NFQ-MGB	34.715	34.475	0.211	1.000			86.318	-3.700		
				10 - FAS - STANDARD													
12	A12	☑		10	FAS	STANDARD	FAM-NFQ-MGB										
				10 - FAS - STANDARD - 10.0													
10	A10	☐		10	FAS	STANDARD	FAM-NFQ-MGB	37.109	37.207	0.138	10.000			85.379	-3.730		
11	A11	☐		10	FAS	STANDARD	FAM-NFQ-MGB	37.305	37.207	0.138	10.000			85.379	-3.730		
				10 - HPRT - STANDARD - 10.0													
34	C10	☐		10	HPRT	STANDARD	FAM-NFQ-MGB	30.982	30.790	0.167	10.000			86.318	-3.700		
35	C11	☐		10	HPRT	STANDARD	FAM-NFQ-MGB	30.706	30.790	0.167	10.000			86.318	-3.700		
36	C12	☐		10	HPRT	STANDARD	FAM-NFQ-MGB	30.683	30.790	0.167	10.000			86.318	-3.700		
				100 - FAS - STANDARD - 100.0													
7	A7	☐		100	FAS	STANDARD	FAM-NFQ-MGB	33.287	33.154	0.160	100.000			85.379	-3.730		
8	A8	☐		100	FAS	STANDARD	FAM-NFQ-MGB	33.197	33.154	0.160	100.000			85.379	-3.730		
9	A9	☐		100	FAS	STANDARD	FAM-NFQ-MGB	32.976	33.154	0.160	100.000			85.379	-3.730		
				100 - HPRT - STANDARD - 100.0													
31	C7	☐		100	HPRT	STANDARD	FAM-NFQ-MGB	27.654	27.593	0.083	100.000			86.318	-3.700		
32	C8	☐		100	HPRT	STANDARD	FAM-NFQ-MGB	27.625	27.593	0.083	100.000			86.318	-3.700		
33	C9	☐		100	HPRT	STANDARD	FAM-NFQ-MGB	27.498	27.593	0.083	100.000			86.318	-3.700		
				1000 - FAS - STANDARD - 1000.0													
4	A4	☐		1000	FAS	STANDARD	FAM-NFQ-MGB	29.403	29.296	0.093	1,000.000			85.379	-3.730		

Tips for analyzing your own experiments

When you analyze your own Relative Standard Curve experiment, look for the following items:

- **Slope/amplification efficiency values**—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. The following factors affect amplification efficiency:
  - Range of standard quantities—For more 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 more accurate efficiency measurements, include replicates to decrease the effects of pipetting inaccuracies.
  - PCR inhibitors—PCR inhibitors in the reaction can alter amplification efficiency.
- **R^2 values (correlation coefficient)**—The R^2 value is a measure of the closeness of fit between the regression line and the individual C_T data points of the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points. An R^2 value >0.99 is desirable.
- **C_T values**—The threshold cycle (C_T) is the PCR cycle number at which the fluorescence level meets the threshold.
  - A C_T value >8 and <35 is desirable.
  - A C_T value <8 indicates that there is too much template in the reaction.
  - A C_T value >35 indicates a low amount of target in the reaction. For C_T values >35, expect a higher standard deviation.

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

- Omit wells (see “Improve CT precision by omitting wells” on page 194).

  Or
- Rerun the experiment.

Assess amplification results using the amplification plot

Amplification plots available for viewing

The **Amplification Plot** screen displays amplification of all samples in the selected wells. There are three plots available:

- **ΔRn vs Cycle**—ΔRn is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. This plot displays ΔRn as a function of cycle number. Use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle**—Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays Rn as a function of cycle number. Use this plot to identify and examine irregular amplification.
- **C_T vs Well**—C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. Use this plot to locate outlying amplification (outliers).

Each plot can be viewed as a linear or log10 graph type.

Purpose

The purpose of viewing the amplification plot for the example experiment is to identify:

- Correct baseline and threshold values
- Outliers

View the amplification plot

1. In the **Experiment Menu** pane, click **Analysis** ▸ **Amplification Plot**.

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

2. Display the FAS wells in the **Amplification Plot** screen.
   a. Click the **Plate Layout** tab.

b. In the **Select Wells** dropdown list, select **Target ▸ FAS**.

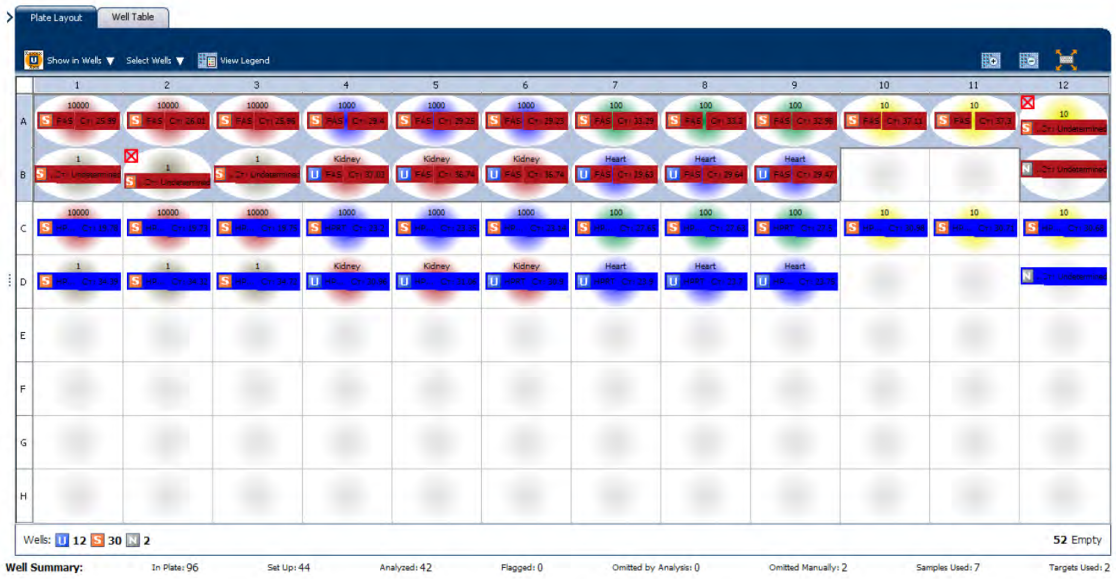


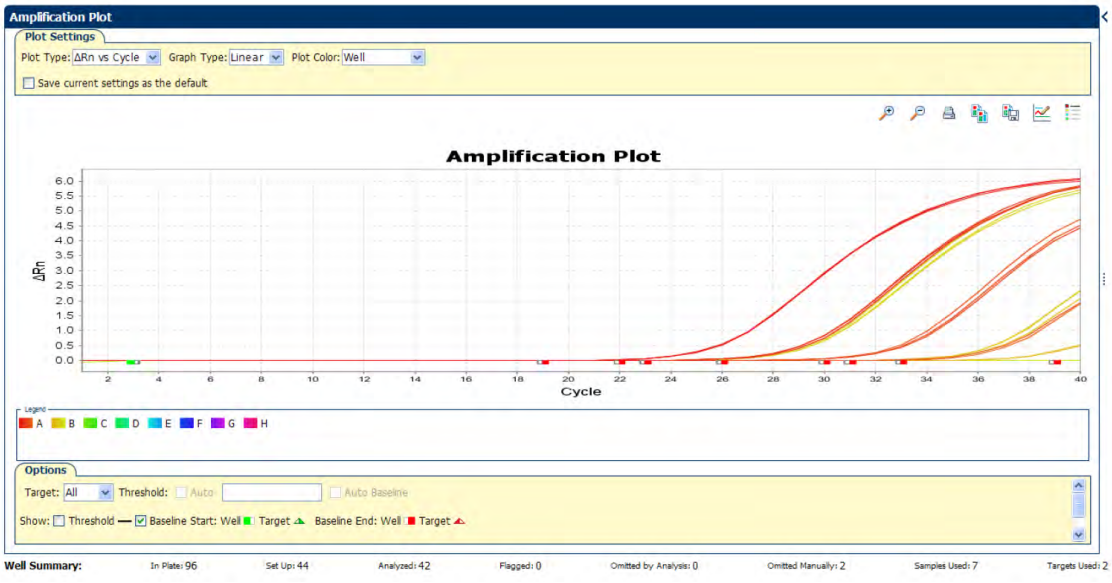
Figure 41 Plate Layout screen

3. In the Amplification Plot screen, select the following.

Item	Select
Plot Type dropdown list	**ΔRn vs Cycle**
Plot Color dropdown list	**Well** (default)
 (This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**).	Check (default)

4. View the baseline values.
   a. From the **Graph Type** dropdown list, select **Linear**.
   b. Select the **Baseline** checkbox to show the start cycle and end cycle.

c. Confirm that the baseline is set correctly. The end cycle should be set a few cycles before the cycle number where significant fluorescent signal is detected. In the example experiment, the baseline is set correctly.



5. View the threshold values.

Item	Select
Graph Type dropdown list	**Log**
Target dropdown list	**RNaseP**

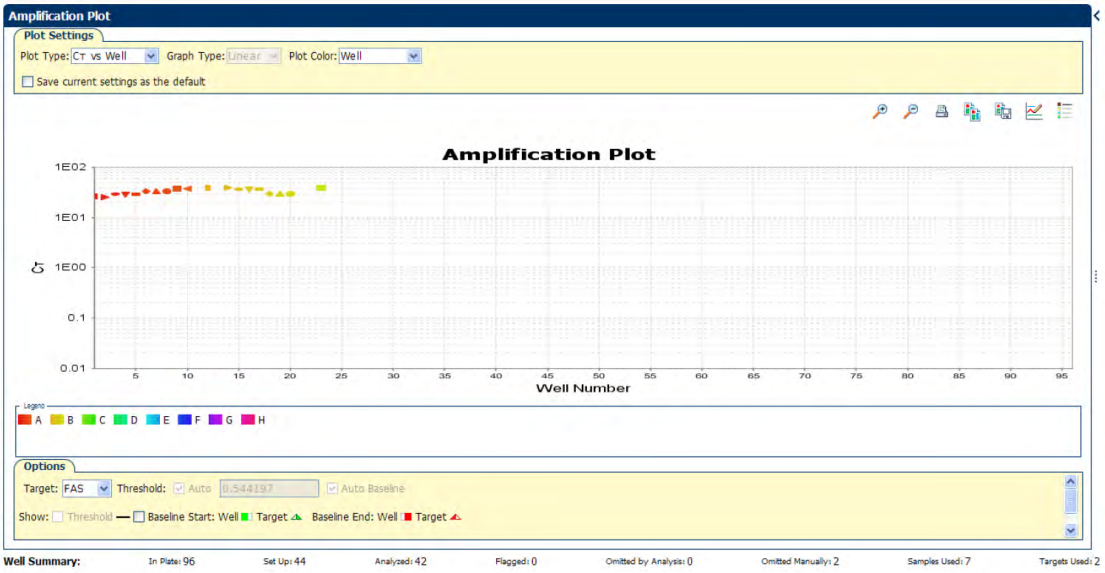
a. Select the **Threshold** check box to show the threshold.

b. Confirm that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.

6. Locate outliers.

a. In the **Plot Type** dropdown list, select **C_T vs. Well**.

b. Look for outliers from the amplification plot. In the example experiment, there are no outliers for FAS.



7. Repeat step 2 to step 6 for the HPRT wells.

Tips for analyzing your own experiments

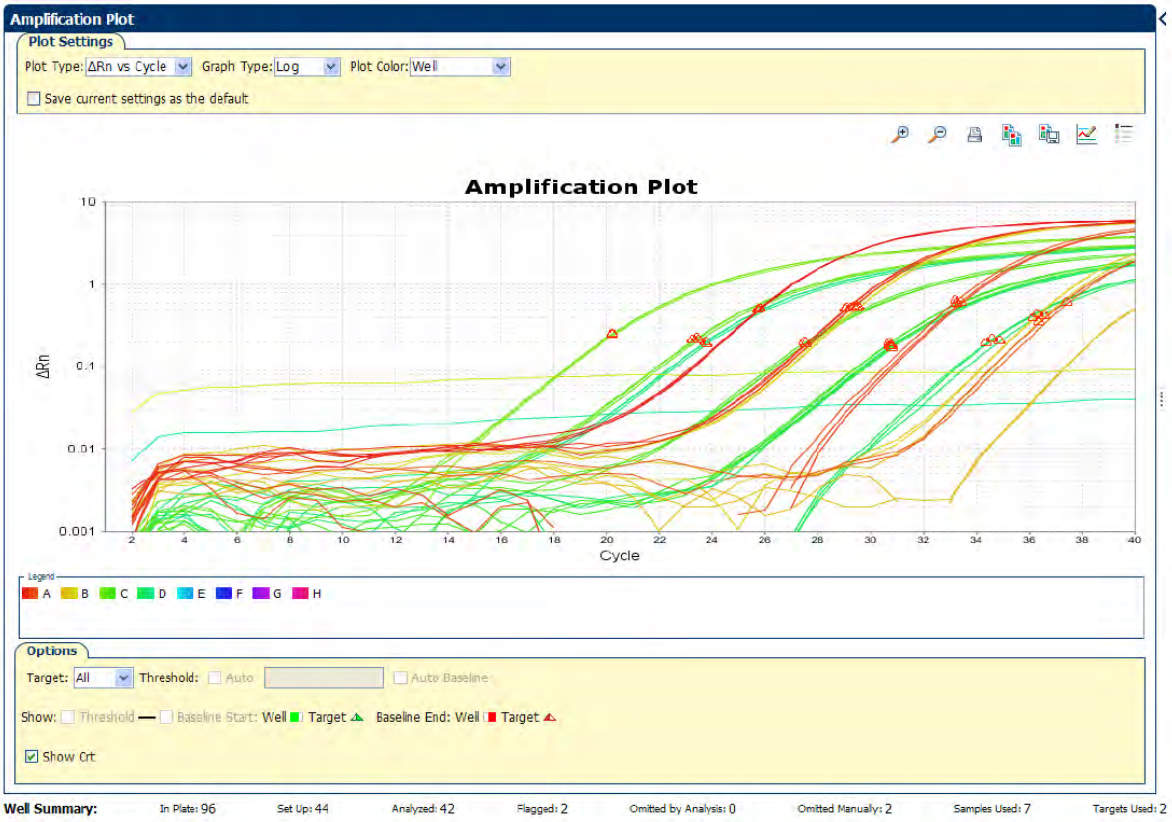
When you analyze your own Relative Standard Curve experiment, look for the following items:

- **Outliers**
- **A typical amplification plot**—The QuantStudio™ 12K Flex Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear phase
  - Exponential (geometric phase)
  - Baseline

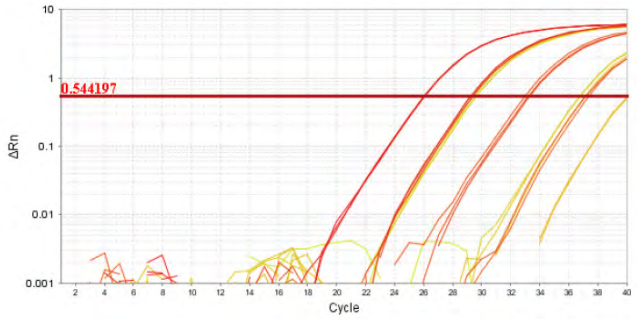
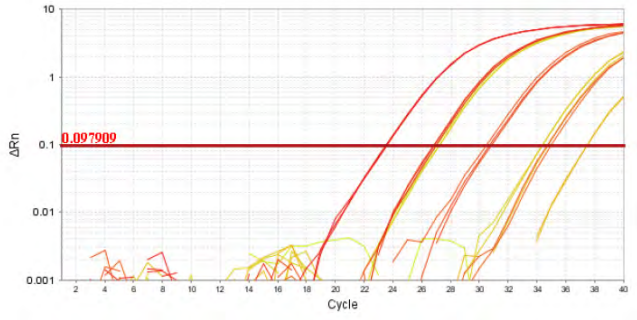
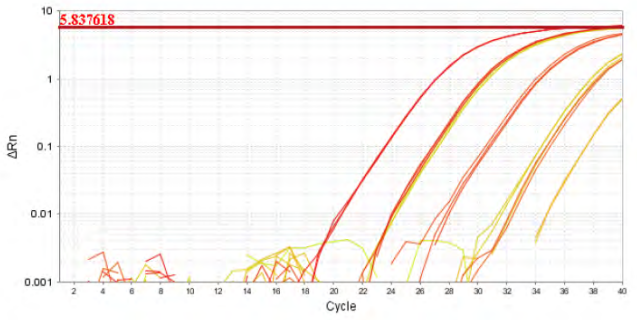
Figure 42 Typical amplification plot

IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudio™ 12K Flex Software. We recommend that you examine the **Amplification Plot** screen and review the assigned baseline and threshold values for each well after analysis completes.

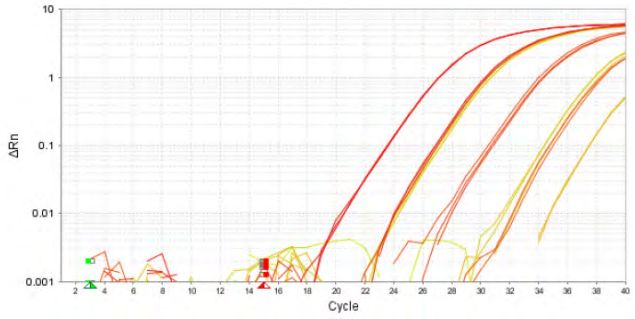
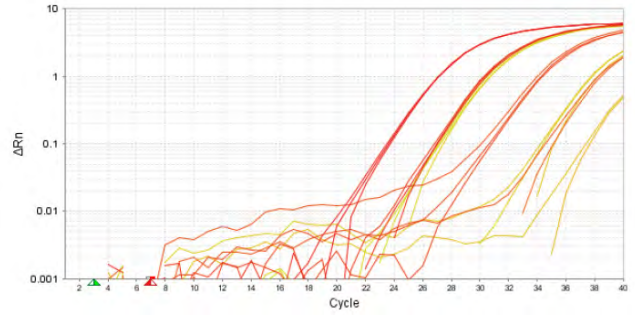
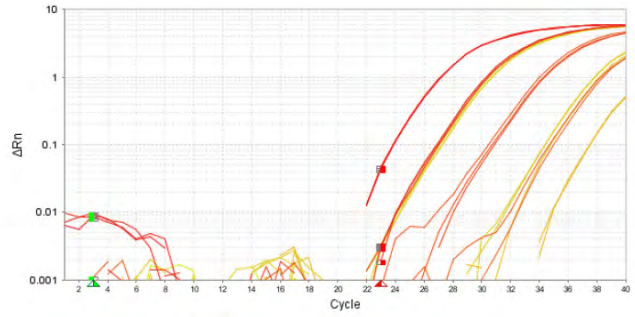
Note: If you use the Relative Threshold algorithm to analyze an experiment that includes amplification, select to view the analysis results using the ΔRn vs Cycle, Rn vs Cycle, or C_{RT} vs Well plot type and Linear or Log graph type. Also select the **Show Crt** checkbox to view the derived fractional cycle on the amplification plot.


Amplification Plot
Plot Settings
Plot Type: ΔRn vs Cycle
Graph Type: Log
Plot Color: Well
Save current settings as the default
Amplification Plot
ΔRn
Cycle
Legend
A B C D E F G H
Options
Target: All
Threshold:
Auto
Auto Baseline
Show: Threshold Baseline Start: Well Target Baseline End: Well Target
Show Crt
Well Summary:
In Plate: 96
Set Up: 44
Analyzed: 42
Flagged: 2
Omitted by Analysis: 0
Omitted Manually: 2
Samples Used: 7
Targets Used: 2

- **Correct threshold values**

Condition	Example
Threshold Set Correctly The threshold is set in the exponential phase of the amplification curve. Threshold settings above or below the optimum increase the standard deviation of the replicate groups.	
Threshold Set Too Low The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the exponential phase of the curve.	
Threshold Set Too High The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar down into the exponential phase of the curve.	

- **Correct baseline values**

Condition	Example
Baseline Set Correctly The amplification curve begins after the maximum baseline.	
Baseline Set Too Low The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.	
Baseline Set Too High The amplification curve begins before the maximum baseline. Decrease the End Cycle value.	

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

- Omit wells (see “Improve CT precision by omitting wells” on page 194).
  Or
- Manually adjust the baseline and/or threshold (see “Adjust analysis settings” on page 189).

Assess the gene expression profile using the gene expression plot

The Gene Expression Plot screen displays the results of relative quantification calculations in the gene expression profile. There are two plots available:

- **RQ vs Target**—Groups the relative quantification (RQ) values by target. Each sample is plotted for each target. You can view the plot as the linear, log10, Ln, and log2 graph types.

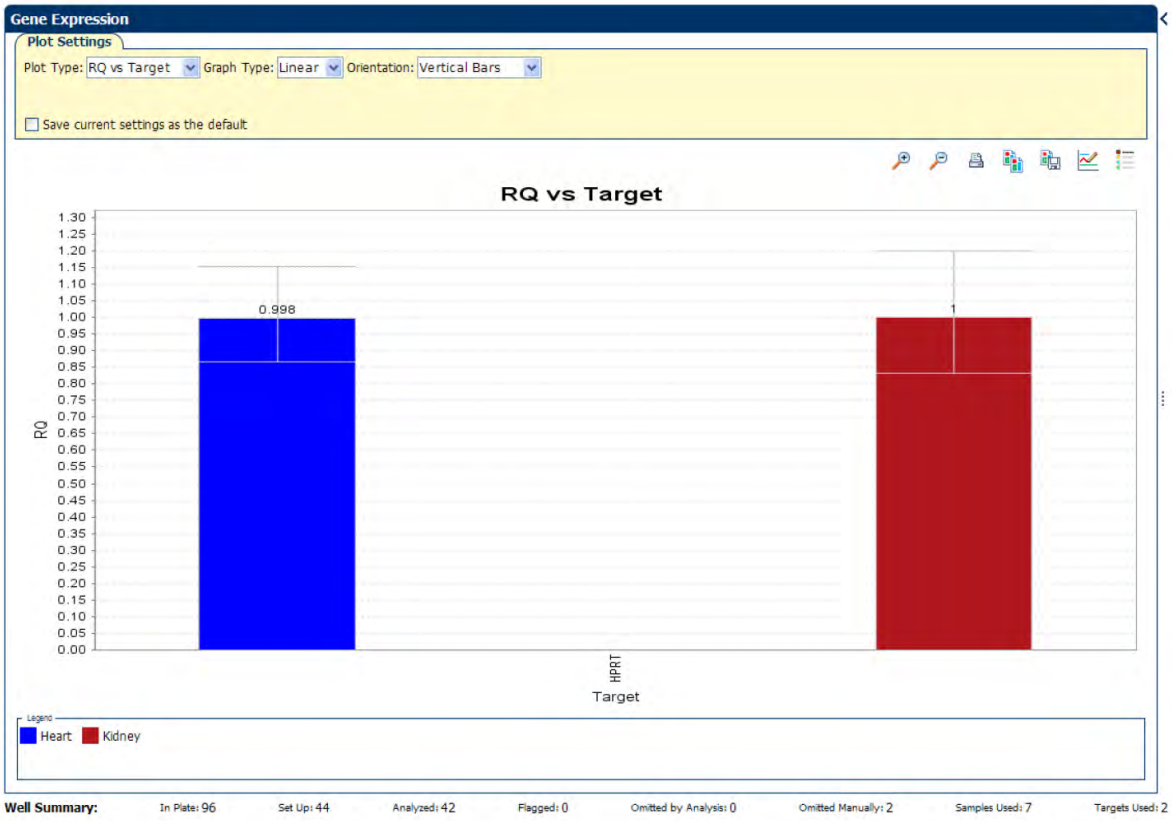


Figure 43 Gene Expression plot when viewed as a linear graph

- **RQ vs Sample**—Groups the relative quantification (RQ) values by sample. Each target is plotted for each sample. You can view the plot as the linear, log10, Ln, and log2 graph types.

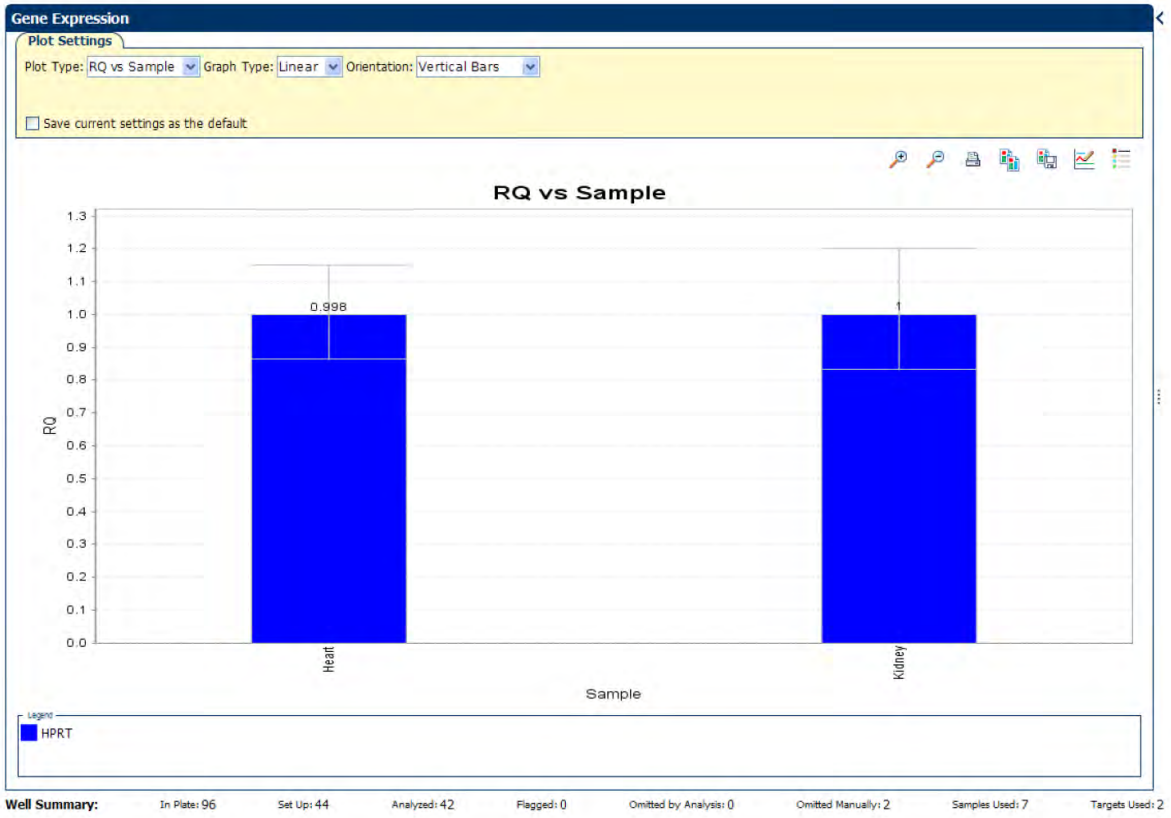


Figure 44 Gene Expression plot when viewed as a linear graph

Example experiment values

Review each target in the **Gene Expression Plot** screen for the expression level (or fold change) of the target sample relative to the reference sample.

View the gene expression plot

1. In the **Experiment Menu** pane, click **Analysis** ▸ **Gene Expression**.

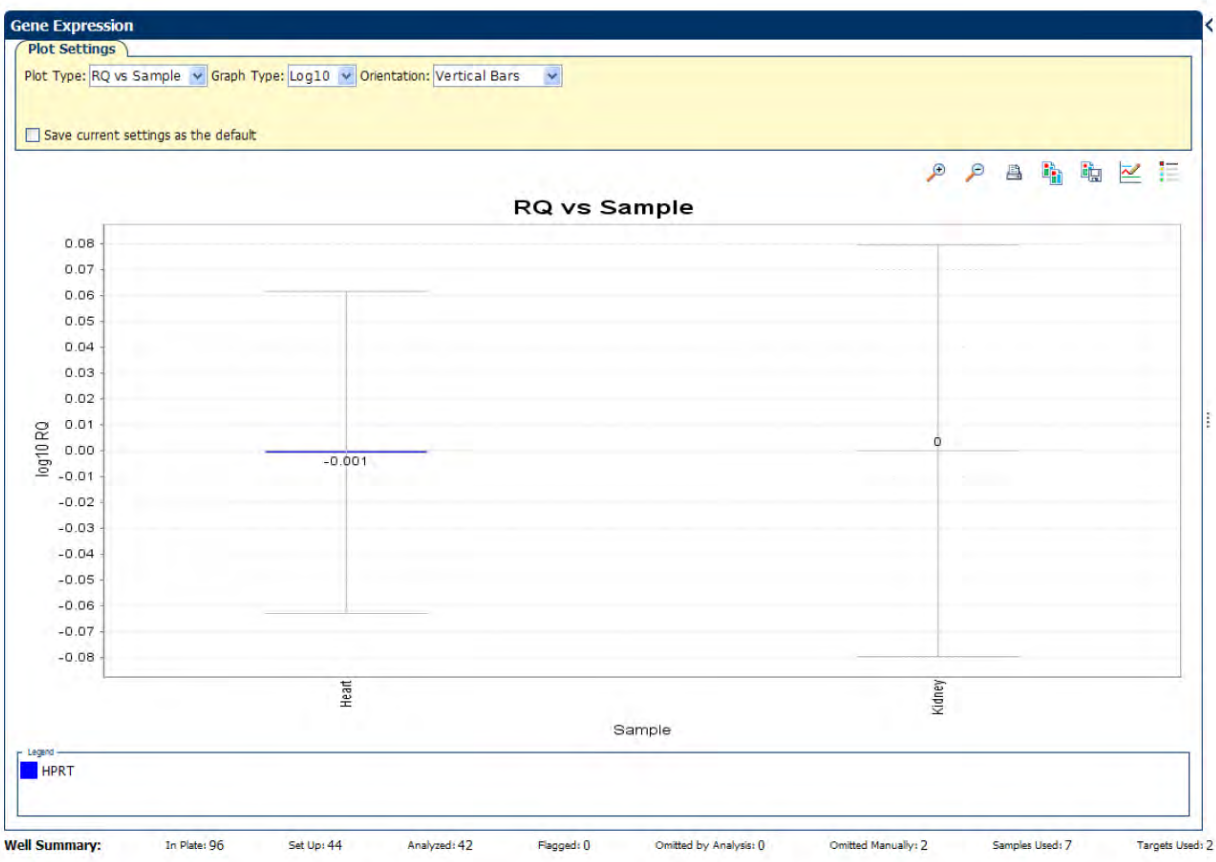
   Note: If no data are displayed, click **Analyze**.

2. Select the following items in the **Gene Expression Plot** screen.
   a. In the **Plot Type** dropdown list, select **RQ vs Sample**.
   b. In the **Graph Type** dropdown list, select **Log10**.
   c. In the **Orientation** dropdown list, select **Vertical Bars**.

3. Click **Show a legend for the plot** (default).

Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

In the example experiment, the expression level of HPRT in heart is displayed relative to its expression level in the reference sample (kidney). Since the reference sample is compared to itself, the relative expression level is 1. When the result is displayed in the Log10 graph type, the expression level of the reference sample appears as 0 in the graph (log10 of 1 = 0).



Assessing the gene expression plot in your own experiments

When you analyze your own Relative Standard Curve experiment, look for differences in gene expression (as a fold change) relative to the reference sample.

Identify well problems using the well table

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

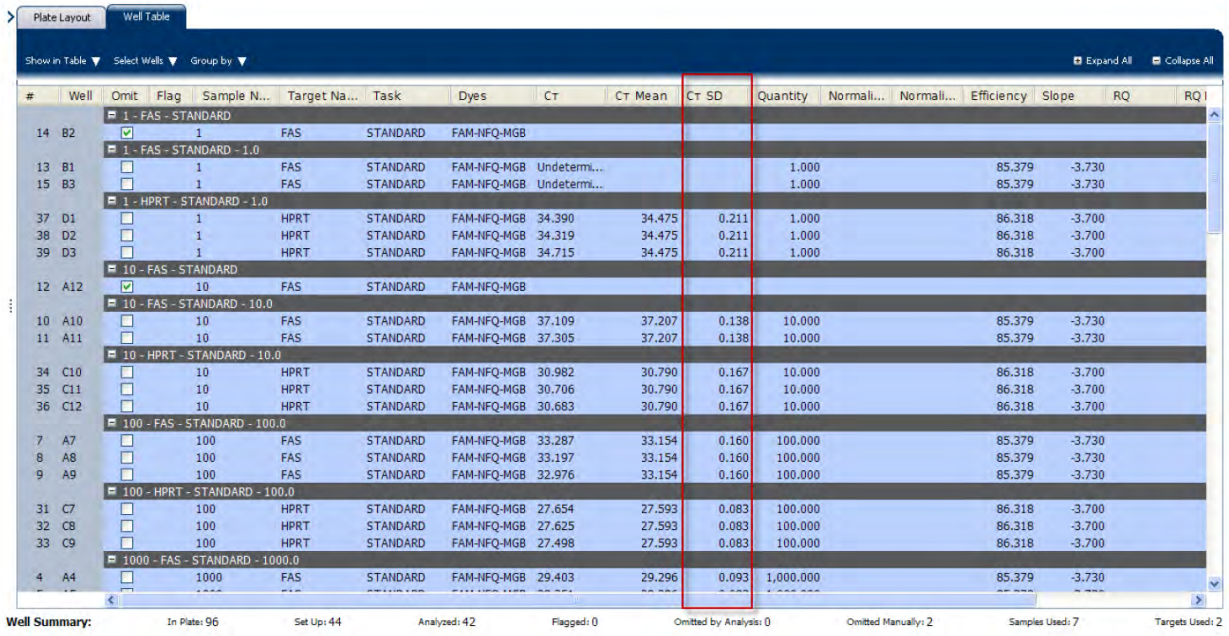
- The sample name, target name, task, and dyes
- The calculated threshold cycle (C_T), normalized fluorescence (Rn), and quantity values
- Flags

Example experiment values and flags

Review the Well Table to evaluate the C_T precision of the replicate groups.

View the well table

1. In the **Experiment Menu** pane, select **Analysis ▸ Amplification Plot,** then click the **Well Table** tab.
2. In the **Group By** dropdown list, select **Replicate**.
3. Look at the C_T SD column to evaluate the C_T precision of the replicate groups.
   In the example experiment, the C_T SD have the expected value of < 0.5.



#	Well	Omit	Flag	Sample N...	Target Na...	Task	Dyes	CT	CT Mean	CT SD	Quantity	Normali...	Normali...	Efficiency	Slope	RQ	RQ I
				1 - FAS - STANDARD													
14	B2	☑	1		FAS	STANDARD	FAM-NFQ-MGB										
				1 - FAS - STANDARD - 1.0													
13	B1	☐	1		FAS	STANDARD	FAM-NFQ-MGB	Undetermi...			1.000			85.379	-3.730		
15	B3	☐	1		FAS	STANDARD	FAM-NFQ-MGB	Undetermi...			1.000			85.379	-3.730		
				1 - HPRT - STANDARD - 1.0													
37	D1	☐	1		HPRT	STANDARD	FAM-NFQ-MGB	34.390	34.475	0.211	1.000			86.318	-3.700		
38	D2	☐	1		HPRT	STANDARD	FAM-NFQ-MGB	34.319	34.475	0.211	1.000			86.318	-3.700		
39	D3	☐	1		HPRT	STANDARD	FAM-NFQ-MGB	34.715	34.475	0.211	1.000			86.318	-3.700		
				10 - FAS - STANDARD													
12	A12	☑	10		FAS	STANDARD	FAM-NFQ-MGB										
				10 - FAS - STANDARD - 10.0													
10	A10	☐	10		FAS	STANDARD	FAM-NFQ-MGB	37.109	37.207	0.138	10.000			85.379	-3.730		
11	A11	☐	10		FAS	STANDARD	FAM-NFQ-MGB	37.305	37.207	0.138	10.000			85.379	-3.730		
				10 - HPRT - STANDARD - 10.0													
34	C10	☐	10		HPRT	STANDARD	FAM-NFQ-MGB	30.982	30.790	0.167	10.000			86.318	-3.700		
35	C11	☐	10		HPRT	STANDARD	FAM-NFQ-MGB	30.706	30.790	0.167	10.000			86.318	-3.700		
36	C12	☐	10		HPRT	STANDARD	FAM-NFQ-MGB	30.683	30.790	0.167	10.000			86.318	-3.700		
				100 - FAS - STANDARD - 100.0													
7	A7	☐	100		FAS	STANDARD	FAM-NFQ-MGB	33.287	33.154	0.160	100.000			85.379	-3.730		
8	A8	☐	100		FAS	STANDARD	FAM-NFQ-MGB	33.197	33.154	0.160	100.000			85.379	-3.730		
9	A9	☐	100		FAS	STANDARD	FAM-NFQ-MGB	32.976	33.154	0.160	100.000			85.379	-3.730		
				100 - HPRT - STANDARD - 100.0													
31	C7	☐	100		HPRT	STANDARD	FAM-NFQ-MGB	27.654	27.593	0.083	100.000			86.318	-3.700		
32	C8	☐	100		HPRT	STANDARD	FAM-NFQ-MGB	27.625	27.593	0.083	100.000			86.318	-3.700		
33	C9	☐	100		HPRT	STANDARD	FAM-NFQ-MGB	27.498	27.593	0.083	100.000			86.318	-3.700		
				1000 - FAS - STANDARD - 1000.0													
4	A4	☐	1000		FAS	STANDARD	FAM-NFQ-MGB	29.403	29.296	0.093	1,000.000			85.379	-3.730		

Note: To show or hide columns in the Well Table, select or deselect respectively the column name from the **Show in Table** dropdown list.

Assess the well table in your own experiments

When you analyze your own Relative Standard Curve experiment, look for standard deviation in the replicate groups (C_T SD values). If needed, omit outliers (“Improve CT precision by omitting wells” on page 194).

Confirm accurate dye signal using the multicomponent plot

The **Multicomponent Plot** screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the Relative Standard Curve example experiment, you review the Multicomponent Plot screen for the following items:

- ROX™ dye (passive reference)
- FAM™ dye (reporter for RNase P)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

View the multicomponent plot

1. In the **Experiment Menu** pane, select **Analysis** ▸ **Multicomponent Plot.**

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

2. Display the unknown and standard wells one at a time in the **Multicomponent Plot** screen.
   a. Click the **Plate Layout** tab.
   b. Select one well in the plate layout. The well is shown in the Multicomponent Plot.

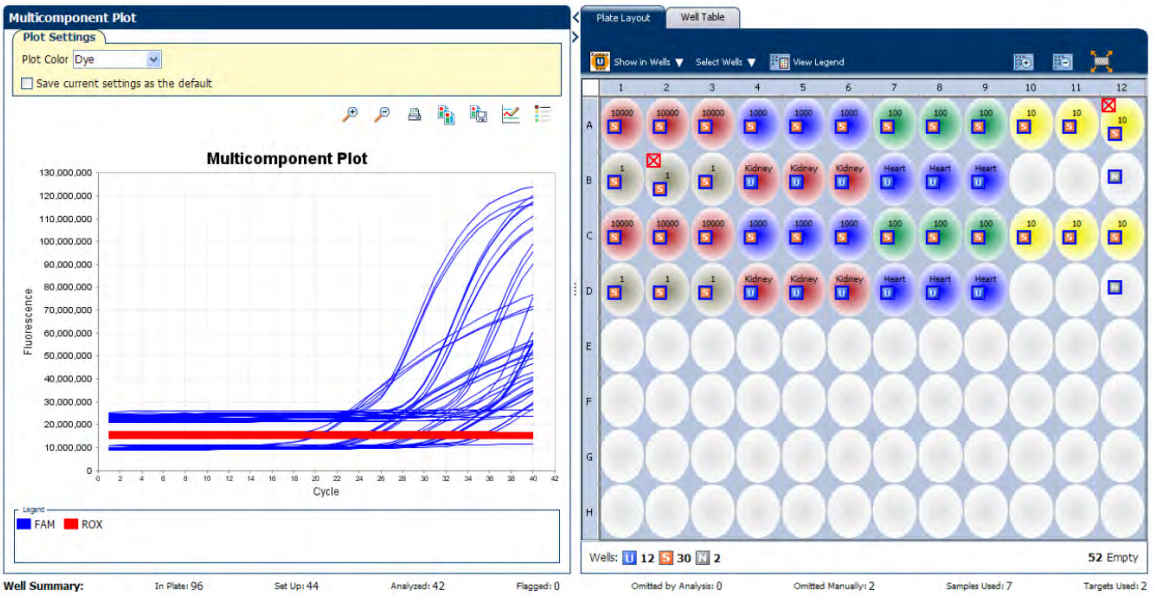
      Note: If you select multiple wells, the Multicomponent Plot displays the data for all selected wells simultaneously.

3. In the **Plot Color** dropdown list, select **Dye**.
4. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

5. Check the FAM™ dye signal.

   In the example experiment, the FAM™ dye signal increases throughout the PCR process, indicating normal amplification.

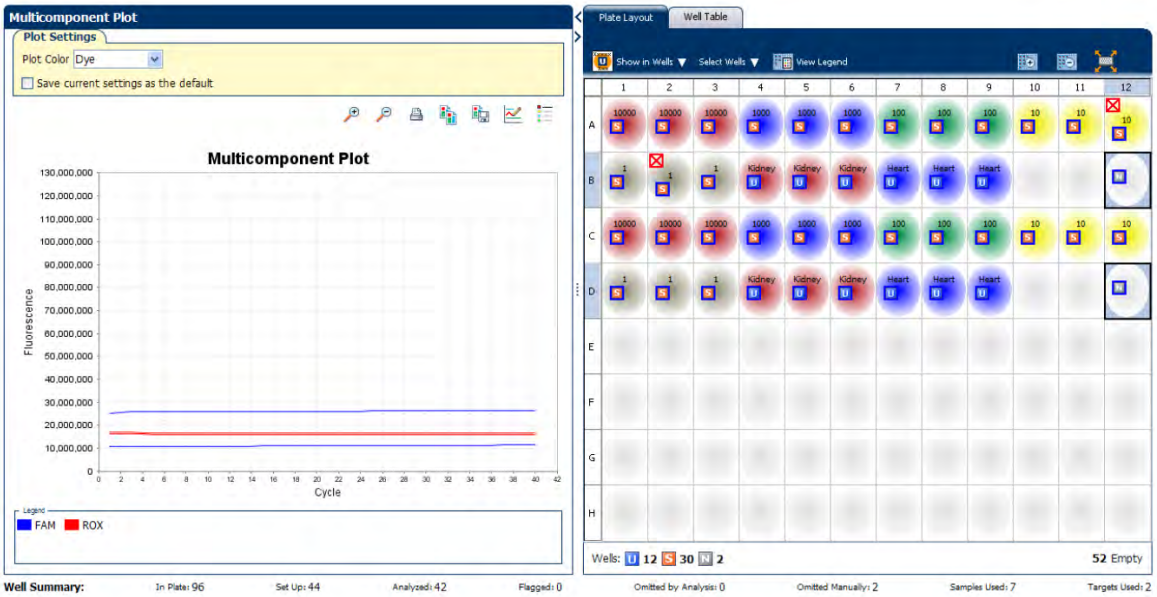


6. Check the ROX™ dye signal.

   In the example experiment, the ROX™ dye signal remains constant throughout the PCR process indicating typical data.

7. Select the negative control wells one at time and check for amplification.

   In the example experiment, there is no amplification in any of the negative control wells.



Tips for confirming dye accuracy in your own experiment

When you analyze your own Relative Standard Curve experiment, look for the following items:

- **Passive reference**—The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye**—The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- **Irregularities in the signal**—There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative Control wells**—There should not be any amplification in the negative control wells.

Determine signal accuracy using the raw data plot

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

About the example experiment

In the Relative Standard Curve example experiment, you review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the raw data plot

1. In the **Experiment Menu** pane, select **Analysis** ▸ **Raw Data Plot**.

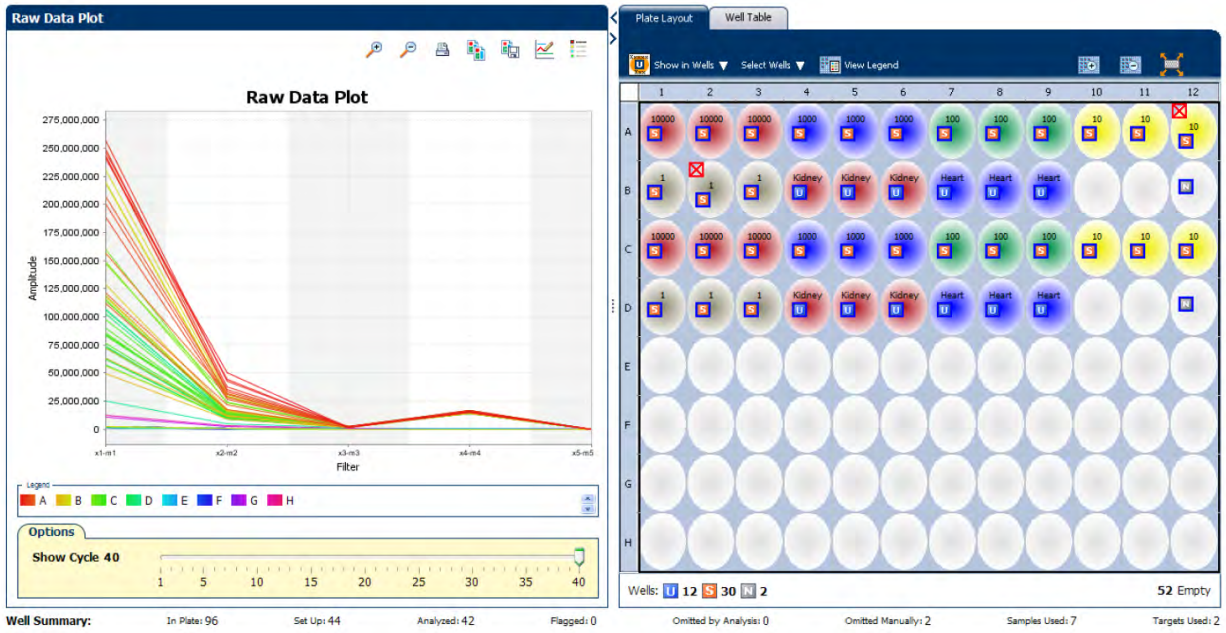
   Note: If no data are displayed, click **Analyze**.

2. Display all 384 wells in the **Raw Data Plot** screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.

3. Click **Show a legend for the plot** (default).

   The legend displays the color code for each row of the reaction plate (see the legend in the Raw Data Plot shown below).

4. Click and drag the Show Cycle pointer from cycle 1 to cycle 40. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.



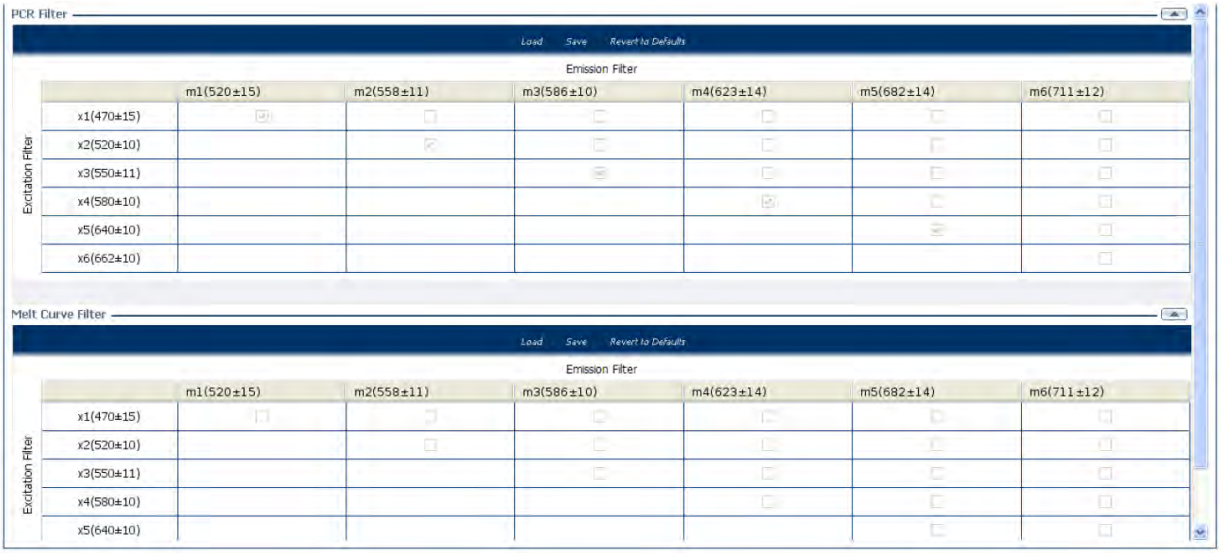


Figure 45 Filters used for the example experiment

Tips for determining signal accuracy in your own experiment

When you analyze your own Relative Standard Curve experiment, look for the following item in each filter:

- Characteristic signal growth
- No abrupt changes or dips

View the endogenous control profile using the QC plot

In the Relative Standard Curve experiment, the **QC Plot** screen displays the Endogenous Control Profile plot for all the targets in the experiment. The QC Plot serves as a tool to help users choose the best endogenous control for that experiment. The endogenous control profile plot is a visual display of the C_T values of the endogenous control across each sample. You can view up to four endogenous controls at a time. The sample is plotted on the X-axis, and the C_T is plotted on the Y-axis. Each candidate control is viewed as a color and shape combination in the plot. Endogenous controls are also known as reference genes.

1. In the **Experiment Menu** pane, select **Analysis** ▸ **QC Plot**.

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

2. In the **QC Plot** screen, click **Target Table** to select a target to profile.
   a. In the **Candidate Control** column, select the check box of the target of the endogenous control profile to plot.

      In the example experiment, the endogenous control is FAS.

   b. Select a color from the **Color** dropdown list.

   c. Select a shape from the **Shape** dropdown list.

3. Click the **View Replicate Results Table** tab.

4. Select the checkbox associated with the samples you want to plot.

5. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

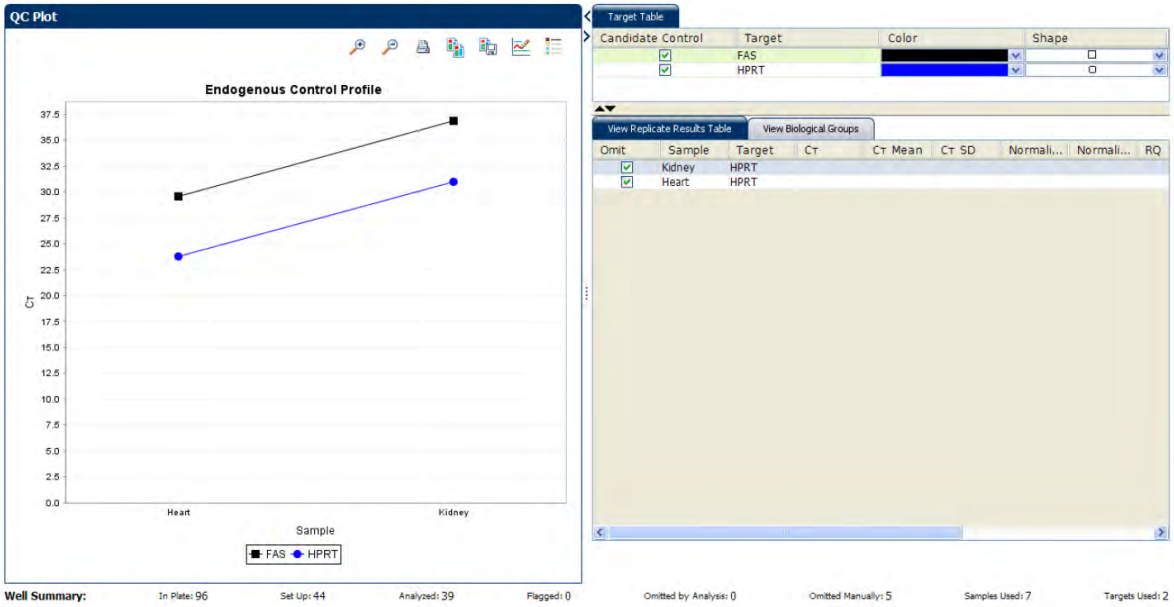


Figure 46 QC Plot in the Relative Standard Curve example experiment
This example experiment does not define Biological Groups.

Review the QC flags in the QC summary

The **QC Summary** screen displays a list of the QuantStudio™ 12K Flex Software flags, including the flag frequency and location for the open experiment.

View the QC summary

1. In the **Experiment Menu** pane, select **Analysis** ▸ **QC Summary**.

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

2. Review the Flags Summary.

   Note: A 0 displayed in the **Frequency** column indicates that the flag does not appear in the experiment. If the frequency is greater than 0, the flag appears somewhere in the experiment. The well position is listed in the **Wells** column.

   In the example experiment, there are no flagged wells.

3. In the **Flag Details** table, click each flag with a frequency greater than 0 to display detailed information about the flag. In the example experiment, the **Frequency** column displays **0** for all flags.

4. *(Optional)* For those flags with frequency greater than 0, click the troubleshooting link to view information about correcting the flag.

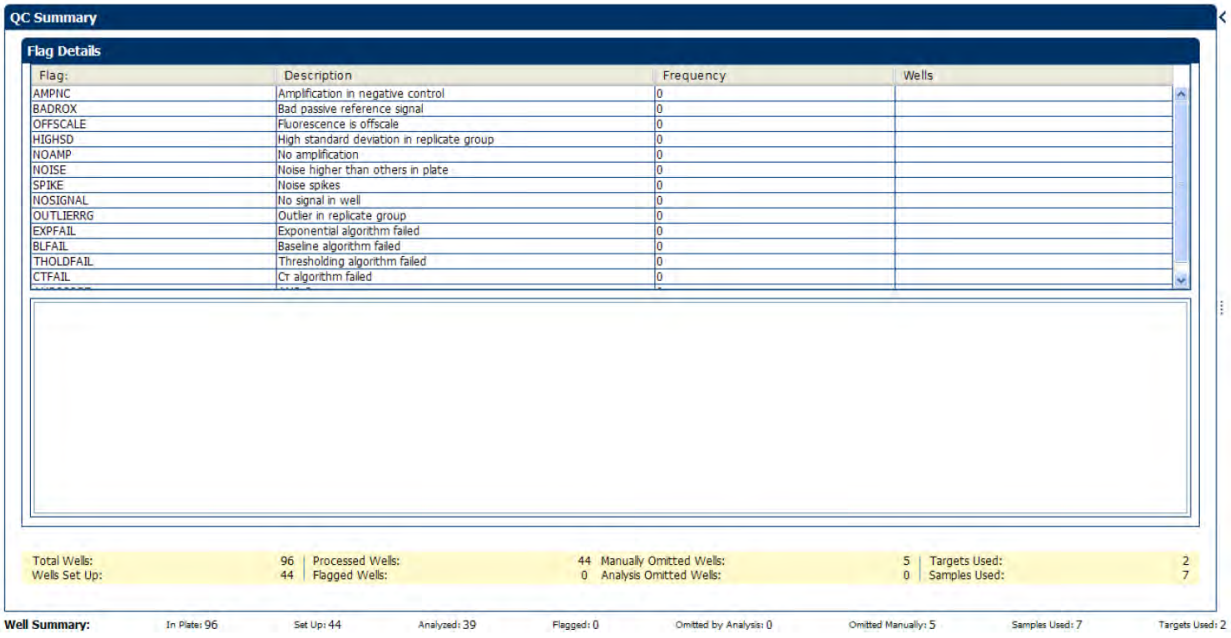


Figure 47 QC Summary screen for the example experiment

Possible flags

The flags listed below may be triggered by the experiment data.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed

(continued)

Flag	Description
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
AMPSCORE	Amplification in the linear region is below a certain threshold, corresponding to the score set in the analysis settings
Secondary analysis flags	
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in the negative control
HIGHSD	High standard deviation in replicate group

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the **Frequency** column for each flag).

More information

Information	Reference
Publishing data	Chapter 1, "General information and instructions"

Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

The **Analysis Settings** dialog box displays the analysis settings for the threshold cycle (C_T), flags, and advanced options.

If the default analysis settings in the QuantStudio™ 12K Flex Software are not suitable for your own experiment, you can change the settings in the **Analysis Settings** dialog box, then reanalyze your experiment.

View the analysis settings

1. In the **Experiment Menu** pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the **Analysis Settings** dialog box.
   In the example experiment, the default analysis settings are used for each tab:
   - C_T Settings
   - Flag Settings
   - Relative Quantification Settings
   - Advanced Settings
   - Standard Curve Settings

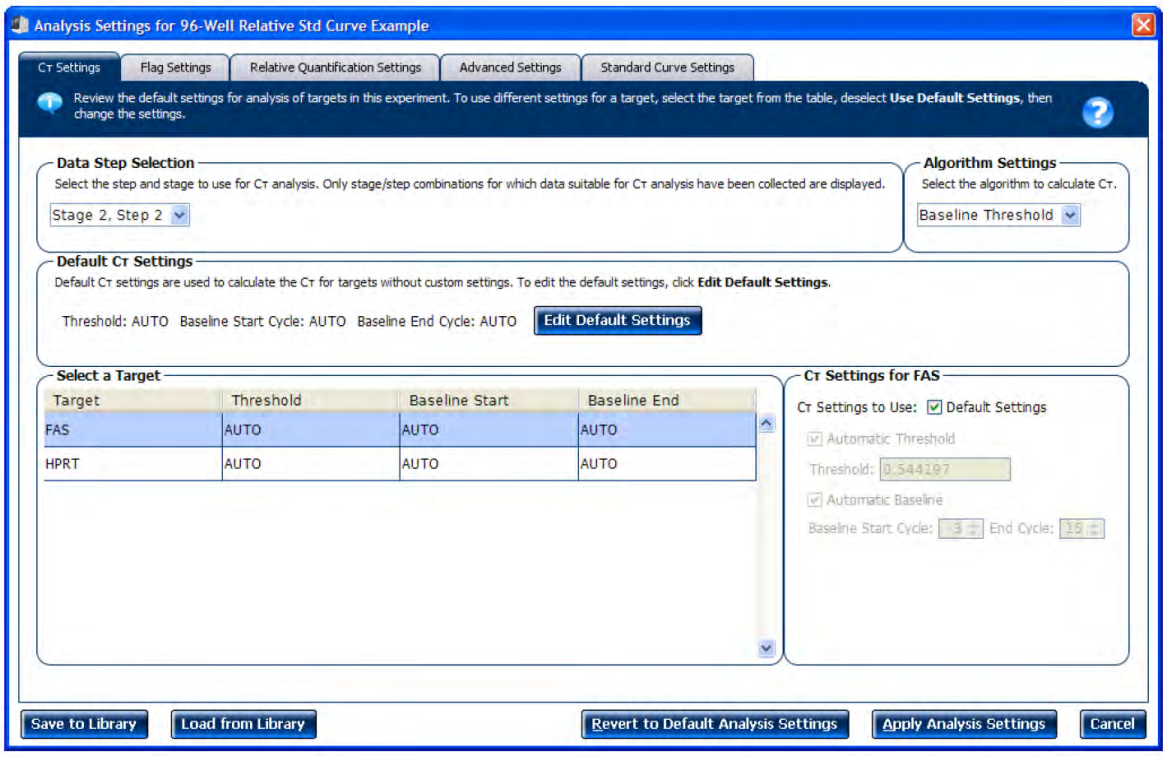


Figure 48 Analysis Settings dialog box for a Relative Standard Curve experiment

3. View and, if necessary, change the analysis settings (see “Adjust analysis settings” on page 191).

Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see “About the analysis settings library” on page 62.

4. Click **Apply Analysis Settings** to apply the current analysis settings.

Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

C_T settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method.

- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

  The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for quantification.

  The Relative Threshold algorithm is a well-based analysis based on the PCR reaction efficiency and fitted to the Amplification curve. This setting is ideal for a single sample across genes with no dependence on targets, thereby reducing variability. It is not necessary to set either a baseline or a threshold when you use the Relative Threshold algorithm, so any settings for baseline or threshold will not affect the analysis.

- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the targets that do not have custom settings. To edit the default settings, click **Edit Default Settings**.

- **C_T Settings for Target**

  When you manually set the threshold and baseline, we recommend the following settings:

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

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag settings

Use the **Flag Settings** tab to perform the following tasks:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the QuantStudio™ 12K Flex Software.

1. In the **Use** column, select the check boxes for flags to apply during analysis.

2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

3. In the **Reject Well** column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations might change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.

4. Click **Apply Analysis Settings** in the **Analysis Settings** dialog box.

   If the run status is complete, the data are reanalyzed.

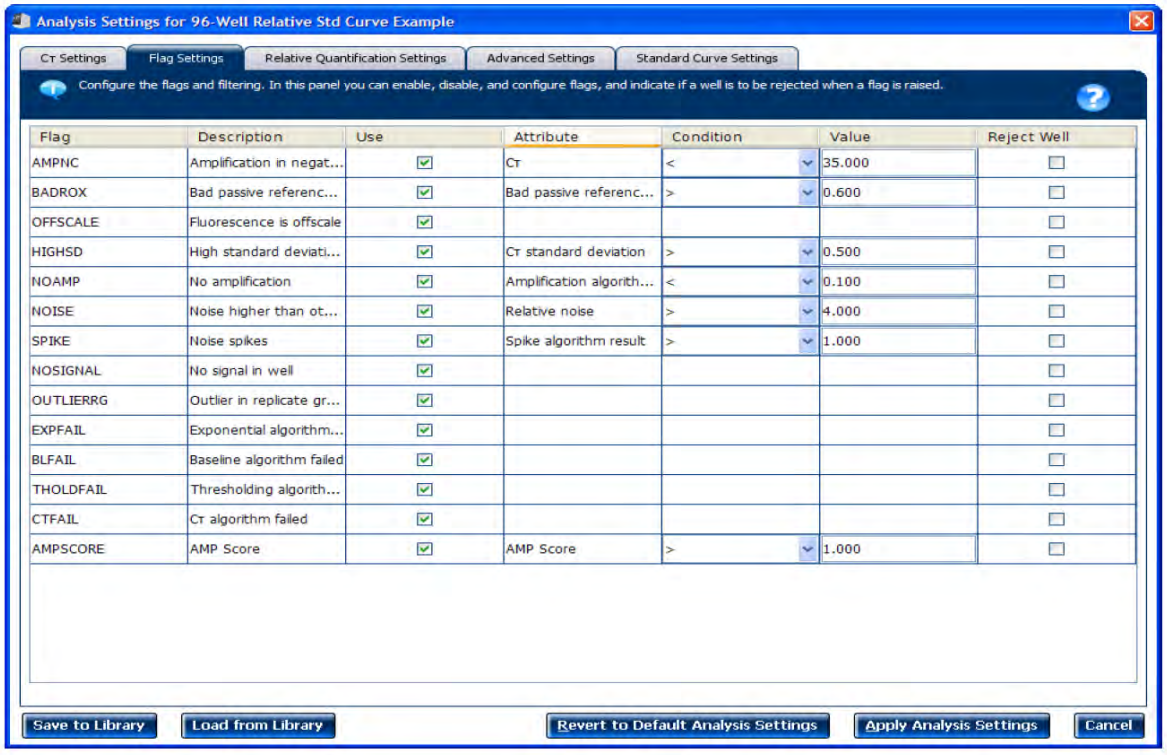


Figure 49 Flag Settings tab

Relative quantification settings

Use the Relative Quantification Settings tab to perform the following tasks:

- Change the type of analysis, singleplex or multiplex.
- Change the reference sample and/or endogenous control.
- Reject Outliers with ΔC_T values less than or equal to the entered value.

  Note: The Outlier Rejection settings apply only to multiplex reactions.

- Select the algorithm to use to determine 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. Select the confidence level to use.
  - **Standard Deviations**—Select to calculate the RQ minimum and maximum values based on the selected number of standard deviations. Select the number of standard deviations to use.

Advanced settings

Use the **Advanced Settings** tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold setting.

The following steps describe the use of custom baseline settings for a well-target combination.

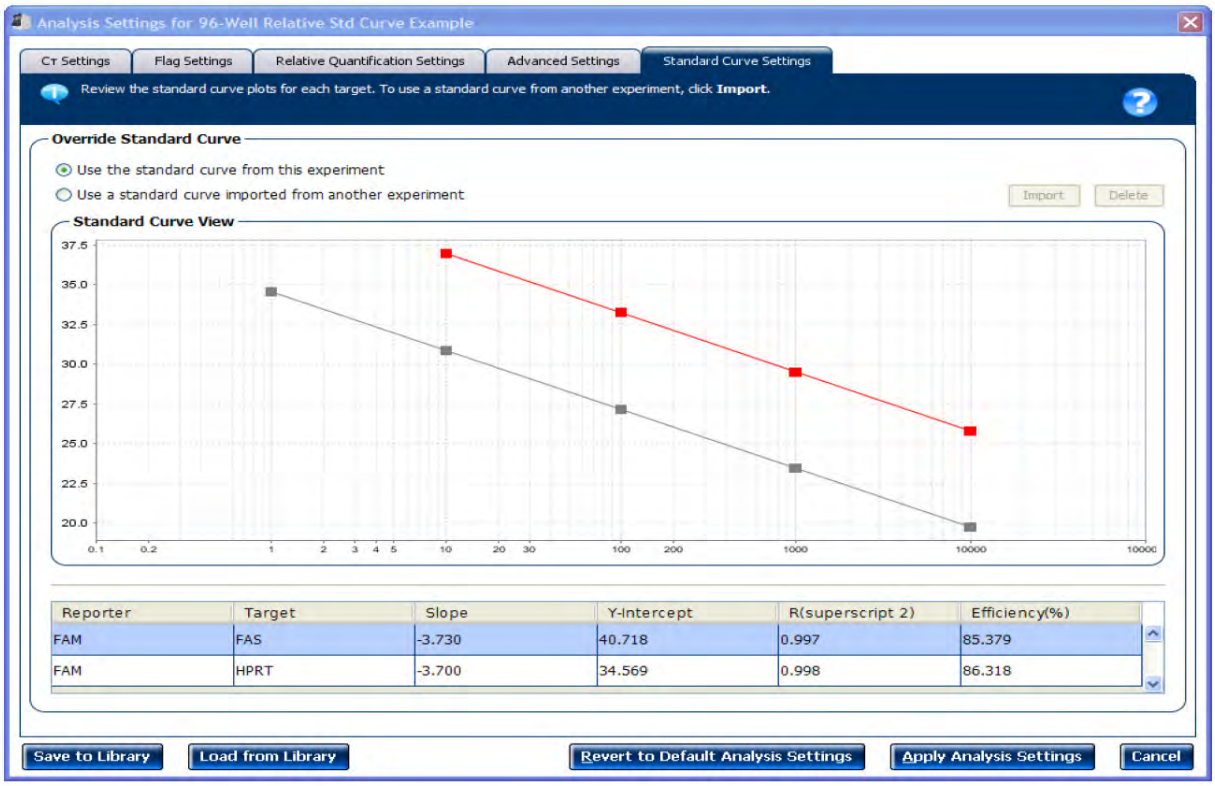
1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** checkbox.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** checkbox.
   - To define the baseline, deselect the **Automatic Baseline** checkbox, then enter the baseline start cycle and baseline end cycle.

Standard curve settings

Use this tab to review the settings of the current standard curve experiment or to import the standard curve from an external experiment (with the same samples and targets) and apply it to this current experiment.

Note: The run method must be the same. We recommend using the standard curve from the current experiment.

For the example experiment, the settings from the current experiment have been used.



Improve C_T precision by omitting wells

Experimental error may cause some wells to be amplified insufficiently or not at all. These wells typically produce C_T values that differ significantly from the average for the associated replicate wells. If included in the calculations, these outliers can result in erroneous measurements. To support C_T precision, omit the outliers from the analysis.

Note: In the Relative Standard Curve example experiment, there are no outliers. No wells need to be removed from analysis.

1. In the **Experiment Menu** pane, select **Analysis** ▸ **Amplification Plot**.

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

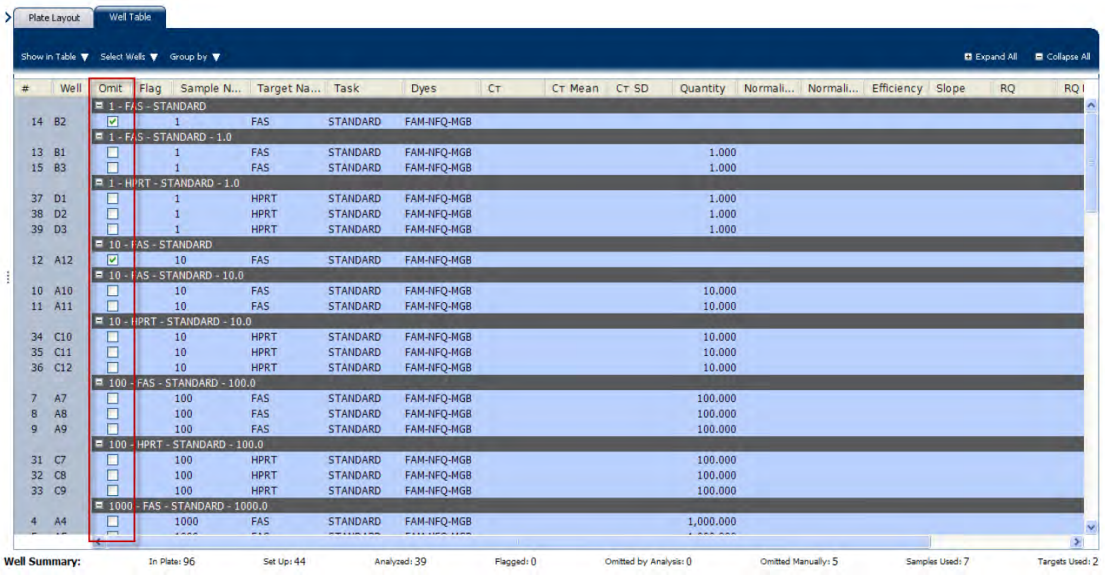
2. In the **Amplification Plot** screen, select **C_T vs Well** from the **Plot Type** dropdown list.

3. Select the **Well Table** tab, select replicates to omit:

4. In the Well Table, view the outliers:
   a. From the **Group By** dropdown list, select **Replicate**.

   b. Look for outliers in the replicate group (make sure they are flagged).

   c. Select the **Omit** checkbox next to outlying well or outlying wells, as shown below.



5. Click **Analyze** to reanalyze the experiment data with the outlying well or outlying wells removed from the analysis.

Note: You can also omit undesirable wells in an experiment from the **Plate Layout** screen. To omit a well from the **Plate Layout** screen, right-click the well, then click **Omit**.

---

14 Export analysis results

1. Open a Relative Standard Curve example experiment file that has been analyzed.

   For information about analysis, see Chapter 13, “Review results and adjust experiment parameters”.

2. In the **Experiment Menu** pane, click **Export**.

   Note: To export data automatically after analysis, select the **Auto Export** checkbox during experiment setup or before running the experiment. The **Auto Export** checkbox is unchecked for the example experiment.

3. In the **Format** dropdown list, select **QuantStudio 12K Flex format**.

   Note: In the **Format** dropdown list, select **7900** if you want to export the Clipped Data.

4. Complete the **Export** dialog box as shown below.

Field or Selection	Entry
Select Data to export/Select Content	Results
Export Data To options	**One File** radio button
Export File Name field	***96-Well Relative Std Curve Example_QuantStudio_export***
File Type dropdown list	***.txt**
Export File Location field	Use the default file location or click **Browse** to select a different location

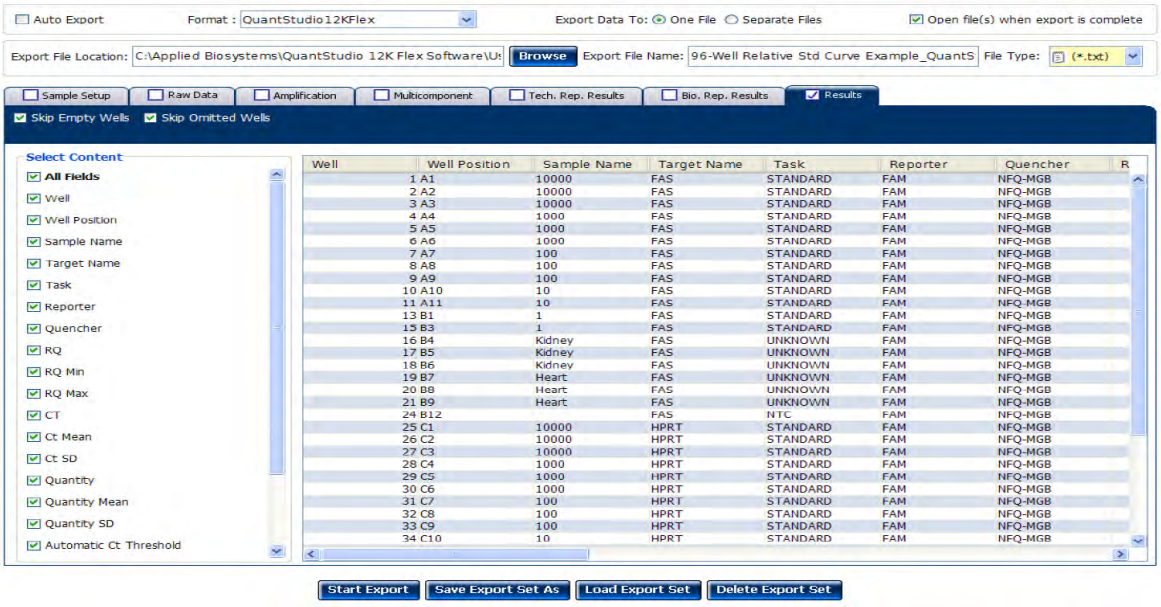


Figure 50 Export screen

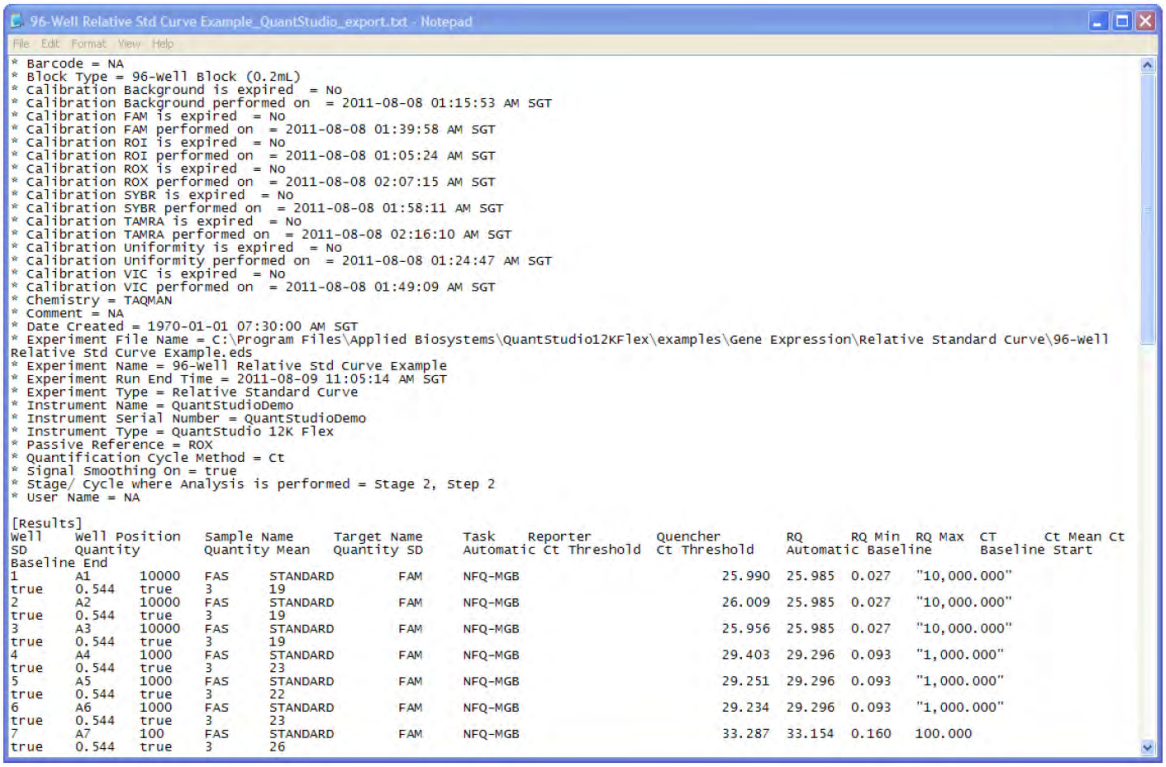


Figure 51 Exported file

Part IV

Running comparative C_t experiments

15 Overview of comparative C_T experiments

IMPORTANT! First-time users of the QuantStudio™ 12K Flex Real–Time PCR System, see the part "Getting started" on page 17 and Appendix A, "Documentation and support". The sections provide information and general instructions that are applicable to all the experiments described in this document.

Note: For more information about any of the topics discussed in this guide, access the Help from within the QuantStudio™ 12K Flex Software by pressing **F1,** clicking in the toolbar, or selecting **Help ▸ QuantStudio 12K Flex Software Help**.

Overview of comparative C_T experiments

The Comparative CT ($\Delta\Delta C_T$) method is used to determine the relative target quantity in samples. With the comparative C_T method, the QuantStudio™ 12K Flex Software measures amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the endogenous control. The software determines the relative quantity of target in each sample by comparing normalized C_T (ΔC_T) in each sample to normalized C_T (ΔC_T) in the reference sample.

Comparative C_T experiments are commonly used to perform the following tasks:

- Compare expression levels of a gene in different tissues
- Compare expression levels of a gene in a treated sample and an untreated sample
- Compare expression levels of wild-type alleles and mutated alleles
- Analyze the gene expression changes over time under specific treatment conditions

Assemble required components

- **Sample**—The tissue group that you are testing for a target gene.
- **Reference sample (also called a calibrator)**—The sample used as the basis for relative quantification results. For example, in a study of drug effects on gene expression, an untreated control is an appropriate reference sample.
- **Endogenous control**—A gene that is used to normalize template input differences, and sample-to-sample or run-to-run variation.
- **Replicates**—The total number of identical reactions containing identical components and identical volumes.
- **Negative Controls**—Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

PCR options

When performing real-time PCR, choose between:

- Singleplex PCR and multiplex PCR (see "Singleplex and multiplex PCR" on page 200)
  and
- 1-step RT-PCR and 2-step RT-PCR (see "1- and 2-step RT-PCR" on page 200
)

Singleplex and multiplex PCR

You can perform a PCR reaction using either:

- **Singleplex PCR**—In singleplex PCR a single primer and probe set is present in the reaction tube or well. Only one target or endogenous control can be amplified per reaction.
  Or
- **Multiplex PCR**—In multiplex PCR, two or more primer and probe sets are present in the reaction tube or well. Each set amplifies a specific target or endogenous control.
  One example is a probe that is labeled with FAM™ dye detects the target and a probe that is labeled with VIC™ dye detects the endogenous control.

IMPORTANT! SYBR™ Green reagents cannot be used for multiplex PCR.

1- and 2-step RT-PCR

You can perform reverse transcription (RT) and PCR in a single reaction (1-step) or in separate reactions (2-step). The reagent configuration you use depends on whether you are performing 1- or 2-step RT-PCR.

- **1-step RT-PCR**—In 1-step RT-PCR, RT and PCR take place in one buffer system. Using one buffer system provides the convenience of a single-tube preparation for RT and PCR amplification. However, you cannot use Fast PCR Master Mix or the carryover prevention enzyme, AmpErase™ UNG (uracil-N-glycosylase), to perform 1-step RT-PCR.
- **2-step RT-PCR**—2-step RT-PCR is performed in two separate reactions. First, total RNA is reverse-transcribed into cDNA, then the cDNA is amplified by PCR. This method is useful for detecting multiple transcripts from a single cDNA template or for storing cDNA aliquots for later use. The AmpErase™ UNG enzyme can be used to prevent carryover contamination.

About the example experiment

To illustrate how to perform comparative C_T experiment, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with the QuantStudio™ 12K Flex Real–Time PCR System.

The objective of the comparative C_T example experiment is to compare the expression of GH1, LPIN1, TGFB1, LIPC, ACTB, and CCKAR in liver, heart, brain, and lung tissues.

- The samples are liver, heart, lung, and brain tissues.
- The targets are GH1, LPIN1, TGFB1, LIPC, ACTB, and CCKAR.
- The reference sample is brain.
- The endogenous control is ACTB.
- The experiment is designed for singleplex PCR, where the targets and endogenous control assays are performed in separate wells.
- Reactions are set up for 2-step RT-PCR. The SuperScript™ VILO™ cDNA Synthesis Kit is used for reverse transcription. The TaqMan™ Fast Universal PCR Master Mix is used for PCR.
- Primer and probe sets are selected from the TaqMan™ Gene Expression Assay product line:
  - GH1 Assay Mix: Hs00236859_m1
  - LPIN1 Assay Mix: Hs00299515_m1
  - LIPC Assay Mix: Hs00165106_m1
  - ACTB Assay Mix: Hs99999903_m1
  - TGFB1 Assay Mix: Hs00998133_m1
  - CCKAR Assay Mix: Hs00167891_m1

16 Design the experiment

This chapter explains how to design the example experiment from the **Setup** menu in the **Experiment Menu** pane.

Note: To automatically export the analyzed data to a specified location, select the **Auto Export** checkbox in the **Export** screen, before running the experiment. For more information on Auto Export, see the part "Getting started" on page 17.

Define the experiment properties

In the **Experiment Menu** pane, click **Setup** ▸ **Experiment Properties**.

Enter or select the following information.

Field or Selection	Entry
Experiment Name field	Enter ***96-Well Fast Comparative CT Example.eds***.
Barcode field	Leave the **Barcode** field empty.
User Name field	Enter ***Example User*** or enter a user name.
Comments field	Enter ***Comparative CT example***.
Block	Select **96-Well (0.2 mL)**.
Experiment Type	Select **Comparative C_T ($\Delta\Delta C_T$)**.
Reagents	Select **TaqMan Reagents**.
Ramp speed	Select **Fast**.

Save the experiment.

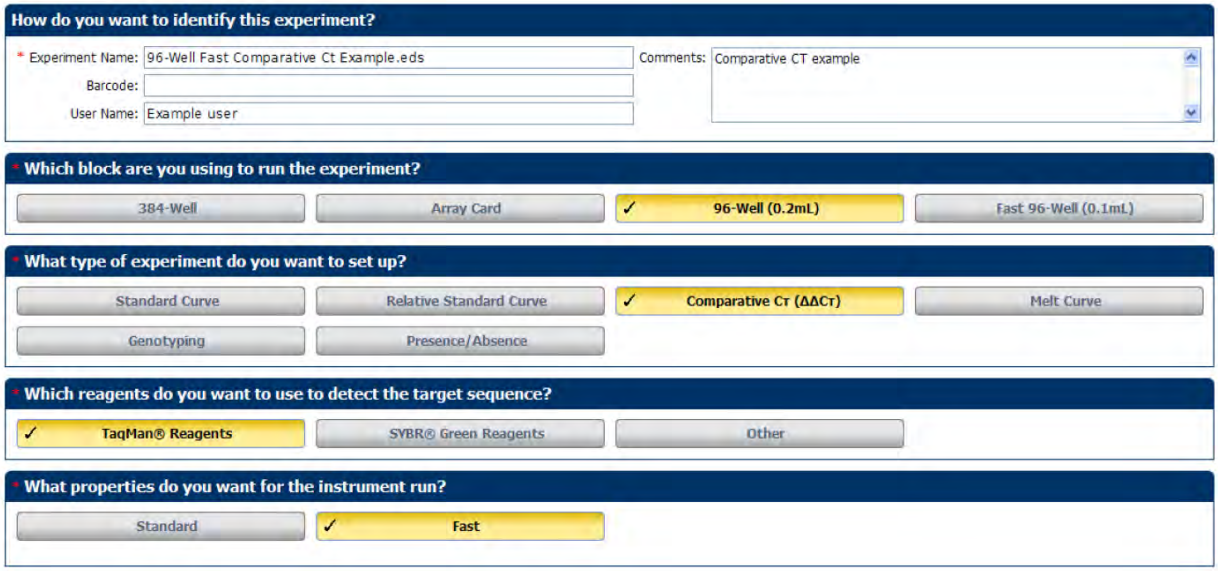


Figure 52 Experiment Properties screen

Define targets, samples and biological replicates

1. Click **Define** to access the **Define** screen.
2. Enter the following target information.

Target name	Reporter	Quencher	Color
GH1	FAM	NFQ-MGB	
LP1N1	FAM	NFQ-MGB	
TGFB1	FAM	NFQ-MGB	
L1PC	FAM	NFQ-MGB	
ACTB	FAM	NFQ-MGB	
CCKAR	FAM	NFQ-MGB	

3. Enter the following sample information.

Sample Name	Color
Brain	
Lung	
Liver	
Heart	

4. In the **Passive Reference** dropdown list, select **ROX** .

5. Enter the following analysis settings.

Field	Select
Reference Sample field	**Brain**
Endogenous Control field	**ACTB**

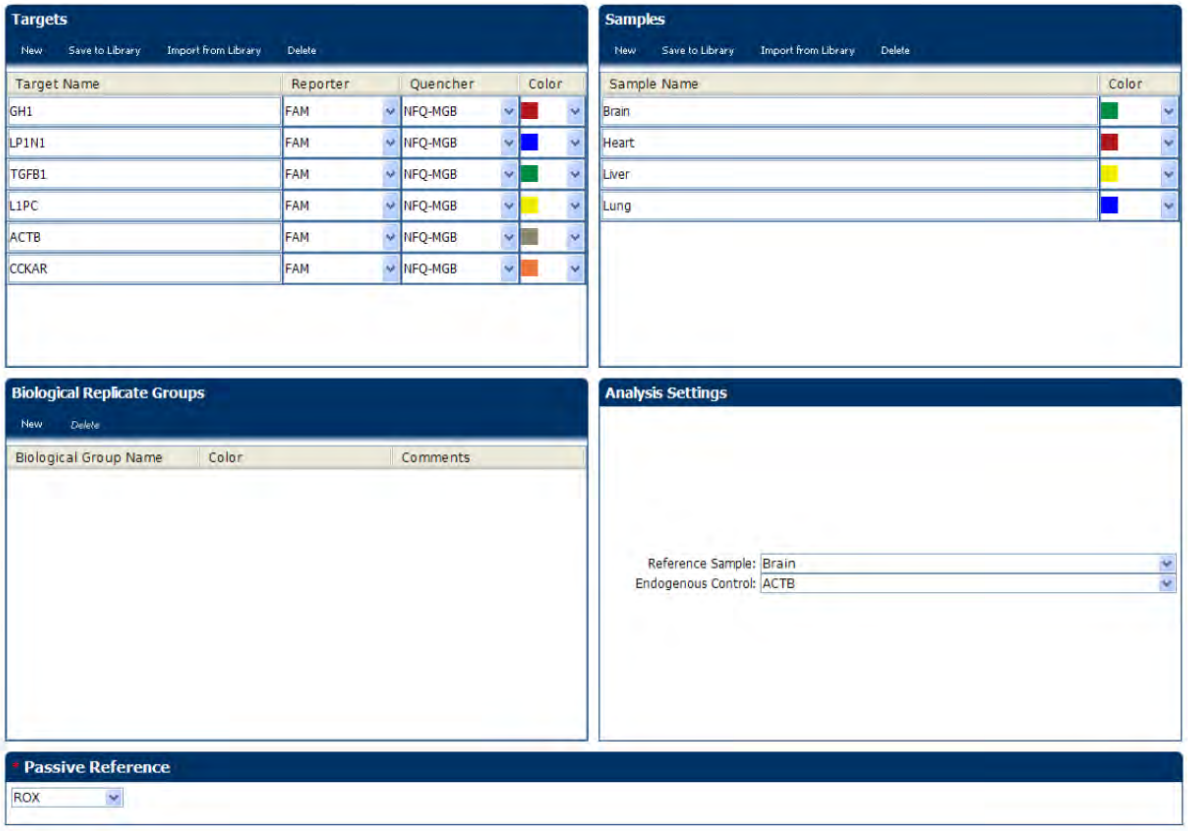


Figure 53 Define screen

Note: This example experiment does not define biological replicate groups. Leave the **Biological Replicate Groups** pane blank.

Assign targets, samples and biological groups

Click **Assign** to access the **Assign** screen.

Enter the following targets and samples.

Target name	Well number	Task	Sample name
GH1	A1, B1, C1	Unknown	Heart
	A2, B2, C2	Unknown	Brain
	E1, F1, G1	Unknown	Lung
	E2, F2, G2	Unknown	Liver
	D1, D2, H1, H2	Negative	Heart, Brain, Lung, Liver
LP1N1	A3, B3, C3	Unknown	Heart
	A4, B4, C4	Unknown	Brain
	E3, F3, G3	Unknown	Lung
	E4, F4, G4	Unknown	Liver
	D3, D4, H3, H4	Negative	Heart, Brain, Lung, Liver
TGFB1	A5, B5, C5	Unknown	Heart
	A6, B6, C6	Unknown	Brain
	E5, F5, G5	Unknown	Lung
	E6, F6, G6	Unknown	Liver
	D5, D6, H5, H6	Negative	Heart, Brain, Lung, Liver
L1PC	A7, B7, C7	Unknown	Heart
	A8, B8, C8	Unknown	Brain
	E7, F7, G7	Unknown	Lung
	E8, F8, G8	Unknown	Liver
	D7, D8, H7, H8	Negative	Heart, Brain, Lung, Liver
ACTB	A9, B9, C9	Unknown	Heart
	A10, B10, C10	Unknown	Brain
	E9, F9, G9	Unknown	Lung
	E10, F10, G10	Unknown	Liver
	D9, D10, H9, H10	Negative	Heart, Brain, Lung, Liver
CCKAR	A11, B11, C11	Unknown	Heart

(continued)

Target name	Well number	Task	Sample name
CCKAR	A12, B12, C12	Unknown	Brain
	E11, F11, G11	Unknown	Lung
	E12, F12, G12	Unknown	Liver
	D11, D12, H11, H12	Negative	Heart, Brain, Lung, Liver

Figure 54 Assign screen

Set up the run method

Click **Run Method** to access the **Run Method** screen. Set the thermal profile under the **Graphical View** tab. Enter the following information:

- Reaction Volume Per Well: ***20 µL***
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Hold Stage	Step 1	2.05°C/s	95°C	20 seconds
PCR Stage	Step 1	2.05°C/s	95°C	1 second
Number of Cycles: 40 Enable AutoDelta: Unchecked (default) Starting Cycle: Disabled when Enable AutoDelta is unchecked	Step 2	1.71°C/s	60°C	22 seconds

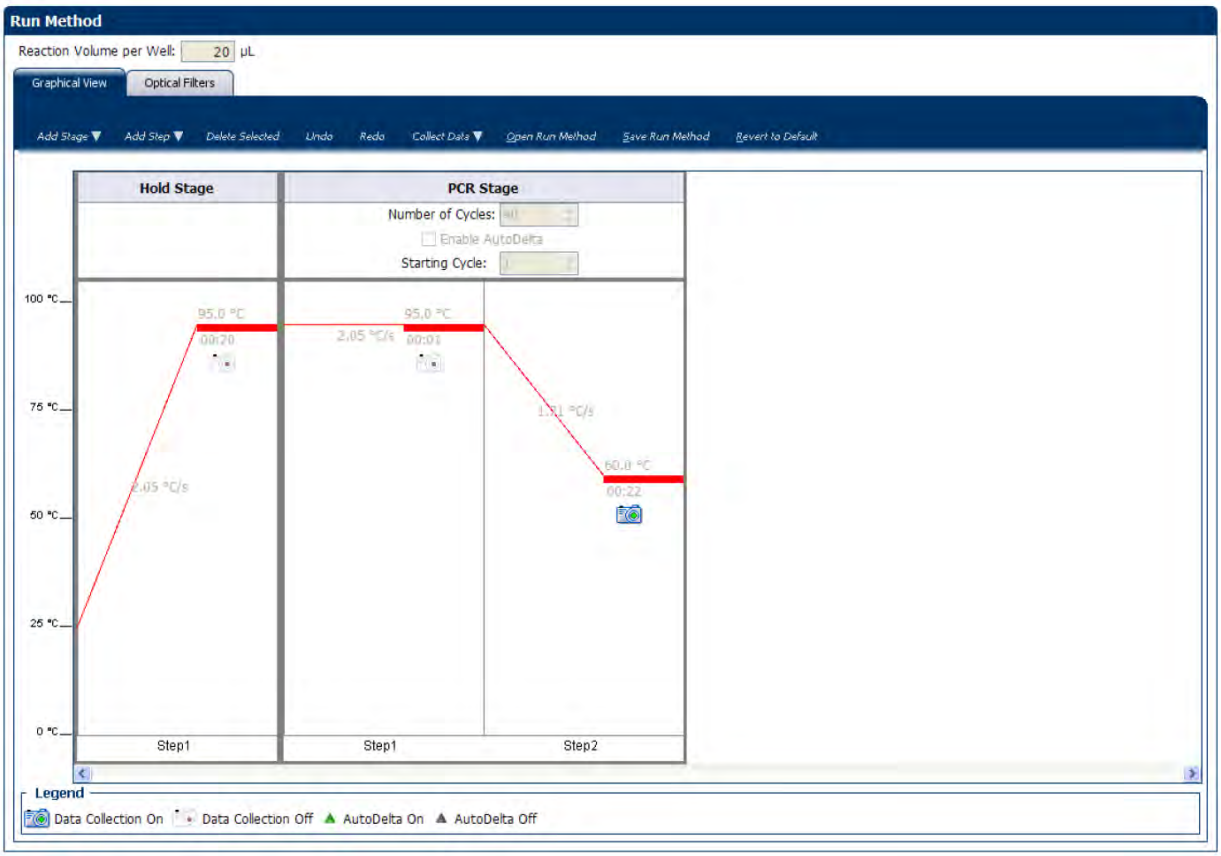


Figure 55 Run Method screen

Tips for designing your own experiment

We recommend the following items for your own experiments:

- Identify each target assay with a unique name and color. You can enter up to 100 characters in the **Target Name** field.
- Identify each sample using a unique name and color. You can enter up to 100 characters in the **Sample Name** field.

- Select an endogenous control for each sample. The endogenous control is a target that is present in all samples under investigation. It should be expressed equally in all sample types, regardless of treatment or tissue origin (examples of endogenous controls are β-actin, GAPDH, and 18S ribosomal RNA [18S rRNA]). The endogenous control is used to normalize the PCR results; the endogenous control corrects for variable sample mass, nucleic acid extraction efficiency, reverse transcription efficiency, and pipette calibration errors. Note that:
  - Each sample type (for example, each tissue in a study comparing multiple tissues) requires an endogenous control.
  - If samples are spread across multiple plates, each plate must have an endogenous control. Additionally, every plate must include an endogenous control for every sample type on the plate.
- Select an endogenous control from your previously defined target assays. Amplification results from the endogenous control are used to normalize the amplification results from the target for differences in the amount of template added to each reaction.
- Select a reference sample from your previously defined samples. Amplification results from the samples and from the reference sample are compared to determine relative expression.

For more information

Information	Reference
Consumables	Chapter 1, “General information and instructions”
Using the Standard Curve quantification methods	“Running standard curve experiments” on page 86
Using the Relative Standard Curve quantification method	“Running relative standard curve experiments” on page 145
Using alternative setup	Chapter 2, “Experiment shortcuts”

17 Prepare the reactions

This chapter explains how to prepare the PCR reactions for the Comparative C_T ($\Delta\Delta C_T$) example experiment.

Assemble required materials

- Items listed in the part "Getting started" on page 17
- Samples—Total RNA isolated from liver, heart, brain, and lung tissues
- Example experiment reaction mix components:
  - TaqMan™ Fast Universal PCR Master Mix (2X)
  - ACTB Assay Mix (20X)
  - TGFB1 Assay Mix (20X)
  - GH1 Assay Mix (20X)
  - LIPN1 Assay Mix (20X)
  - LIPC Assay Mix (20X)
  - CCKAR Assay Mix (20X)

Prepare the template

Prepare the template for the PCR reactions using the High-Capacity cDNA Reverse Transcription Kit or the SuperScript™ VILO™ cDNA Synthesis Kit to perform reverse transcription.

Example experiment settings

For the Comparative C_T example experiment, the template for the PCR reactions is cDNA reverse-transcribed from total RNA samples.

Prepare the template

Use the SuperScript™ VILO™ cDNA Synthesis Kit (Cat. No. 11754250). Follow the procedures for the kit.

1. Prepare the RT master mix.
2. Prepare the cDNA reactions.
3. Perform reverse transcription on a thermal cycler.

Prepare the sample dilutions

For the Comparative C_T example experiment, no more than 10% of your reaction should consist of the undiluted RT product.

1. Label a separate microcentrifuge tube for each diluted sample.
   - Liver
   - Heart
   - Brain
   - Lung
2. Add the required volume of water (diluent) to each empty tube.

Tube	Sample name	Diluent volume
1	Liver	19 µL
2	Heart	19 µL
3	Brain	19 µL
4	Lung	19 µL

3. Add the required volume of cDNA sample stock (100 ng/µL) to each empty tube.

Tube	Sample name	Volume
1	Liver	1.0 µL
2	Heart	1.0 µL
3	Brain	1.0 µL
4	Lung	1.0 µL

4. Vortex each diluted sample for 3 to 5 seconds, then centrifuge the tubes briefly.
5. Place the diluted samples on ice until you prepare the reaction plate.

Prepare the reaction mix

1. Label an appropriately sized tube for each reaction mix.
   - ACTB Reaction Mix
   - TGFB1 Reaction Mix
   - GH1 Reaction Mix
   - LPIN1 Reaction Mix
   - LIPC Reaction Mix
   - CCKAR Reaction Mix

2. For the ACTB assay, add the required volumes of each component to the ACTB Reaction Mix tube.

Component	Volume for 1 reaction	Volume for 16 reactions (plus 10% overage)
TaqMan™ Fast Universal PCR Master Mix (2×)	10.0 µL	176.0 µL
ACTB Assay Mix (20×)	1.0 µL	17.6 µL
Water	8 µL	140.8 µL
Total reaction mix volume	**19.0 µL**	**158.4 µL**

3. Mix the reaction mix in each tube by gently pipetting up and down, then cap each tube.
4. Centrifuge the tubes briefly to remove air bubbles.
5. Place the reaction mixes on ice until you prepare the reaction plate.
6. Repeat step 2 to step 5 for the TGFB1, GH1, LPIN1, LIPC, and CCKAR assays.

Note: Do not add the sample at this time.

Prepare the reaction plate

Example experiment reaction plate components

The reaction plate for the Comparative C_T example experiment contains:

- A MicroAmp™ Optical 96-Well Reaction Plate
- Reaction volume: 20 µL/well
- The reaction plate contains:
  - 72 Unknown wells U
  - 24 Negative Control wells N
  - 0 Empty wells

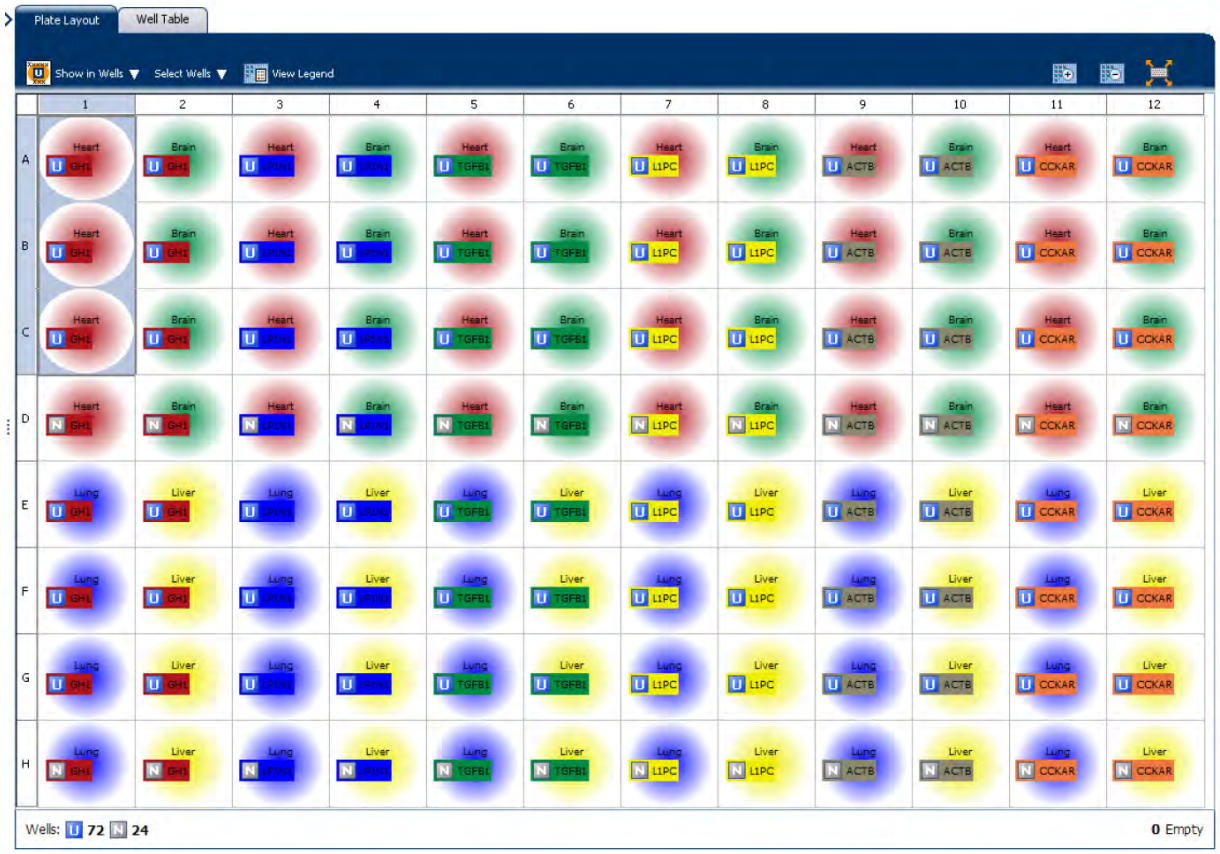


Figure 56 Plate layout for example experiment

Prepare the reaction plate components

1. Add 1 µL of each cDNA to the appropriate wells.
2. Pipette 1 µL of sterile water into the NTC wells.
3. Add 19 µL of the appropriate assay-specific reaction mix to the wells.
4. Seal the reaction plate with optical adhesive film.
5. Centrifuge the reaction plate briefly to remove air bubbles.
6. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.
7. Until you are ready to perform the run, place the reaction plate at 4°C, in the dark.

Tips for preparing reactions for your own experiments

Tips for preparing templates

When you prepare your own Comparative C_T experiment, we recommend the following templates:

- **Complementary DNA (cDNA)**—cDNA reverse-transcribed from total RNA samples.
- **Genomic DNA (gDNA)**—Purified gDNA already extracted from tissue or sample.

Tips for preparing the reaction mix

If your experiment includes more than one target assay, prepare the reaction mix for each target assay separately.

Tips for preparing the reaction plate

When you prepare your own Comparative C_T experiment, make sure the arrangement of the PCR reactions matches the plate layout displayed in the QuantStudio™ 12K Flex Software.

For more information

Information	Reference
Assigning the reaction plate components	Chapter 1, “General information and instructions”
Sealing the reaction plate	Chapter 1, “General information and instructions”

18 Run the experiment

This chapter explains how to run the example experiment on the QuantStudio™ 12K Flex Real-Time PCR Instrument.

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the instrument is in operation.

Start the run

1. Open the Comparative C_T example file that you created.
   See Chapter 16, “Design the experiment”.
2. Load the reaction plate into the instrument.
3. Start the run.

Monitor the run

You can monitor an experiment run in three ways:

- From the **Run** screen of the QuantStudio™ 12K Flex Software, while the experiment is in progress
- From the **Instrument Console** screen of the QuantStudio™ 12K Flex Software, to monitor an experiment started from another computer or from the instrument touchscreen (see “Monitor a run from the software Instrument Console screen” on page 215)
- From the instrument touchscreen (see “Monitor a run from the instrument touchscreen” on page 220)

Monitor a run from the software Instrument Console screen

1. In the **Instrument Console** screen, select the icon of the instrument that you are using to run the experiment.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. In the **Instrument Manager** screen, click **Monitor Running Experiment** to access the **Run** screen.

View the amplification plot

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the QuantStudio™ 12K Flex Software for potential problems.

Click **Amplification Plot** from the **Run Experiment** menu, select the **Plate Layout** tab, then select the wells to view.

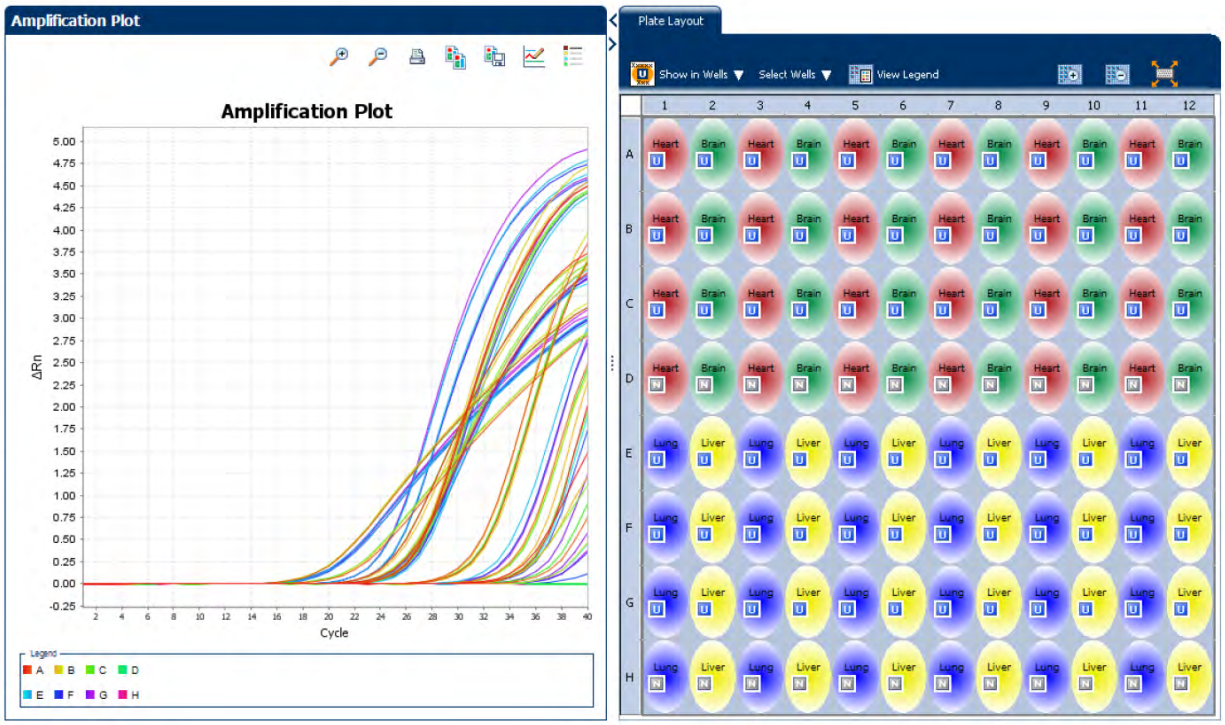


Figure 57 The Amplification Plot screen as it appears at the end of the example experiment

View the temperature plot

Click **Temperature Plot** from the **Run Experiment** menu.

Figure 58 The Temperature Plot screen as it appears during the example experiment

Note: The sample temperature displayed in the **Current Temperatures** group is an estimated value.

View the run method

Click **Run Method** from the **Run Experiment** menu.

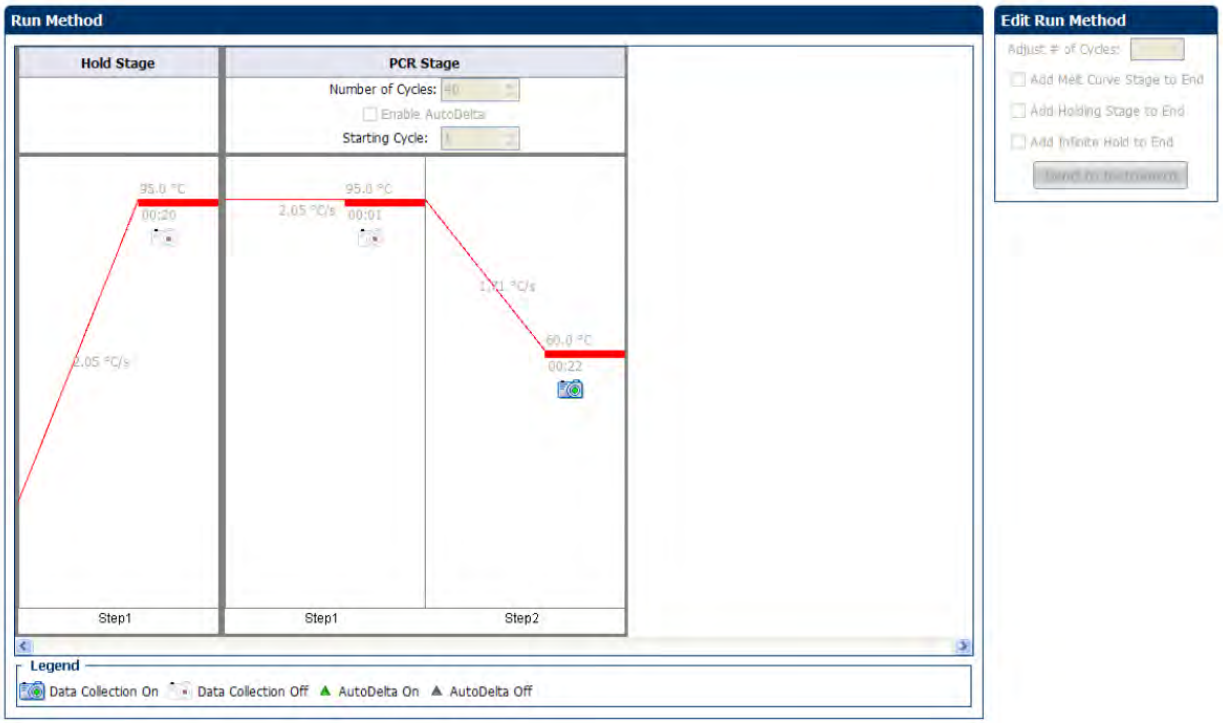


Figure 59 The Run Method screen as it appears in the example experiment

View run data

Click **View Run Data** from the **Run Experiment** menu.

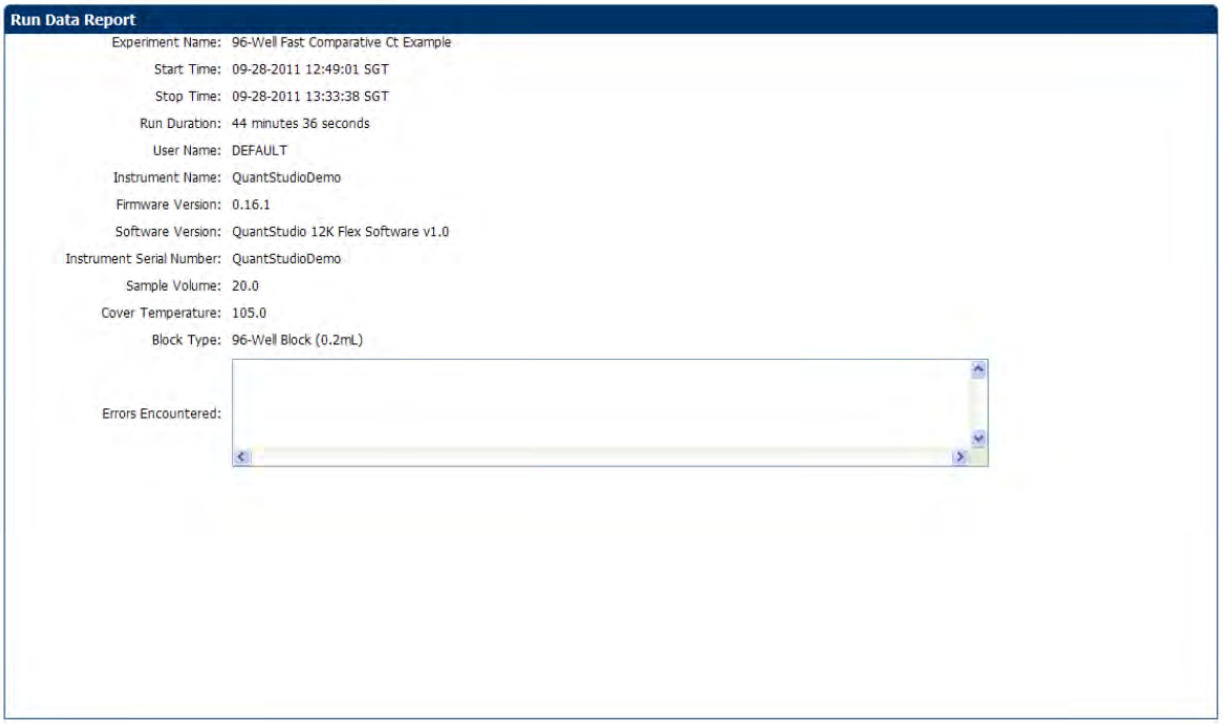


Figure 60 The View Run Data screen as it appears in the example experiment

Monitor a run from the instrument touchscreen

You can view the progress of the run from the instrument touchscreen.

The following figures are for visual representation only. Actual results vary with the experiment.

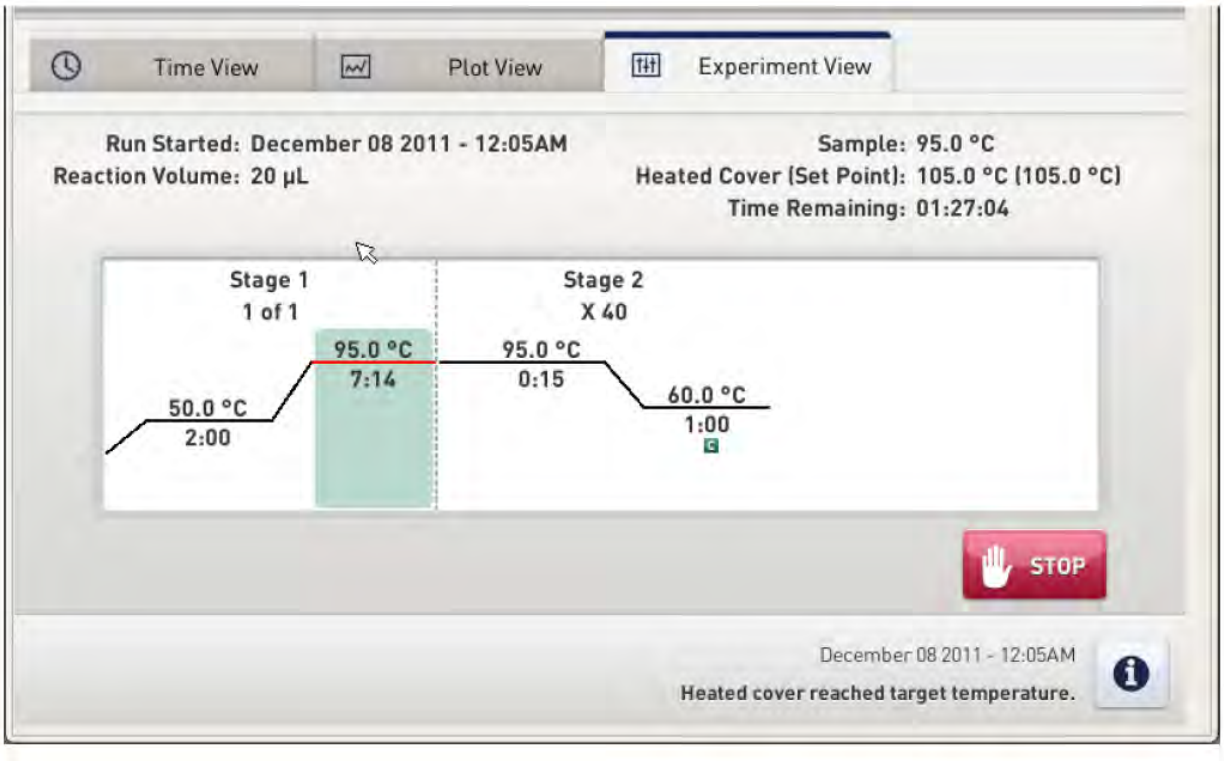


Figure 61 Experiment View tab

Figure 62 Time View tab

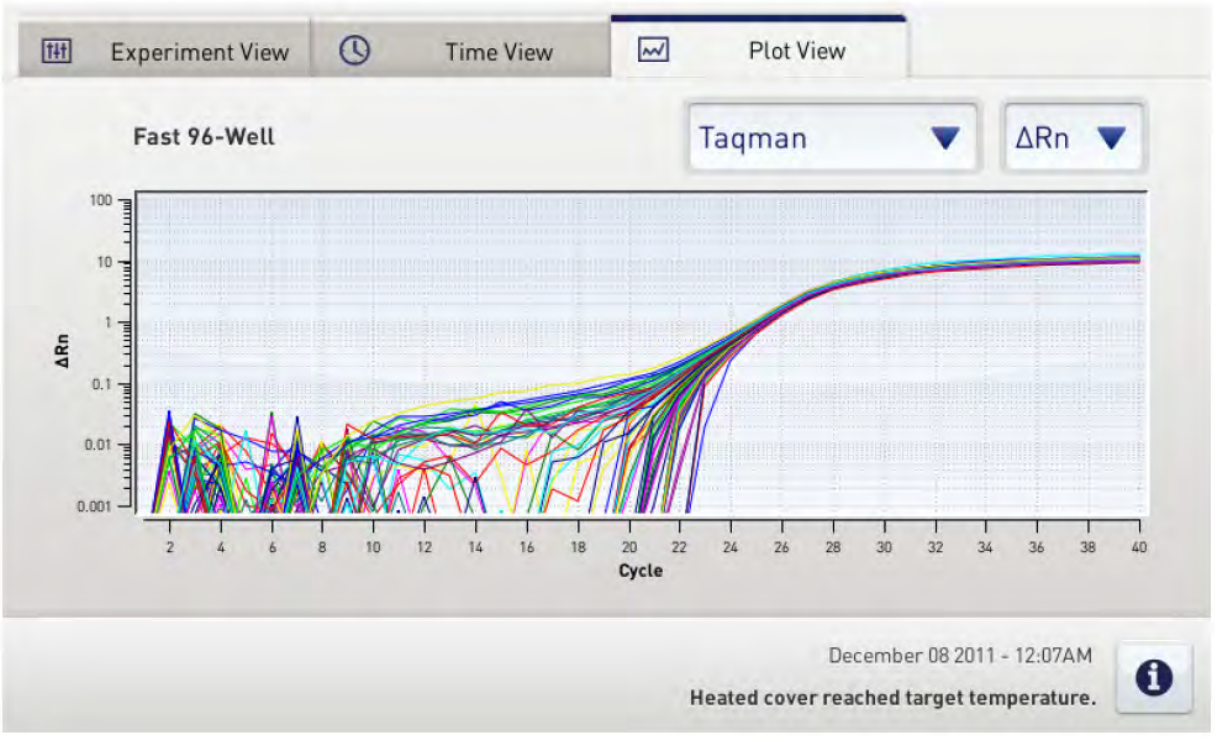


Figure 63 Plot View tab

19 Review results and adjust experiment parameters

The following topics are covered in this chapter:

- Review the analyzed data using several of the analysis screens and publish the data (see “Review results” on page 222)
- Modify experiment parameters to troubleshoot problems with experiment results before rerunning an experiment (see “Adjust parameters for re-analysis of your own experiments” on page 244)

Review results

Analyze the example experiment

1. Open the example experiment file.
   See Chapter 18, “Run the experiment”.
2. Click **Analyze**.

   Note: You can also access the experiment to analyze from the **Home** screen.

The software analyzes the data using the default analysis settings.

Assess the gene expression profile using the gene expression plot

The **Gene Expression Plot** screen displays the results of relative quantification calculations in the gene expression profile. There are two plots available:

- **RQ vs Target**—Groups the relative quantification (RQ) values by target. Each sample is plotted for each target. You can view the plot as the linear, log10, Ln, and log2 graph types.

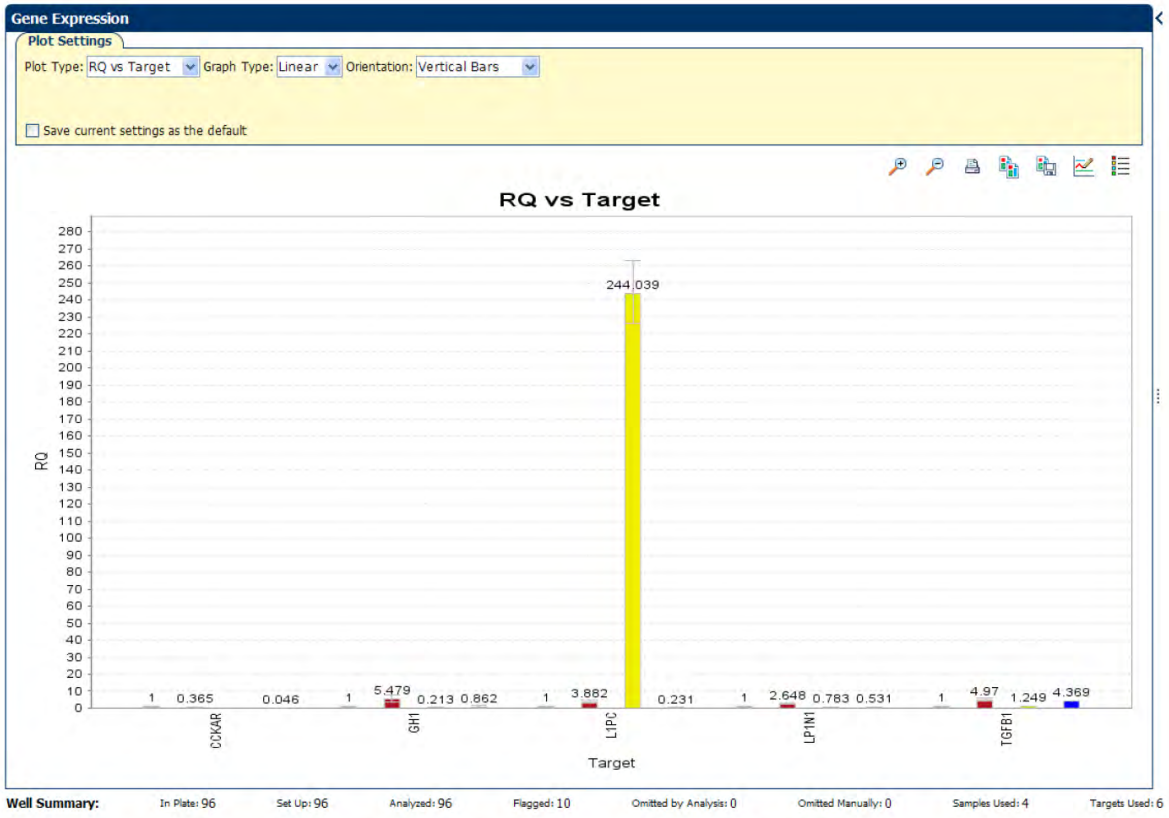


Figure 64 Gene Expression plot when viewed as a linear graph

- **RQ vs Sample**—Groups the relative quantification (RQ) values by sample. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.

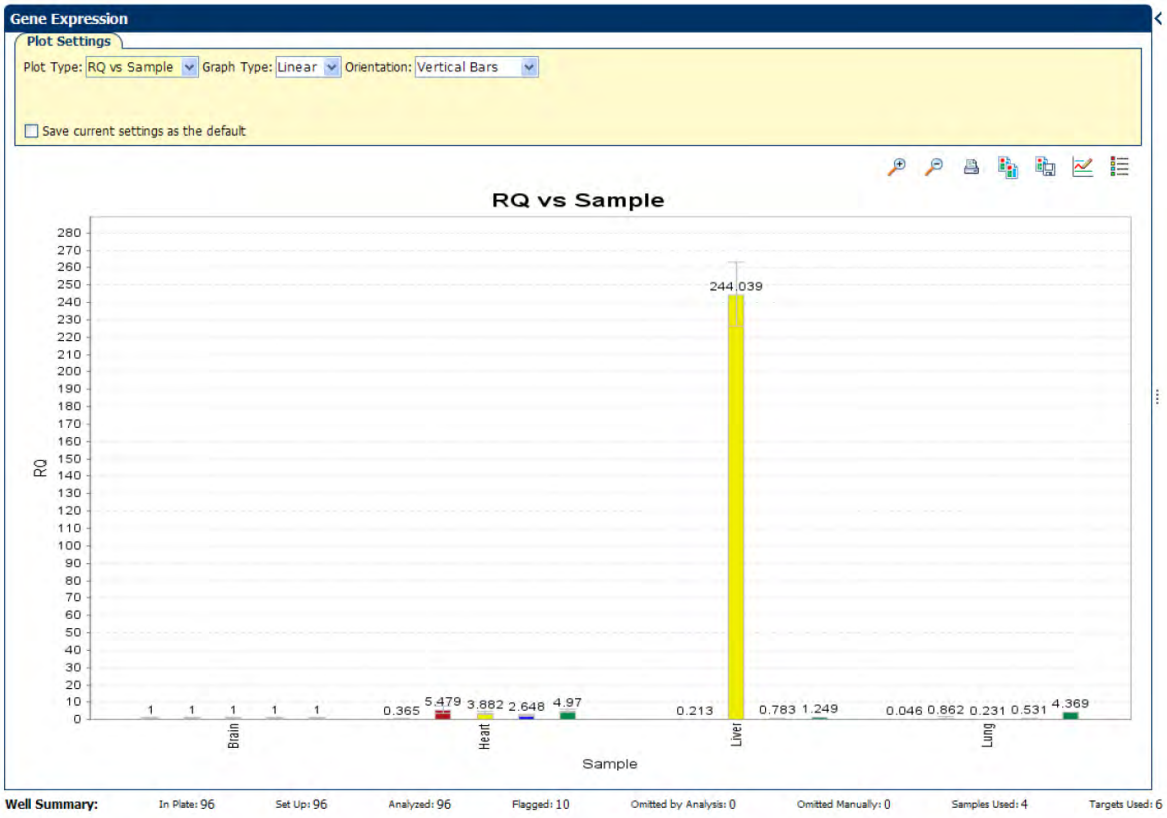


Figure 65 Gene Expression plot when viewed as a linear graph

Example experiment values

Review each target in the **Gene Expression Plot** screen for the expression level (or fold change) of the target sample relative to the reference sample.

View the gene expression plot

1. In the **Experiment Menu** pane, click **Analysis** ▸ **Gene Expression**.

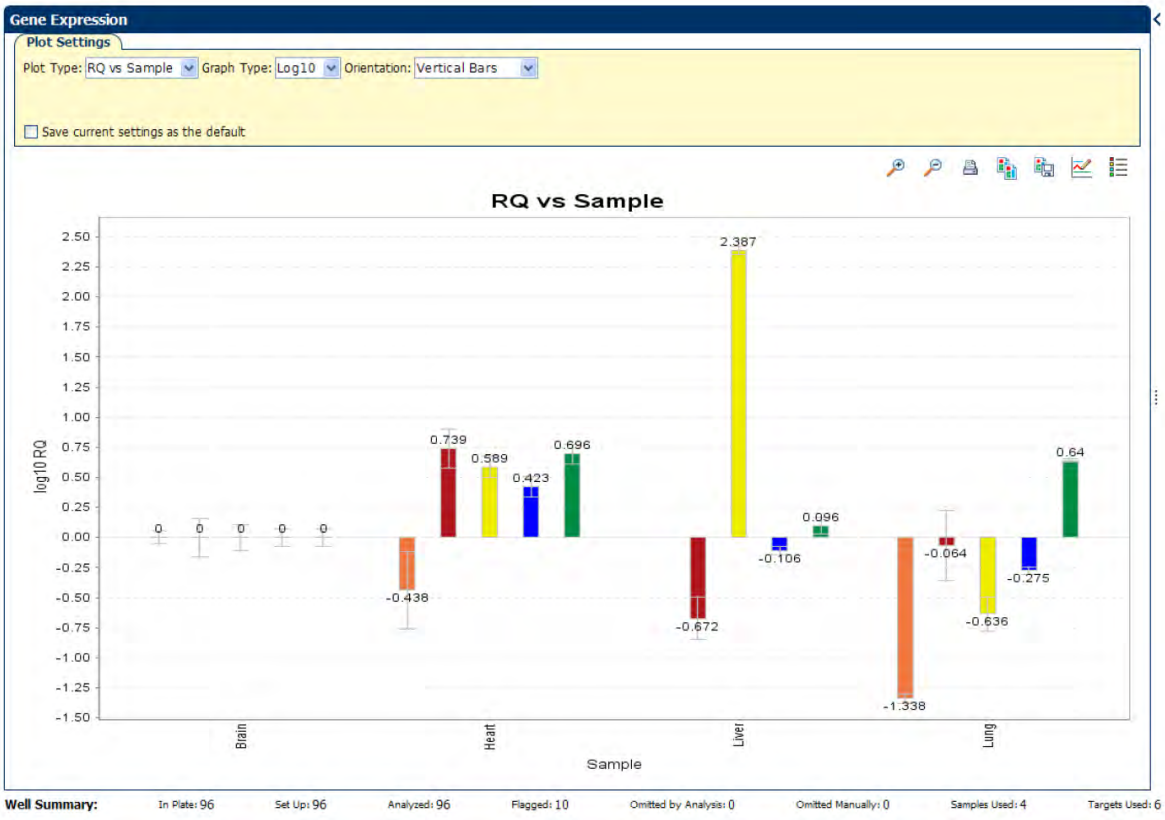
   Note: If no data are displayed, click **Analyze**.

2. Select the following items in the **Gene Expression Plot** screen.
   a. In the **Plot Type** dropdown list, select **RQ vs Sample**.
   b. In the **Graph Type** dropdown list, select **Log10**.
   c. In the **Orientation** dropdown list, select **Vertical Bars**.

3. Click **Show a legend for the plot** (default).

Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

In the example experiment, as shown below, the expression level of each target gene in liver, heart, and lung is displayed relative to its respective expression level in the reference sample (brain). Since the reference sample is compared to itself, the relative expression level is 1. When the result is displayed in the Log10 graph type, the expression level of the reference sample appears as 0 in the graph (log10 of 1 = 0).



Assessing the gene expression plot your own experiments

When you analyze your own Comparative C_T experiment, look for differences in gene expression (as a fold change) relative to the reference sample.

Identify well problems using the well table

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

- The sample name, target name, task, and dyes
- The calculated threshold cycle (C_T), normalized fluorescence (Rn), and quantity values
- Flags

Example experiment values and flags

Review the Well Table to evaluate the C_T precision of the replicate groups.

View the well table

1. In the **Experiment Menu** pane, select **Analysis ▸ Amplification Plot,**, then click the **Well Table** tab.
2. In the **Group By** dropdown list, select **Replicate**.
3. Look at the C_T SD column to evaluate the C_T precision of the replicate groups.

   In the example experiment, there are ten outliers. These wells are omitted in the troubleshooting section (“Improve CT precision by omitting wells” on page 248).

#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	CT	CT Mean	CT SD	ΔCT	ΔCT Me...	ΔCT SE	ΔΔCT	RQ
Brain - L1PC															
8	A8	☐		Brain	L1PC	UNKNOWN	FAM-NFQ-...	31.479	31.637	0.217		12.054	0.130	0.000	
20	B8	☐		Brain	L1PC	UNKNOWN	FAM-NFQ-...	31.548	31.637	0.217		12.054	0.130	0.000	
32	C8	☐		Brain	L1PC	UNKNOWN	FAM-NFQ-...	31.884	31.637	0.217		12.054	0.130	0.000	
44	D8	☐		Brain	L1PC	NTC	FAM-NFQ-...	Undetermi...							
Brain - LP1N1															
4	A4	☐		Brain	LP1N1	UNKNOWN	FAM-NFQ-...	24.883	24.955	0.133		5.372	0.085	0.000	
16	B4	☐		Brain	LP1N1	UNKNOWN	FAM-NFQ-...	25.108	24.955	0.133		5.372	0.085	0.000	
28	C4	☐		Brain	LP1N1	UNKNOWN	FAM-NFQ-...	24.873	24.955	0.133		5.372	0.085	0.000	
40	D4	☐		Brain	LP1N1	NTC	FAM-NFQ-...	Undetermi...							
Brain - TGFB1															
6	A6	☐		Brain	TGFB1	UNKNOWN	FAM-NFQ-...	26.743	26.793	0.144		7.210	0.091	0.000	
18	B6	☐		Brain	TGFB1	UNKNOWN	FAM-NFQ-...	26.680	26.793	0.144		7.210	0.091	0.000	
30	C6	☐		Brain	TGFB1	UNKNOWN	FAM-NFQ-...	26.956	26.793	0.144		7.210	0.091	0.000	
42	D6	☐		Brain	TGFB1	NTC	FAM-NFQ-...	Undetermi...							
Heart - ACTB															
9	A9	☐		Heart	ACTB	UNKNOWN	FAM-NFQ-...	22.101	21.917	0.168					
21	B9	☐		Heart	ACTB	UNKNOWN	FAM-NFQ-...	21.880	21.917	0.168					
33	C9	☐		Heart	ACTB	UNKNOWN	FAM-NFQ-...	21.771	21.917	0.168					
45	D9	☐		Heart	ACTB	NTC	FAM-NFQ-...	Undetermi...							
Heart - CCKAR															
11	A11	☐	1	Heart	CCKAR	UNKNOWN	FAM-NFQ-...	37.170	37.383	0.649		15.465	0.387	1.453	
23	B11	☐	1	Heart	CCKAR	UNKNOWN	FAM-NFQ-...	38.111	37.383	0.649		15.465	0.387	1.453	
35	C11	☐	1	Heart	CCKAR	UNKNOWN	FAM-NFQ-...	36.867	37.383	0.649		15.465	0.387	1.453	
47	D11	☐		Heart	CCKAR	NTC	FAM-NFQ-...	Undetermi...							
Heart - GH1															
1	A1	☐		Heart	GH1	UNKNOWN	FAM-NFQ-...	34.655	34.427	0.292		12.509	0.194	-2.454	
13	B1	☐		Heart	GH1	UNKNOWN	FAM-NFQ-...	34.098	34.427	0.292		12.509	0.194	-2.454	
25	C1	☐		Heart	GH1	UNKNOWN	FAM-NFQ-...	34.527	34.427	0.292		12.509	0.194	-2.454	
37	D1	☐		Heart	GH1	NTC	FAM-NFQ-...	Undetermi...							
Heart - L1PC															
7	A7	☐		Heart	L1PC	UNKNOWN	FAM-NFQ-...	31.978	32.015	0.073		10.097	0.106	-1.957	
19	B7	☐		Heart	L1PC	UNKNOWN	FAM-NFQ-...	32.099	32.015	0.073		10.097	0.106	-1.957	
31	C7	☐		Heart	L1PC	UNKNOWN	FAM-NFQ-...	31.967	32.015	0.073		10.097	0.106	-1.957	
43	D7	☐		Heart	L1PC	NTC	FAM-NFQ-...	Undetermi...							

Note: To show or hide columns in the Well Table, select or deselect respectively the column name from the **Show in Table** dropdown list.

Assessing the well table in your own experiments

When you analyze your own Comparative C_T experiment, look for standard deviation in the replicate groups (C_T SD values). If needed, omit outliers (see “Improve CT precision by omitting wells” on page 248).

Assess amplification results using the amplification plot

Amplification plots available for viewing

The **Amplification Plot** screen displays amplification of all samples in the selected wells. There are three plots available:

- **ΔRn vs Cycle**—ΔRn is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. This plot displays ΔRn as a function of cycle number. Use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle**—Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays Rn as a function of cycle number. Use this plot to identify and examine irregular amplification.
- **C_T vs Well**—C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. Use this plot to locate outlying amplification (outliers).

Each plot can be viewed as a linear or log10 graph type.

Purpose

The purpose of viewing the amplification plot for the example experiment is to identify:

- Correct baseline and threshold values
- Outliers

View the amplification plot

1. In the **Experiment Menu** pane, click **Analysis** ▸ **Amplification Plot**.

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

2. Display the LP1N1 wells in the **Amplification Plot** screen.
   a. Click the **Plate Layout** tab.

b. In the **Select Wells** dropdown list, select **Target ▸ LP1N1**.

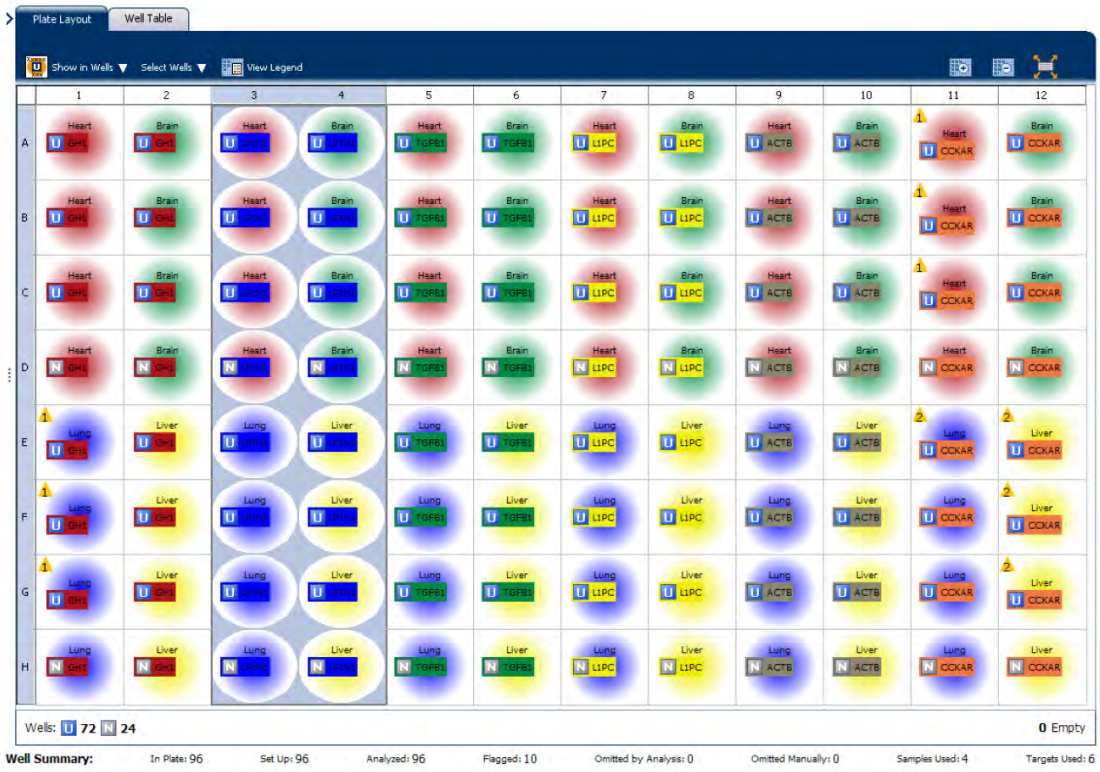


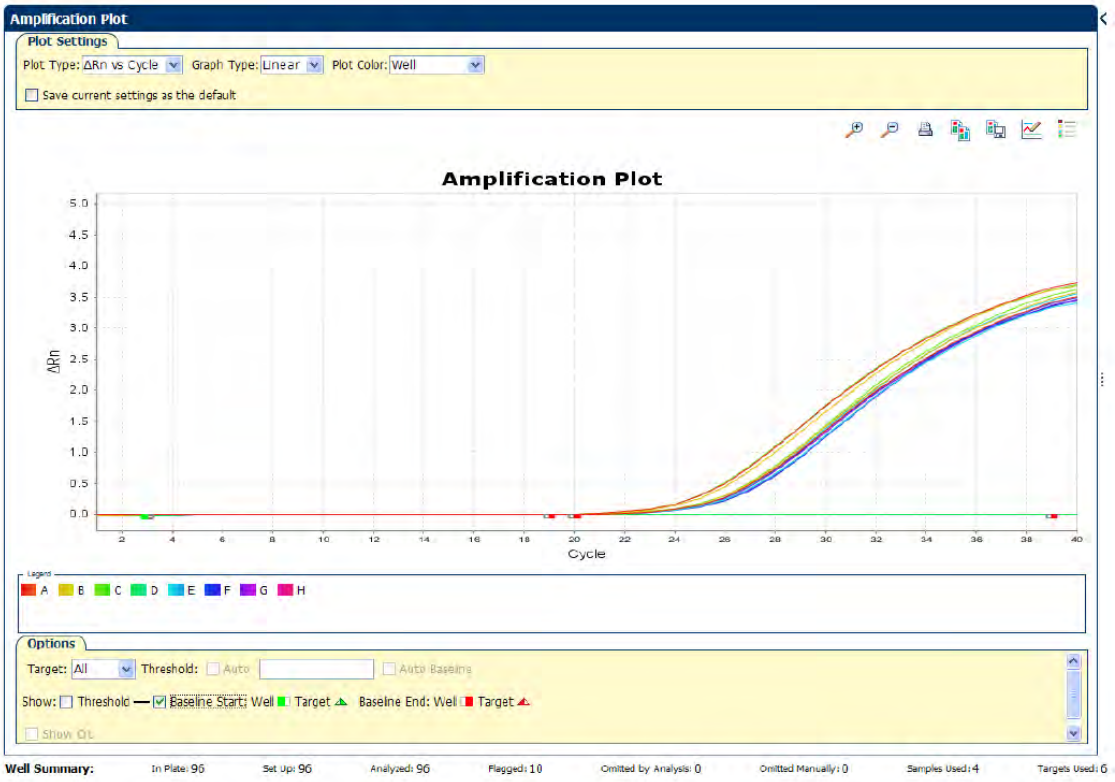
Figure 66 Plate Layout screen

3. In the Amplification Plot screen, select the following.

Item	Select
Plot Type dropdown list	**ΔRn vs Cycle**
Plot Color dropdown list	**Well** (default)
 (This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**).	Check (default)

4. View the baseline values.
   a. From the **Graph Type** dropdown list, select **Linear**.
   b. Select the **Baseline** checkbox to show the start cycle and end cycle.

c. Confirm that the baseline is set correctly. The end cycle should be set a few cycles before the cycle number where significant fluorescent signal is detected. In the example experiment, the baseline is set correctly.



5. View the threshold values.

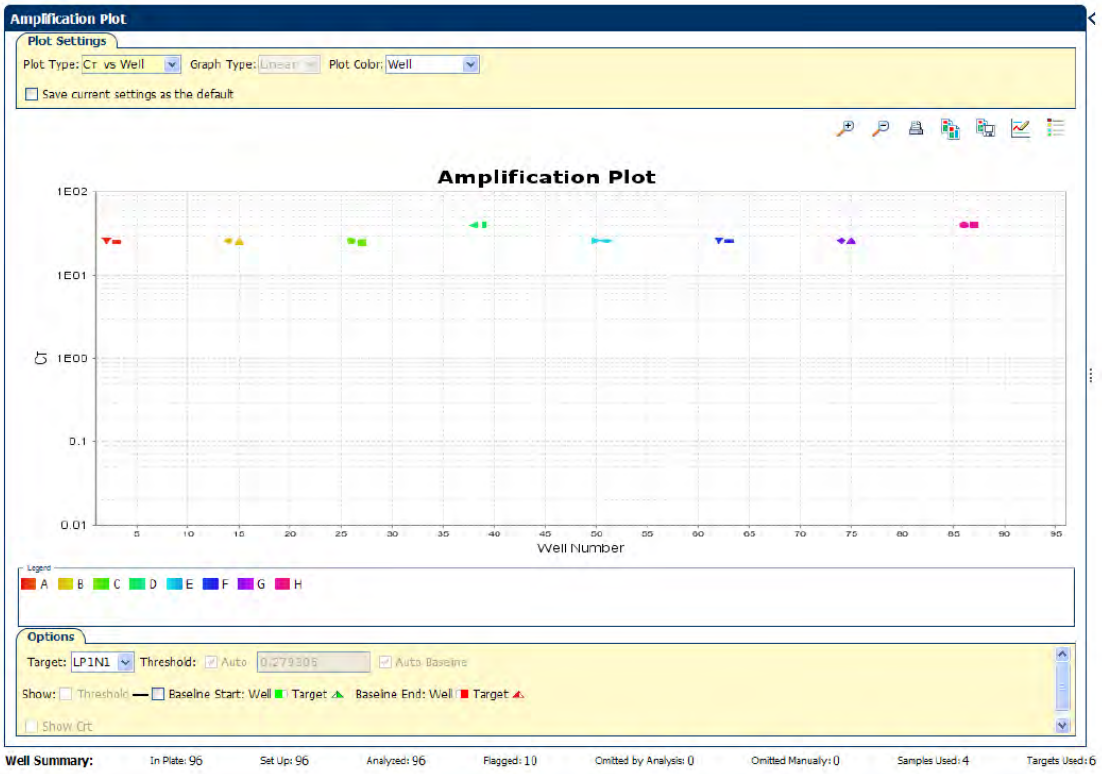
Item	Select
Graph Type dropdown list	**Log**
Target dropdown list	**LP1N1**

a. Select the **Threshold** check box to show the threshold.

b. Confirm that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.

6. Locate outliers.
   a. In the **Plot Type** dropdown list, select **C_T vs. Well**.

b. Look for outliers from the amplification plot. In the example experiment, there are no outliers for LP1N1.



7. Repeat step 2 to step 6 for the GH1, TGFB1, LIPC, ACTB, and CCKAR wells.

   In the example experiment, there is seven outliers for CCKAR and three outliers for GH1. These wells are omitted in the troubleshooting section (“Improve CT precision by omitting wells” on page 248).

Tips for analyzing your own experiments

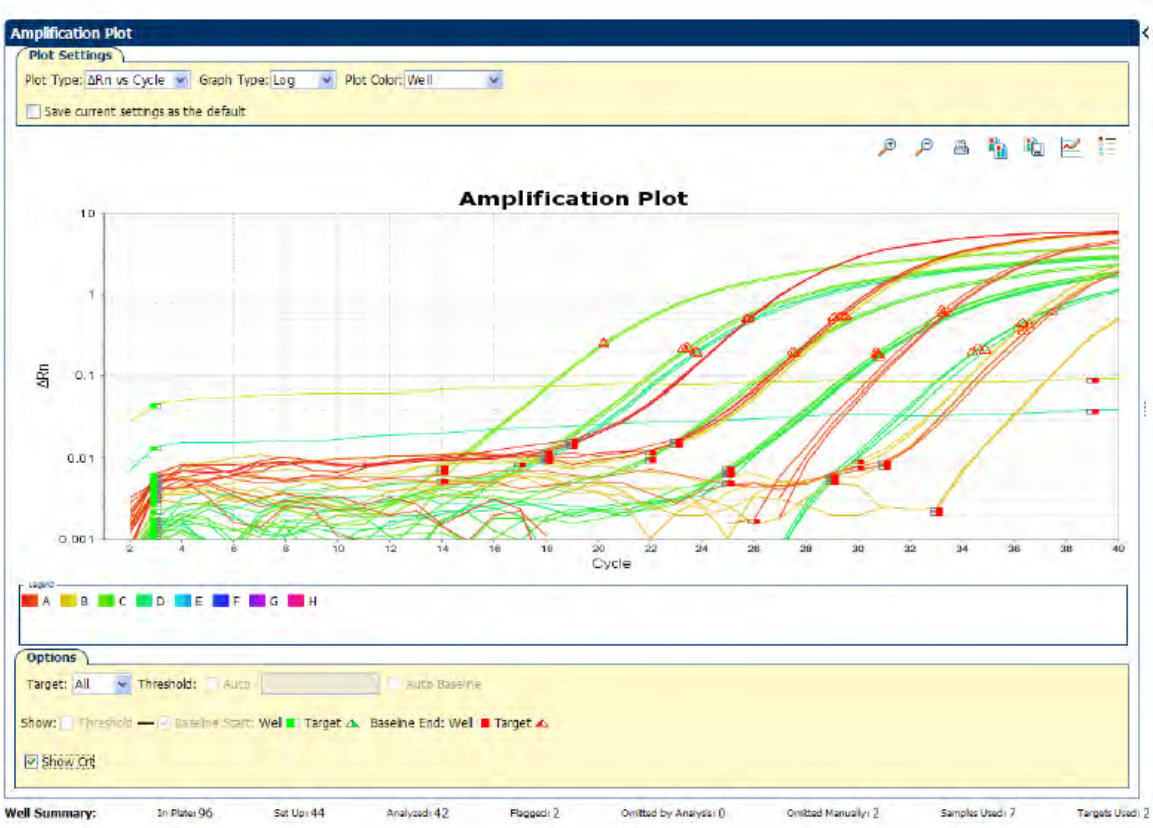
When you analyze your own Comparative C_T experiment, look for the following items:

- **Outliers**
- **A typical amplification plot**—The QuantStudio™ 12K Flex Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear phase
  - Exponential (geometric phase)
  - Baseline

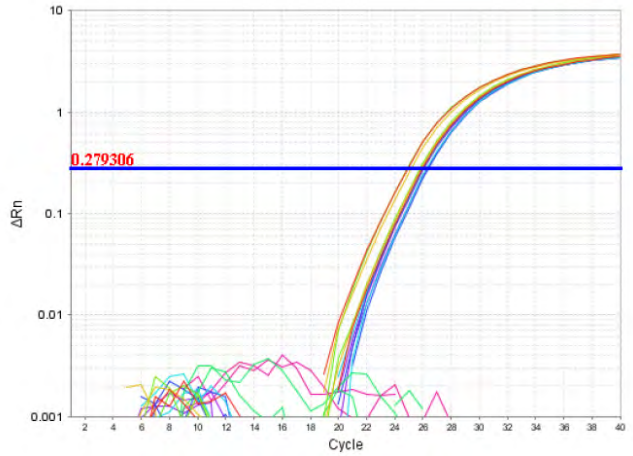
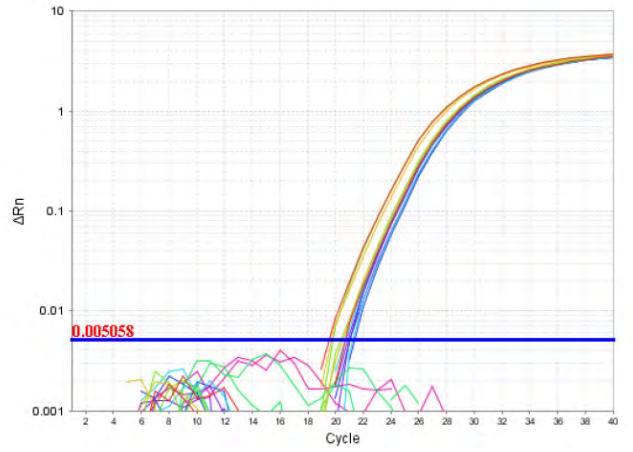
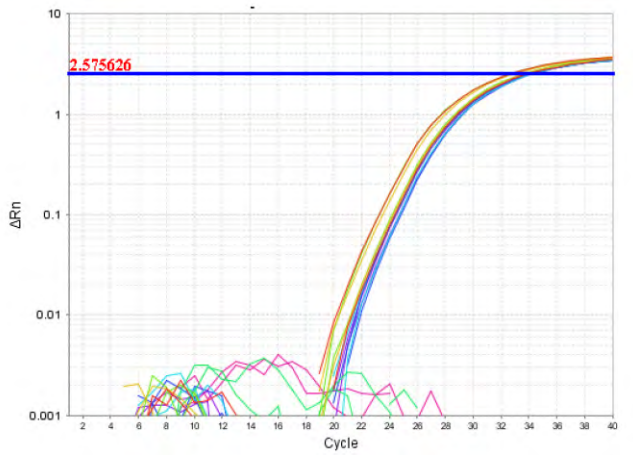
Figure 67 Typical amplification plot

IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudio™ 12K Flex Software. We recommend that you examine the **Amplification Plot** screen and review the assigned baseline and threshold values for each well after analysis completes.

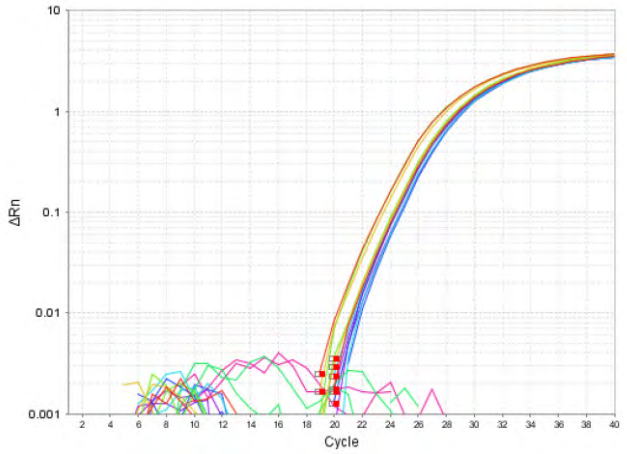
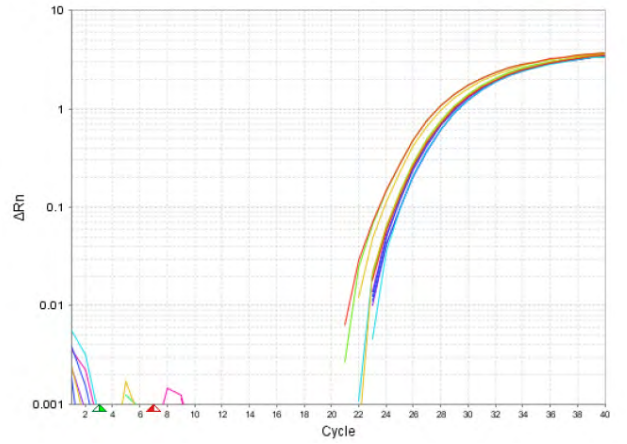
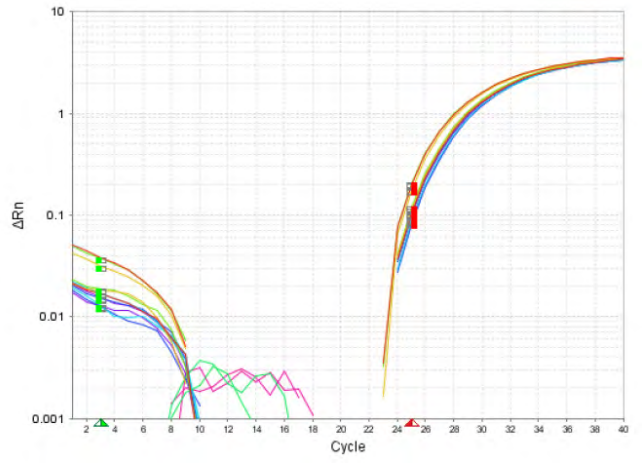
Note: If you use the Relative Threshold algorithm to analyze an experiment that includes amplification, select to view the analysis results using the ΔRn vs Cycle, Rn vs Cycle, or C_{RT} vs Well plot type and Linear or Log graph type. Also select the **Show Crt** check box to view the derived fractional cycle on the amplification plot.


Amplification Plot
Plot Settings
Plot Type: ΔRn vs Cycle
Graph Type: Log
Plot Color: Well
Save current settings as the default
Amplification Plot
ΔRn
Cycle
Options
Target: All
Threshold:
Auto Baseline
Show: Threshold
Baseline Start: Well
Target
Baseline End: Well
Target
Show Crt
Well Summary:
In Plate: 96
Set Up: 44
Analyzed: 42
Flagged: 2
Omitted by Analysis: 0
Omitted Manually: 2
Samples Used: 7
Targets Used: 2

- **Correct threshold values**

Condition	Example
Threshold Set Correctly The threshold is set in the exponential phase of the amplification curve. Threshold settings above or below the optimum increase the standard deviation of the replicate groups.	
Threshold Set Too Low The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the exponential phase of the curve.	
Threshold Set Too High The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar down into the exponential phase of the curve.	

- **Correct baseline values**

Condition	Example
Baseline Set Correctly The amplification curve begins after the maximum baseline.	
Baseline Set Too Low The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.	
Baseline Set Too High The amplification curve begins before the maximum baseline. Decrease the End Cycle value.	

- View the analyzed data using the relative threshold settings

  The QuantStudio™ 12K Flex Software provides the Relative Threshold method to view the analyzed data. The relative threshold algorithm lets you compare the data per well and per target. These options allow analysis of a single gene across samples or, alternatively, a single sample across genes with no dependency on targets, thereby reducing variability.

  To view the analyzed data using the relative threshold settings, see “Adjust analysis settings” on page 244.

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

- Omit wells (see “Improve CT precision by omitting wells” on page 248).

  Or
- Manually adjust the baseline and/or threshold (see “Adjust analysis settings” on page 244).

Confirm accurate dye signal using the multicomponent plot

The **Multicomponent Plot** screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the Comparative C_T example experiment, you review the Multicomponent Plot screen for the following items:

- ROX™ dye (passive reference)
- FAM™ dye (reporter)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

View the multicomponent plot

1. In the **Experiment Menu** pane, select **Analysis** ▸ **Multicomponent Plot.**

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

2. Display the unknown and standard wells one at a time in the **Multicomponent Plot** screen.
   a. Click the **Plate Layout** tab.
   b. Select one well in the plate layout. The well is shown in the Multicomponent Plot.

      Note: If you select multiple wells, the Multicomponent Plot displays the data for all selected wells simultaneously.

3. In the **Plot Color** dropdown list, select **Dye**.
4. Click **Show a legend for the plot** (default).

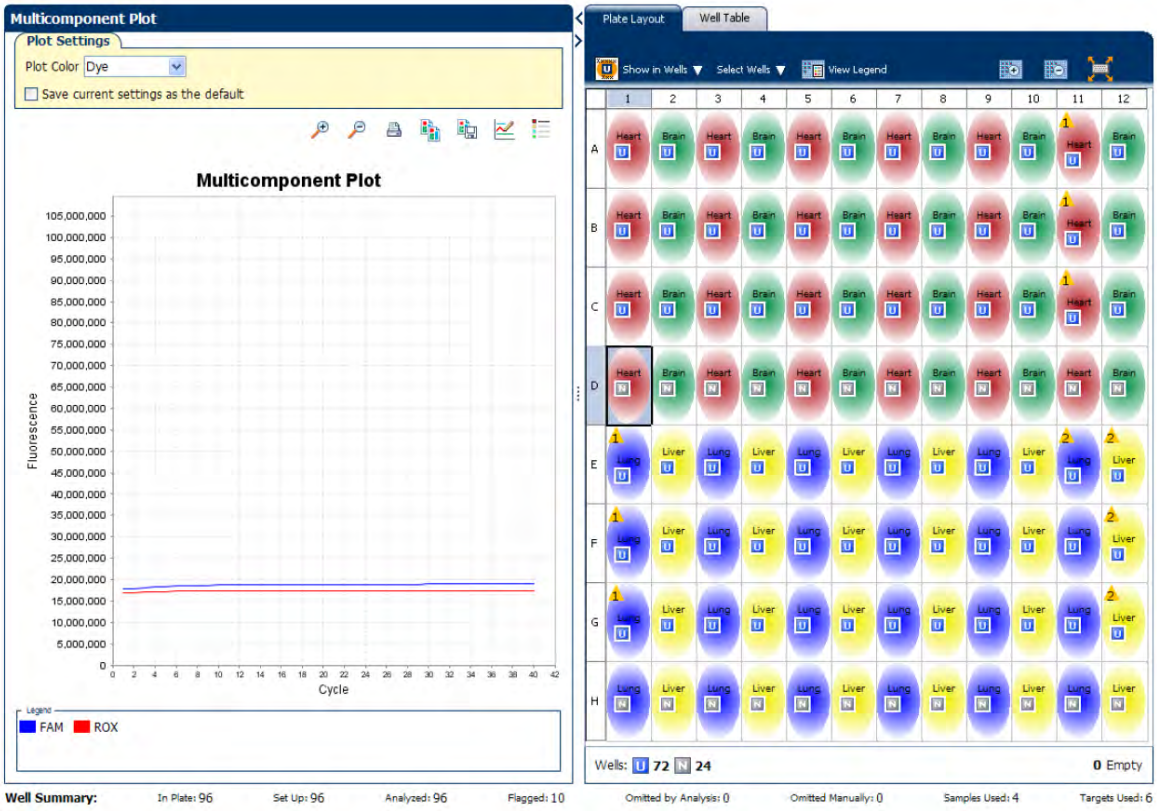
   Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

5. Check the FAM™ dye signals.

   In the example experiment, the FAM™ dye signal increases throughout the PCR process, indicating normal amplification.

6. Select the negative control wells one at time and check for amplification.

   In the example experiment, there is no amplification in the negative control wells.



Tips for confirming dye accuracy in your own experiment

When you analyze your own Comparative C_T experiment, look for the following items:

- **Passive reference**—The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye**—The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- **Irregularities in the signal**—There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative Control wells**—There should not be any amplification in the negative control wells.

Determine signal accuracy using the raw data plot

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

About the example experiment

In the Comparative C_T example experiment, you review the **Raw Data Plot** screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the raw data plot

1. In the **Experiment Menu** pane, select **Analysis** ▸ **Raw Data Plot**.

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

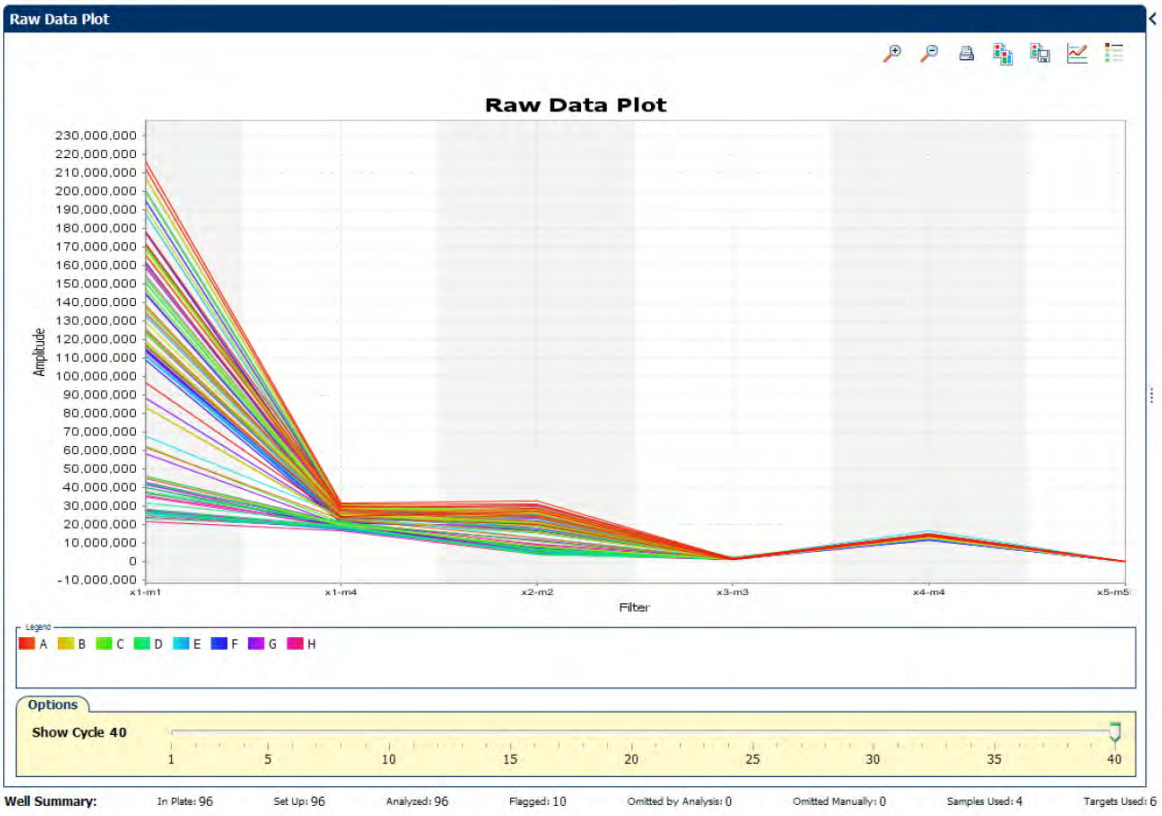
2. Display all 48 wells in the **Raw Data Plot** screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.

3. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

   Note: The legend displays the color code for each row of the reaction plate (see the legend in the Raw Data Plot shown below).

4. Click and drag the Show Cycle pointer from cycle 1 to cycle 40. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.



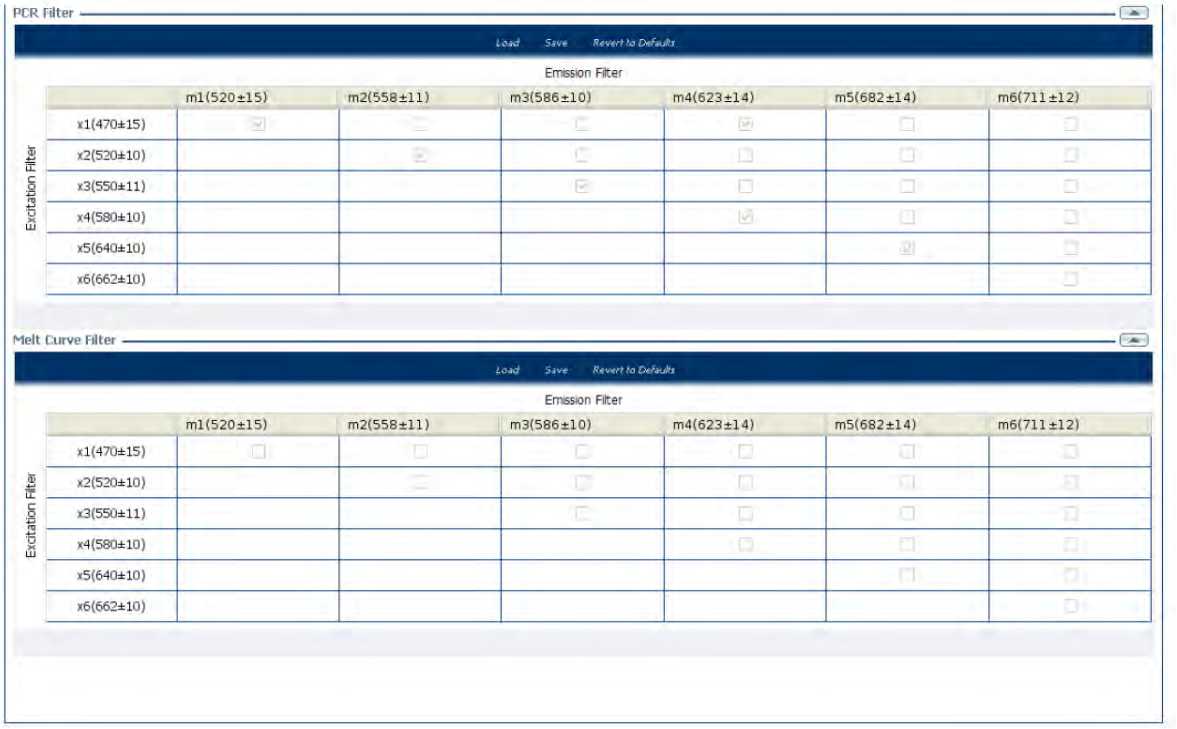


Figure 68 Filters used for the example experiment

Tips for determining signal accuracy in your own experiment

When you analyze your own Comparative C_T experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

View the endogenous control profile using the QC plot

In the Comparative C_T experiment, the **QC Plot** screen displays the Endogenous Control Profile plot for all the targets in the experiment. The QC Plot serves as a tool to help you choose the best endogenous control for that experiment. The endogenous control profile plot is a visual display of the C_T level of the endogenous control across the sample. You can view up to four endogenous controls at a time. The sample is plotted on the X-axis, and C_T is plotted on the Y-axis. The expression is viewed as a color and shape combination in the plot. Endogenous controls are also known as reference genes.

Example experiment settings

In the example experiment, you can view the endogenous control profile of GH1, LP1N1, TGFB1, L1PC, ACTB, and CCKAR in the **QC Plot** screen.

View the QC plot

1. In the **Experiment Menu** pane, select **Analysis** ▸ **QC Plot**.

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

2. In the **QC Plot** screen, click **Target Table** to select a target to profile.
   a. In the **Candidate Control** column, select the check box of the target of the endogenous control profile to plot.
      In the example experiment, the endogenous controls selected are GH1, LP1N1, TGFB1, and L1PC.
   b. Select a color from the **Color** dropdown list.
   c. Select a shape from the **Shape** dropdown list.
3. Click the **View Replicate Results Table** tab.
4. Select the check box of the samples to plot.
   In the example experiment, all the four samples, Brain, Heart, Liver, and Lung are selected.
5. Click **Show a legend for the plot** (default).

Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

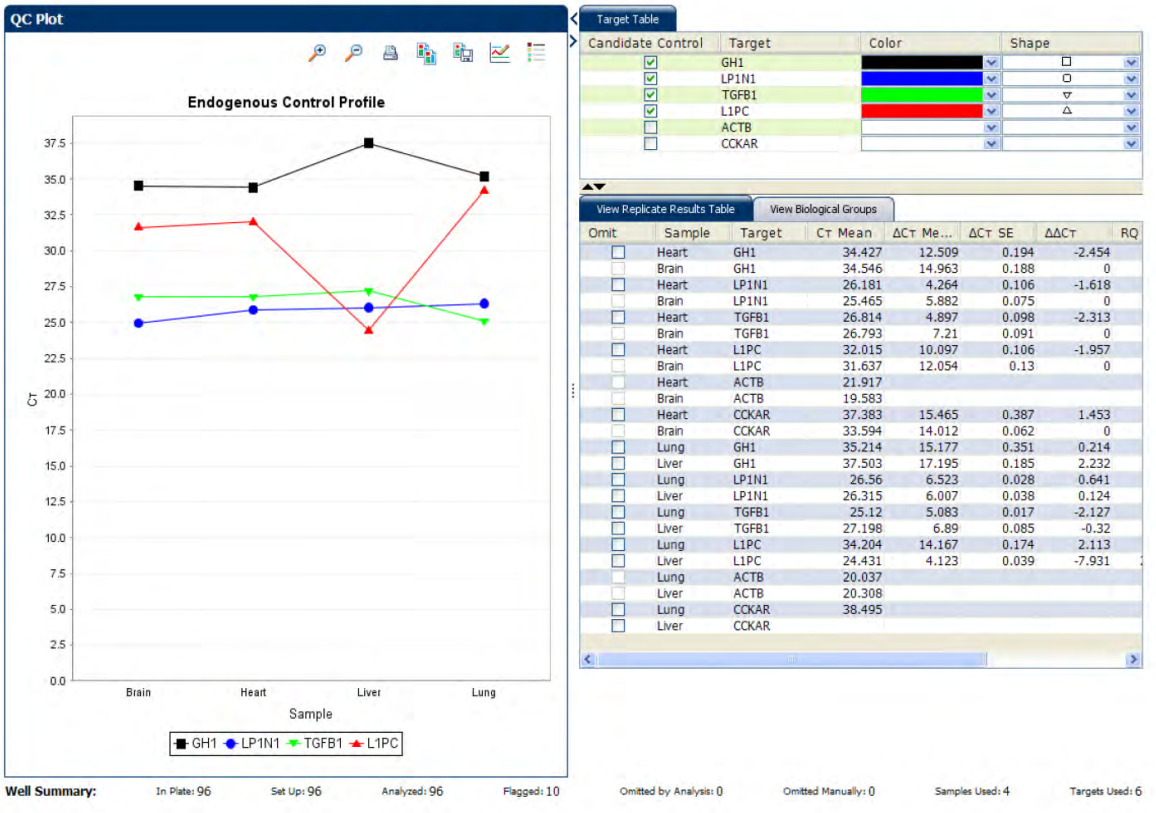


Figure 69 QC Plot in the Comparative Ct example experiment
This example experiment does not define Biological Groups.

Review the flags in the QC summary

The **QC Summary** screen displays a list of the QuantStudio™ 12K Flex Software flags, including the flag frequency and location for the open experiment.

Review the **QC Summary** screen in the Comparative C_T example experiment for any flags triggered by the experiment data. Wells A11, B11, C11, E1, F1, and G1 have data that triggered the HIGHSD flag. Wells E11, E12, F11, and F12 have data that triggered the NOAMP flag. Wells E11, E12, F12, and G12 have data that triggered the EXPFAIL flag.

View the QC summary

1. In the **Experiment Menu** pane, select **Analysis** ▸ **QC Summary**.

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

2. Review the Flags Summary.

   Note: A 0 displayed in the **Frequency** column indicates that the flag does not appear in the experiment. If the frequency is greater than 0, the flag appears somewhere in the experiment. The well position is listed in the **Wells** column.

   In the example experiment, there are ten flagged wells.

3. In the **Flag Details** table, click each flag with a frequency greater than 0 to display detailed information about the flag.

   In the example experiment. The HIGHSD flag appears six times, in the wells A11, B11, C11, E1, F1, and G1, indicating high standard deviation in the replicate group. The NOAMP flag appears four times, in the wells E11, E12, F11, and F12, indicating no amplification in the replicate group. The EXPFAIL flag appears in the wells E11, E12, F12, and G12, indicating that the exponential algorithm failed.

   Note: The HIGHSD flag appears because the C_T values exceed the expected range due to low expression of the CCKAR gene in the Heart sample and the GH1 gene in the Lung sample.

4. *(Optional)* For those flags with frequency greater than 0, click the troubleshooting link to view information on correcting the flag.

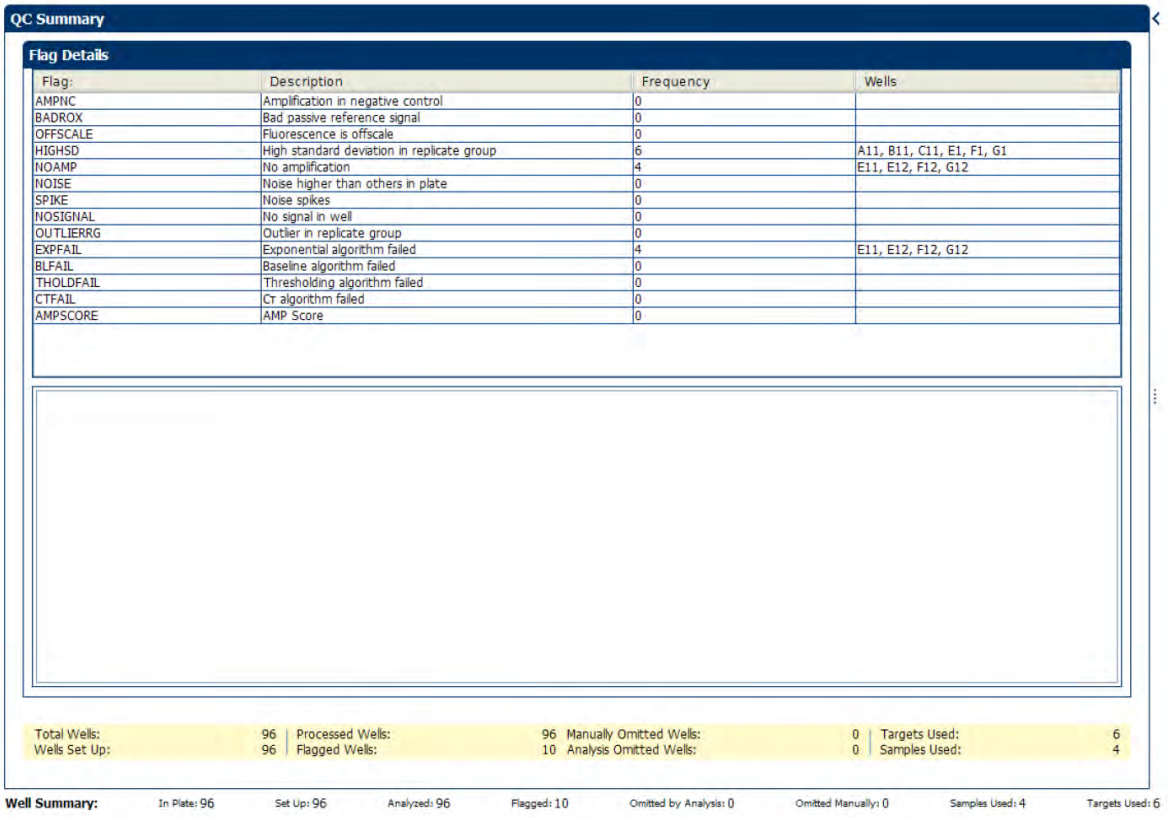


Figure 70 QC Summary screen for the example experiment

Possible flags

The flags listed below may be triggered by the experiment data.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed

(continued)

Flag	Description
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
AMPSCORE	Amplification in the linear region is below a certain threshold, corresponding to the score set in the analysis settings
Secondary analysis flags	
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in the negative control
HIGHSD	High standard deviation in replicate group

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the **Frequency** column for each flag).

For more information

Information	Reference
Publishing data	Chapter 1, “General information and instructions”

Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

The **Analysis Settings** dialog box displays the analysis settings for the threshold cycle (C_T), flags, and advanced options.

If the default analysis settings in the QuantStudio™ 12K Flex Software are not suitable for your own experiment, you can change the settings in the **Analysis Settings** dialog box, then reanalyze your experiment.

View the analysis settings

1. In the **Experiment Menu** pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the **Analysis Settings** dialog box.
   In the example experiment, the default analysis settings are used for each tab:
   - C_T Settings
   - Flag Settings
   - Relative Quantification Settings
   - Advanced Settings

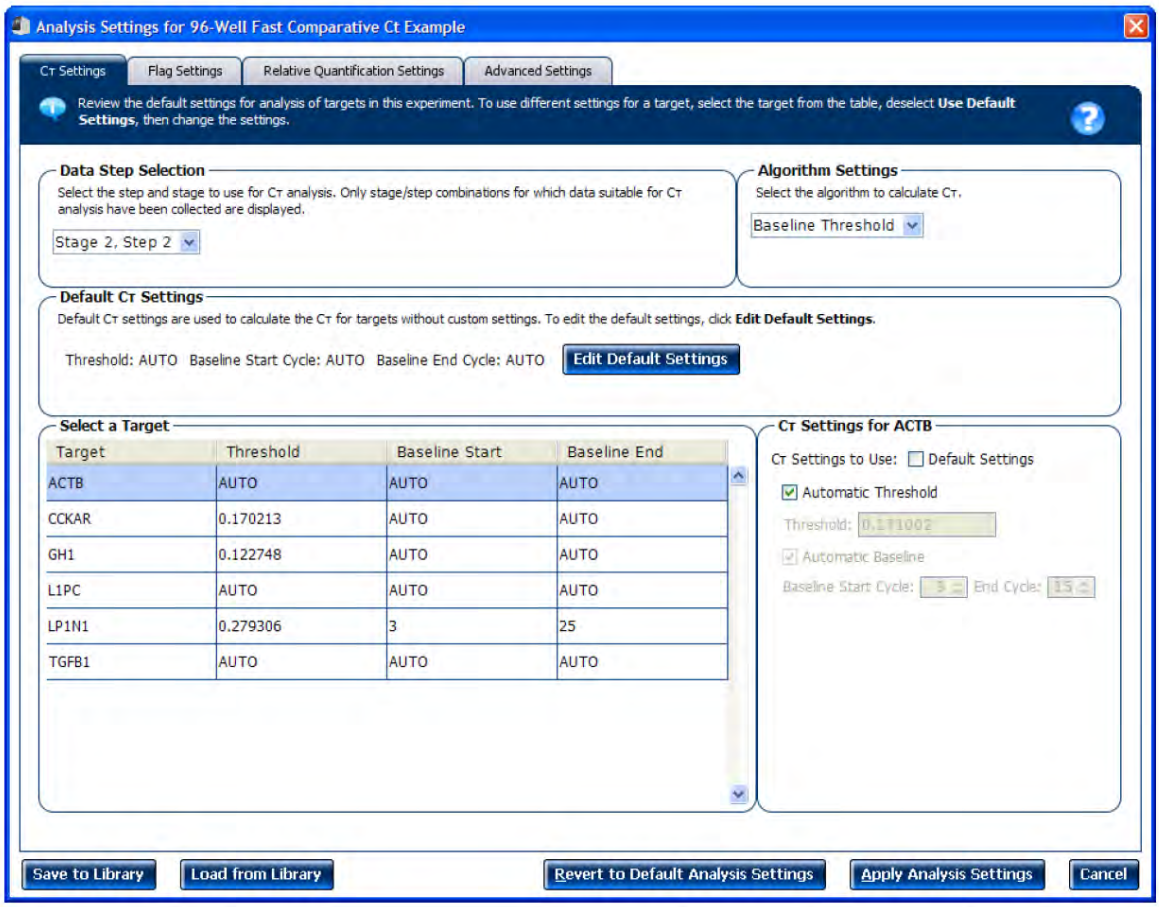


Figure 71 Analysis Settings dialog box for a Comparative C_T experiment

3. View and, if necessary, change the analysis settings (see “Adjust analysis settings” on page 246).

Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see “About the analysis settings library” on page 62.

4. Click **Apply Analysis Settings** to apply the current analysis settings.

Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

C_T settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method.

- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

  The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for quantification.

  The Relative Threshold algorithm is a well-based analysis based on the PCR reaction efficiency and fitted to the Amplification curve. This setting is ideal for a single sample across genes with no dependence on targets, thereby reducing variability. It is not necessary to set either a baseline or a threshold when you use the Relative Threshold algorithm, so any settings for baseline or threshold will not affect the analysis.

- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the targets that do not have custom settings. To edit the default settings, click **Edit Default Settings**.

- **C_T Settings for Target**

  When you manually set the threshold and baseline, we recommend the following settings:

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

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag settings

Use the **Flag Settings** tab to perform the following tasks:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the QuantStudio™ 12K Flex Software.

1. In the **Use** column, select the check boxes for flags to apply during analysis.

2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

3. In the **Reject Well** column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations may change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.

4. Click **Apply Analysis Settings** in the **Analysis Settings** dialog box.

   If the run status is complete, the data are reanalyzed.

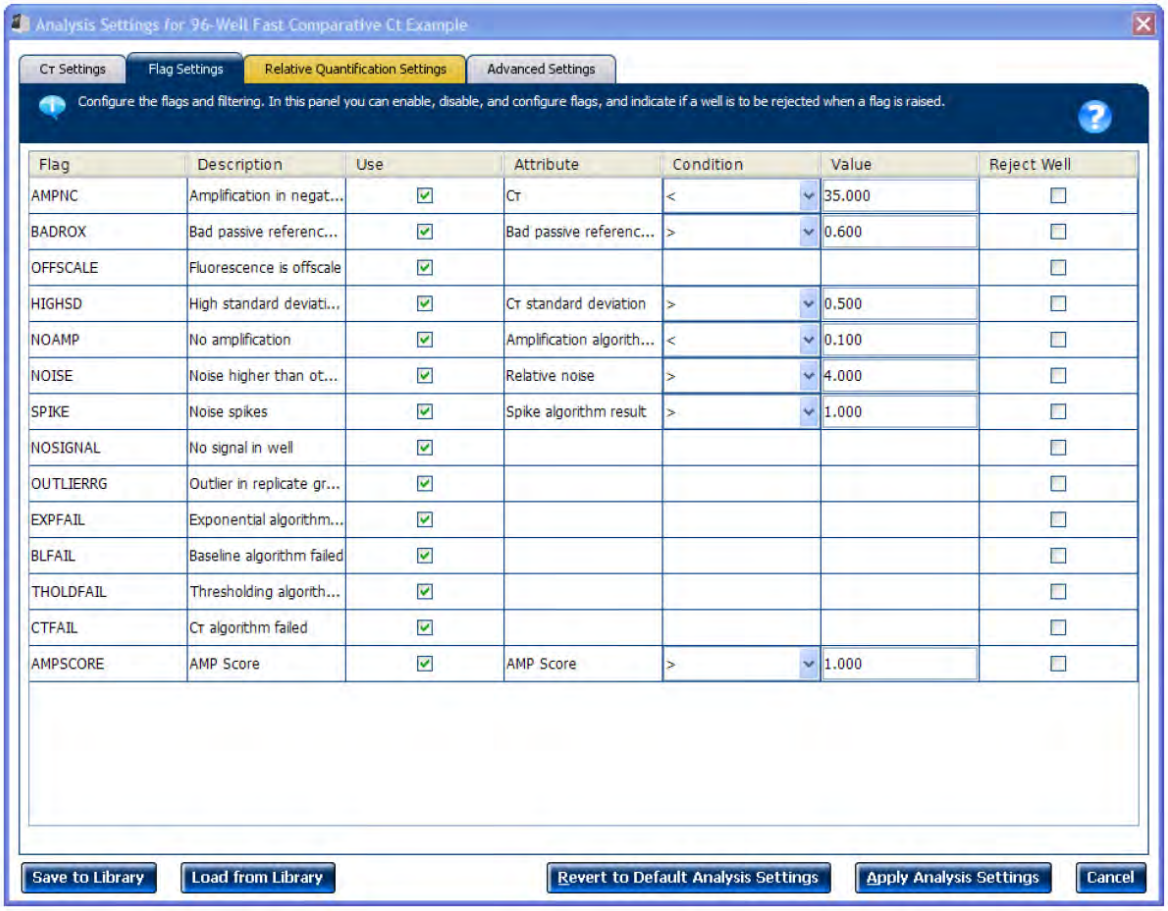


Figure 72 Flag Settings tab

Relative quantification settings

Use the **Relative Quantification Settings** tab to:

- Change the type of analysis, singleplex or multiplex.
- Change the reference sample and/or endogenous control.
- Reject Outliers with ΔC_T values less than or equal to the entered value.

  Note: The Outlier Rejection settings apply only to multiplex reactions.

- Select the algorithm to use to determine 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. Select the confidence level to use.
  - **Standard Deviations**—Select to calculate the RQ minimum and maximum values based on the selected number of standard deviations. Select the number of standard deviations to use.

Advanced settings

Use the **Advanced Settings** tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold setting.

The following steps describe the use of custom baseline settings for a well-target combination.

1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** checkbox.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** checkbox.
   - To define the baseline, deselect the **Automatic Baseline** checkbox, then enter the baseline start cycle and baseline end cycle.

Improve C_T precision by omitting wells

Experimental error may cause some wells to be amplified insufficiently or not at all. These wells typically produce C_T values that differ significantly from the average for the associated replicate wells. If included in the calculations, these outliers can result in erroneous measurements. To support C_T precision, omit the outliers from the analysis.

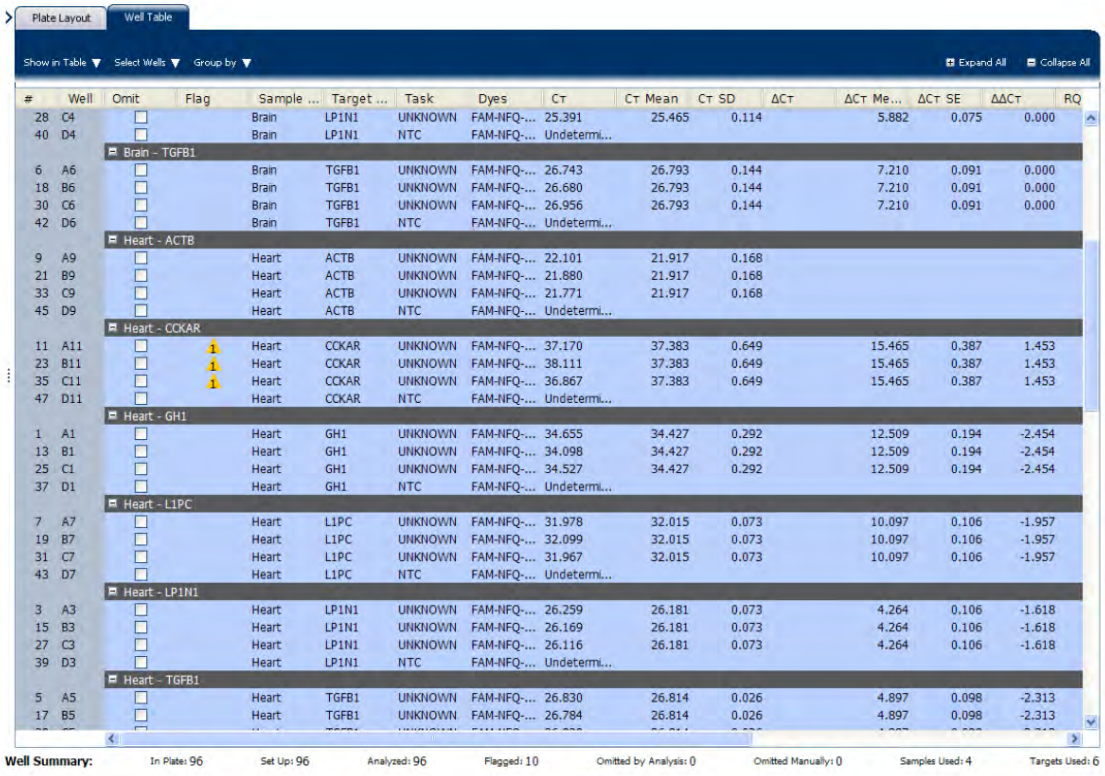
In the Comparative C_T example experiment, there are seven outliers.

1. In the **Experiment Menu** pane, select **Analysis** ▸ **Amplification Plot**.

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

2. In the **Amplification Plot** screen, select **C_T vs Well** from the **Plot Type** dropdown lit.
3. Select the **Well Table** tab.

4. In the Well Table, identify outliers.
   a. From the **Group By** dropdown list, select **Replicate**.
   b. Look for outliers in the replicate group (make sure they are flagged). In the example experiment, wells A11, B11, C11, E1, F1, E11, E12, F11, F12, and G12 have outliers.



#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	Ct	Ct Mean	Ct SD	ΔCt	ΔCt Me...	ΔCt SE	ΔΔCt	RQ
28	C4	☐		Brain	LP1N1	UNKNOWN	FAM-NFQ-...	25.391	25.465	0.114		5.882	0.075	0.000	
40	D4	☐		Brain	LP1N1	NTC	FAM-NFQ-...	Undetermi...							
		Brain - TGFB1													
6	A6	☐		Brain	TGFB1	UNKNOWN	FAM-NFQ-...	26.743	26.793	0.144		7.210	0.091	0.000	
18	B6	☐		Brain	TGFB1	UNKNOWN	FAM-NFQ-...	26.680	26.793	0.144		7.210	0.091	0.000	
30	C6	☐		Brain	TGFB1	UNKNOWN	FAM-NFQ-...	26.956	26.793	0.144		7.210	0.091	0.000	
42	D6	☐		Brain	TGFB1	NTC	FAM-NFQ-...	Undetermi...							
		Heart - ACTB													
9	A9	☐		Heart	ACTB	UNKNOWN	FAM-NFQ-...	22.101	21.917	0.168					
21	B9	☐		Heart	ACTB	UNKNOWN	FAM-NFQ-...	21.880	21.917	0.168					
33	C9	☐		Heart	ACTB	UNKNOWN	FAM-NFQ-...	21.771	21.917	0.168					
45	D9	☐		Heart	ACTB	NTC	FAM-NFQ-...	Undetermi...							
		Heart - CCKAR													
11	A11	☐	ℹ	Heart	CCKAR	UNKNOWN	FAM-NFQ-...	37.170	37.383	0.649		15.465	0.387	1.453	
23	B11	☐	ℹ	Heart	CCKAR	UNKNOWN	FAM-NFQ-...	38.111	37.383	0.649		15.465	0.387	1.453	
35	C11	☐	ℹ	Heart	CCKAR	UNKNOWN	FAM-NFQ-...	36.867	37.383	0.649		15.465	0.387	1.453	
47	D11	☐		Heart	CCKAR	NTC	FAM-NFQ-...	Undetermi...							
		Heart - GH1													
1	A1	☐		Heart	GH1	UNKNOWN	FAM-NFQ-...	34.655	34.427	0.292		12.509	0.194	-2.454	
13	B1	☐		Heart	GH1	UNKNOWN	FAM-NFQ-...	34.098	34.427	0.292		12.509	0.194	-2.454	
25	C1	☐		Heart	GH1	UNKNOWN	FAM-NFQ-...	34.527	34.427	0.292		12.509	0.194	-2.454	
37	D1	☐		Heart	GH1	NTC	FAM-NFQ-...	Undetermi...							
		Heart - L1PC													
7	A7	☐		Heart	L1PC	UNKNOWN	FAM-NFQ-...	31.978	32.015	0.073		10.097	0.106	-1.957	
19	B7	☐		Heart	L1PC	UNKNOWN	FAM-NFQ-...	32.099	32.015	0.073		10.097	0.106	-1.957	
31	C7	☐		Heart	L1PC	UNKNOWN	FAM-NFQ-...	31.967	32.015	0.073		10.097	0.106	-1.957	
43	D7	☐		Heart	L1PC	NTC	FAM-NFQ-...	Undetermi...							
		Heart - LP1N1													
3	A3	☐		Heart	LP1N1	UNKNOWN	FAM-NFQ-...	26.259	26.181	0.073		4.264	0.106	-1.618	
15	B3	☐		Heart	LP1N1	UNKNOWN	FAM-NFQ-...	26.169	26.181	0.073		4.264	0.106	-1.618	
27	C3	☐		Heart	LP1N1	UNKNOWN	FAM-NFQ-...	26.116	26.181	0.073		4.264	0.106	-1.618	
39	D3	☐		Heart	LP1N1	NTC	FAM-NFQ-...	Undetermi...							
		Heart - TGFB1													
5	A5	☐		Heart	TGFB1	UNKNOWN	FAM-NFQ-...	26.830	26.814	0.026		4.897	0.098	-2.313	
17	B5	☐		Heart	TGFB1	UNKNOWN	FAM-NFQ-...	26.784	26.814	0.026		4.897	0.098	-2.313	

c. Select the **Omit** checkbox next to outlying well or outlying wells.

#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	CT	CT Mean	CT SD	ΔCT	ΔCT Me...	ΔCT SE	ΔΔCT	RQ
28	C4	☐		Brain	LP1N1	UNKNOWN	FAM-NFQ-...	25.391	25.465	0.114		5.882	0.075	0.000	
40	D4	☐		Brain	LP1N1	NTC	FAM-NFQ-...	Undetermi...							
		Brain - TGFB1													
6	A6	☐		Brain	TGFB1	UNKNOWN	FAM-NFQ-...	26.743	26.793	0.144		7.210	0.091	0.000	
18	B6	☐		Brain	TGFB1	UNKNOWN	FAM-NFQ-...	26.680	26.793	0.144		7.210	0.091	0.000	
30	C6	☐		Brain	TGFB1	UNKNOWN	FAM-NFQ-...	26.956	26.793	0.144		7.210	0.091	0.000	
42	D6	☐		Brain	TGFB1	NTC	FAM-NFQ-...	Undetermi...							
		Heart - ACTB													
9	A9	☐		Heart	ACTB	UNKNOWN	FAM-NFQ-...	22.101	21.917	0.168					
21	B9	☐		Heart	ACTB	UNKNOWN	FAM-NFQ-...	21.880	21.917	0.168					
33	C9	☐		Heart	ACTB	UNKNOWN	FAM-NFQ-...	21.771	21.917	0.168					
45	D9	☐		Heart	ACTB	NTC	FAM-NFQ-...	Undetermi...							
		Heart - CCKAR													
11	A11	☐	1	Heart	CCKAR	UNKNOWN	FAM-NFQ-...	37.170	37.383	0.649		15.465	0.387	1.453	
23	B11	☐	1	Heart	CCKAR	UNKNOWN	FAM-NFQ-...	38.111	37.383	0.649		15.465	0.387	1.453	
35	C11	☐	1	Heart	CCKAR	UNKNOWN	FAM-NFQ-...	36.867	37.383	0.649		15.465	0.387	1.453	
47	D11	☐		Heart	CCKAR	NTC	FAM-NFQ-...	Undetermi...							
		Heart - GH1													
1	A1	☐		Heart	GH1	UNKNOWN	FAM-NFQ-...	34.655	34.427	0.292		12.509	0.194	-2.454	
13	B1	☐		Heart	GH1	UNKNOWN	FAM-NFQ-...	34.098	34.427	0.292		12.509	0.194	-2.454	
25	C1	☐		Heart	GH1	UNKNOWN	FAM-NFQ-...	34.527	34.427	0.292		12.509	0.194	-2.454	
37	D1	☐		Heart	GH1	NTC	FAM-NFQ-...	Undetermi...							
		Heart - L1PC													
7	A7	☐		Heart	L1PC	UNKNOWN	FAM-NFQ-...	31.978	32.015	0.073		10.097	0.106	-1.957	
19	B7	☐		Heart	L1PC	UNKNOWN	FAM-NFQ-...	32.099	32.015	0.073		10.097	0.106	-1.957	
31	C7	☐		Heart	L1PC	UNKNOWN	FAM-NFQ-...	31.967	32.015	0.073		10.097	0.106	-1.957	
43	D7	☐		Heart	L1PC	NTC	FAM-NFQ-...	Undetermi...							
		Heart - LP1N1													
3	A3	☐		Heart	LP1N1	UNKNOWN	FAM-NFQ-...	26.259	26.181	0.073		4.264	0.106	-1.618	
15	B3	☐		Heart	LP1N1	UNKNOWN	FAM-NFQ-...	26.169	26.181	0.073		4.264	0.106	-1.618	
27	C3	☐		Heart	LP1N1	UNKNOWN	FAM-NFQ-...	26.116	26.181	0.073		4.264	0.106	-1.618	
39	D3	☐		Heart	LP1N1	NTC	FAM-NFQ-...	Undetermi...							
		Heart - TGFB1													
5	A5	☐		Heart	TGFB1	UNKNOWN	FAM-NFQ-...	26.830	26.814	0.026		4.897	0.098	-2.313	
17	B5	☐		Heart	TGFB1	UNKNOWN	FAM-NFQ-...	26.784	26.814	0.026		4.897	0.098	-2.313	

5. Click **Analyze** to reanalyze the experiment data with the outlying well or outlying wells removed from the analysis.

Note: You can also omit undesirable wells in an experiment from the **Plate Layout** screen. To omit a well from the **Plate Layout** screen, right-click the well, then click **Omit**.

---

20 Export analysis results

1. Open the Comparative C_T example experiment file that has been analyzed.

   For information about analysis, see Chapter 19, “Review results and adjust experiment parameters”.

2. In the **Experiment Menu** pane, click **Export**.

   Note: To export data automatically after analysis, select the **Auto Export** checkbox during experiment setup or before running the experiment. The **Auto Export** checkbox is unchecked for the example experiment.

3. In the **Format** dropdown list, select **QuantStudio 12K Flex format**.

   Note: In the **Format** dropdown list, select **7900** if you want to export the Clipped Data.

4. Complete the **Export** dialog box as shown below.

Field or Selection	Entry
Select Data to export/Select Content	Results
Export Data To options	**One File** radio button
Export File Name field	***96-Well Fast Comparative CT Example_QuantStudio_export***
File Type dropdown list	***.txt**
Export File Location field	Use the default file location or click **Browse** to select a different location

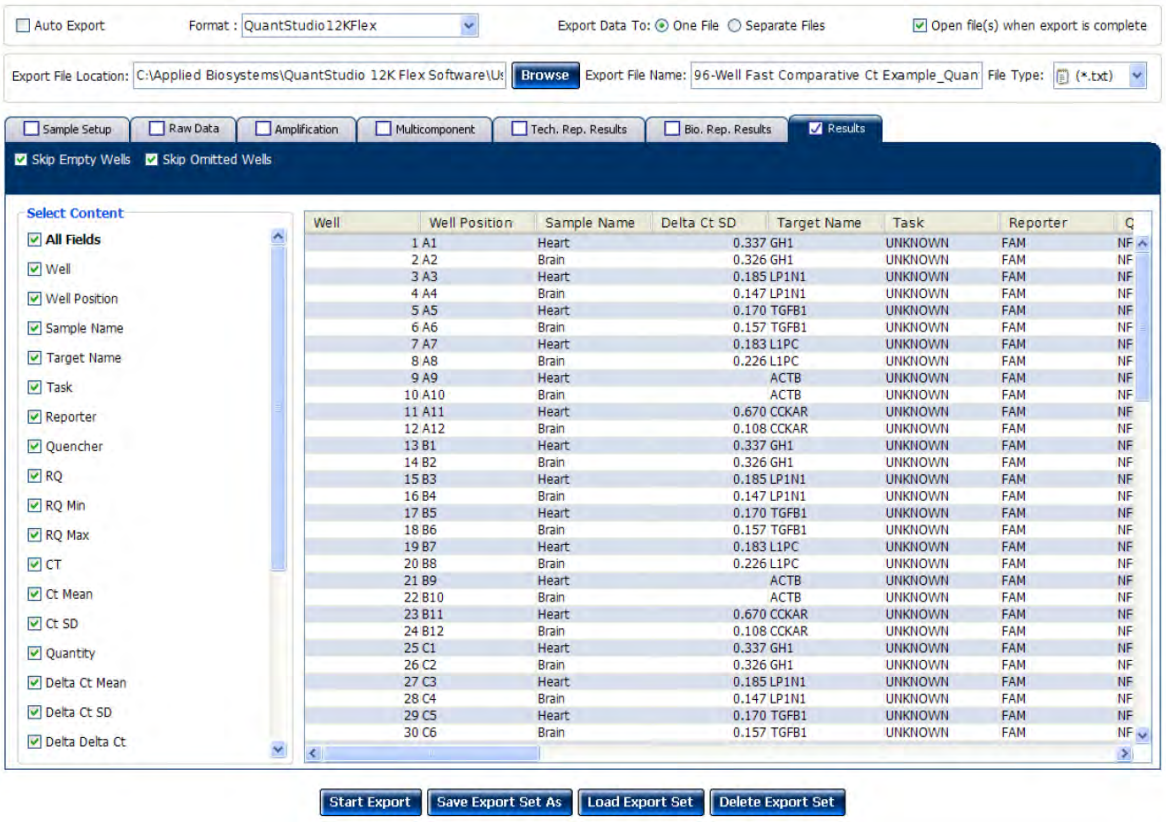


Figure 73 Export screen

96-Well Fast Comparative Ct Example_QuantStudio_export.txt - Notepad

File Edit Format View Help

```
* Barcode = NA
* Block Type = 96-Well Block (0.2mL)
* Calibration Background is expired  = No
* Calibration Background performed on  = 2011-08-08 01:15:53 AM SGT
* Calibration FAM is expired  = No
* Calibration FAM performed on  = 2011-08-08 01:39:58 AM SGT
* Calibration ROI is expired  = No
* Calibration ROI performed on  = 2011-08-08 01:05:24 AM SGT
* Calibration ROX is expired  = No
* Calibration ROX performed on  = 2011-08-08 02:07:15 AM SGT
* Calibration SYBR is expired  = No
* Calibration SYBR performed on  = 2011-08-08 01:58:11 AM SGT
* Calibration TAMRA is expired  = No
* Calibration TAMRA performed on  = 2011-08-08 02:16:10 AM SGT
* Calibration Uniformity is expired  = No
* Calibration Uniformity performed on  = 2011-08-08 01:24:47 AM SGT
* Calibration VIC is expired  = No
* Calibration VIC performed on  = 2011-08-08 01:49:09 AM SGT
* Chemistry = TAQMAN
* Comment = NA
* Date Created = 2011-12-14 12:54:29 PM SGT
* Experiment File Name = C:\Program Files\Applied Biosystems\QuantStudio12KFlex\examples\Gene Expression\Comparative Ct\96-Well Fast
Comparative Ct Example.eds
* Experiment Name = 96-Well Fast Comparative Ct Example
* Experiment Run End Time = 2011-09-28 13:33:38 PM SGT
* Experiment Type = Comparative CT (ΔΔCT)
* Instrument Name = QuantStudioDemo
* Instrument Serial Number = QuantStudioDemo
* Instrument Type = QuantStudio 12K Flex
* Passive Reference = ROX
* Quantification Cycle Method = Ct
* Signal Smoothing On = true
* Stage/ Cycle where Analysis is performed = Stage 2, Step 2
* User Name = NA

[Results]
Well    Well Position   Sample Name     Target Name     Task    Reporter        Quencher        RQ      RQ Min  RQ Max  CT      Ct Mean Ct
SD      Quantity        Delta Ct Mean   Delta Ct SD     Delta Delta Ct  Automatic Ct Threshold  Ct Threshold    Automatic Baseline
Baseline Start  Baseline End    Efficiency      EXPFAIL HIGHSD  NOAMP
1       A1      Heart   GH1     UNKNOWN FAM     NFQ-MGB 5.479   3.769   7.965   34.655  34.427  0.292           12.509  0.337   -2.454
false   0.123   true    3       29      1.000   N       N       N
2       A2      Brain   GH1     UNKNOWN FAM     NFQ-MGB 1.000   0.696   1.436   34.186  34.546  0.320           14.963  0.326   0.000
false   0.123   true    3       30      1.000   N       N       N
3       A3      Heart   LP1N1   UNKNOWN FAM     NFQ-MGB 2.648   2.157   3.252   25.965  25.884  0.076           3.967   0.185   -1.405
true    0.279   true    3       20      1.000   N       N       N
4       A4      Brain   LP1N1   UNKNOWN FAM     NFQ-MGB 1.000   0.850   1.177   24.883  24.955  0.133           5.372   0.147   0.000
true    0.279   true    3       19      1.000   N       N       N
5       A5      Heart   TGFB1   UNKNOWN FAM     NFQ-MGB 4.970   4.114   6.005   26.830  26.814  0.026           4.897   0.170   -2.313
true    0.376   true    3       21      1.000   N       N       N
6       A6      Brain   TGFB1   UNKNOWN FAM     NFQ-MGB 1.000   0.840   1.191   26.743  26.793  0.144           7.210   0.157   0.000
true    0.376   true    3       21      1.000   N       N       N
7       A7      Heart   L1PC    UNKNOWN FAM     NFQ-MGB 3.882   3.166   4.760   31.978  32.015  0.073           10.097  0.183   -1.957
true    0.422   true    3       25      1.000   N       N       N
```

Figure 74 Exported file

Part V

Running genotyping experiments

21 Overview of genotyping experiments

IMPORTANT! First-time users of the QuantStudio™ 12K Flex Real–Time PCR System, see the part "Getting started" on page 17 and Appendix A, "Documentation and support". The sections provide information and general instructions that are applicable to all the experiments described in this document.

Note: For more information about any of the topics discussed in this guide, access the Help from within the QuantStudio™ 12K Flex Software by pressing **F1,** clicking in the toolbar, or selecting **Help ▸ QuantStudio 12K Flex Software Help**.

Overview of data collection

Genotyping experiments are performed to detect single nucleotide polymorphism (SNP) variants of a target nucleic acid sequence in samples. The PCR reactions contain primers designed to amplify the sequence containing the SNP and reagents to detect two different alleles.

You can collect the results of a genotyping experiment in two different ways: At the end of the experiment, or continuously during the experiment. Data collection at the end of the experiment is called end-point data collection. Data collection during the experiment run is considered real-time PCR. The real-time data helps further data analysis.

In end-point data collection, the normalized intensity of the reporter dye, or Rn, is the data collected. Some end-point experiments also include pre-PCR (data collected before the amplification process) data collection. The system calculates the delta Rn (ΔRn) value per the following formula:

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

Overview of the TaqMan™ SNP Genotyping Assay

A genotyping assay detects variants of a single nucleic acid sequence, without quantifying the target. The presence of two probes in each reaction allows genotyping of the two possible variants at the single nucleotide polymorphism (SNP) site in a target sequence.

Each TaqMan™ SNP Genotyping Assay consists of a single, ready-to-use tube containing the following items:

- Two sequence-specific primers for amplifying the polymorphism of interest
- Two allele-specific TaqMan™ MGB probes for detecting the alleles for the specific polymorphism of interest

Overview of TaqMan™ MGB probes

Each allele-specific TaqMan™ MGB probe has:

- A reporter dye at its 5' end:
  - VIC™ dye is linked to the 5' end of the Allele 1 probe.
  - FAM™ dye is linked to the 5' end of the Allele 2 probe.

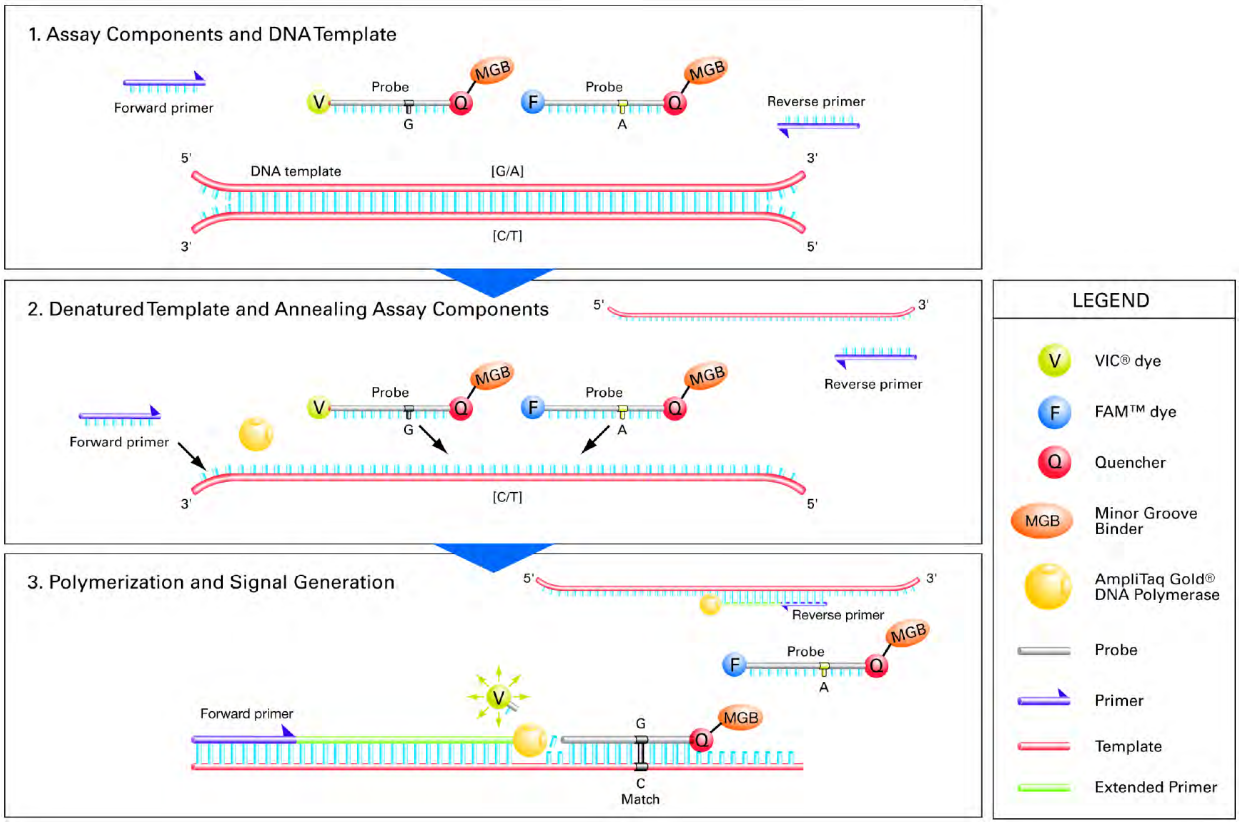
The Allele 1 VIC™ dye-labeled probe corresponds to the first nucleotide inside the square brackets of the context sequence in the assay information file (AIF) shipped with each order. The Allele 2 FAM™ dye-labeled probe corresponds to the second nucleotide inside the square brackets of the context sequence in the AIF. For the context sequence ATCGATT[G/T]ATCC, the VIC™ dye-labeled probe binds to the G allele, and the FAM™ dye-labeled probe to the T allele.

- A minor groove binder (MGB), which increases the melting temperature (T_m) for a given probe length and allows the design of shorter probes. The use of shorter probes results in greater differences in T_m values between matched and mismatched probes, and more robust genotyping.
- A non-fluorescent quencher (NFQ) at its 3' end, which allows for detection of the reporter dye fluorescence with greater sensitivity than with a fluorescent quencher.

Overview of the 5' nuclease assay

The figure below is a schematic depiction of the 5' nuclease assay. During PCR:

- Each TaqMan™ MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites.
- When the oligonucleotide probe is intact, the proximity of the quencher dye to the reporter dye quenches the reporter signal.
- AmpliTaq Gold™ DNA polymerase extends the primers bound to the genomic DNA template.
- AmpliTaq Gold™ DNA polymerase (with its 5' nuclease activity) cleaves probes that are hybridized to the target sequence.
- Cleavage of the probes hybridized to the target sequence separates the quencher dye from the reporter dye, resulting in increased fluorescence by the reporter. The fluorescence signal generated by PCR amplification indicates which alleles are present in the sample.



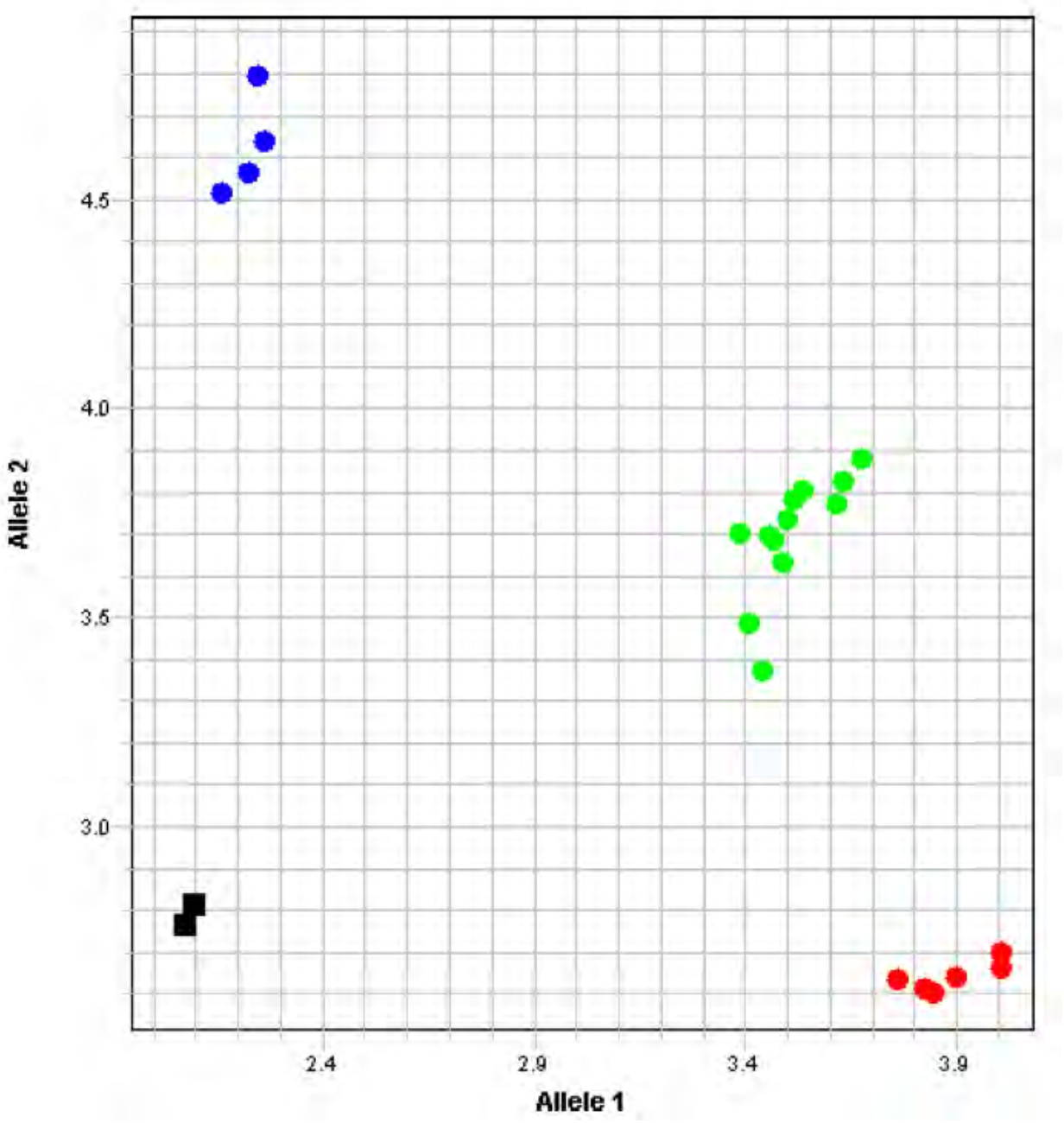
Minimizing non-specific fluorescence

In TaqMan™ assays, fluorescence from nonspecifically bound probes is reduced, because nucleotide mismatches between a probe and a sequence reduce the chances that the probe will be cleaved. The short length of the probe means that a one-base-pair mismatch will a have a larger negative effect on the binding. The mismatched probe will not bind tightly to the allele. The AmpliTaq Gold™ DNA polymerase will likely displace the probe without cleaving the dye.

Reading and analyzing the plates

The QuantStudio™ 12K Flex Software genotypes the DNA samples from the reaction plate simultaneously. First, the software normalizes the fluorescence of the reporter dyes to the fluorescence of the passive reference dye in each well. Next, the software plots the normalized intensities (Rn) of the reporter dyes in each sample well on an Allelic Discrimination Plot, which contrasts the reporter dye intensities of the allele-specific probes. Finally, the QuantStudio™ 12K Flex Software algorithmically clusters the sample data, and assigns a genotype call to the samples of each cluster according to its position on the plot.

Note: The QuantStudio™ 12K Flex Software clustering algorithm does not call genotypes when only one genotype is present in an experiment.



The clustering of datapoints can vary along the horizontal axis (Allele 1), vertical axis (Allele 2), or diagonal (Allele 1/Allele 2). This variation results from differences in the extent of reporter dye fluorescent intensity after PCR amplification. The table below shows the correlation between fluorescence signals and sequences in a sample.

Observation	Indication
VIC™ dye-labeled probe fluorescence only	Homozygosity for Allele 1
FAM™ dye-labeled probe fluorescence only	Homozygosity for Allele 2
Both VIC™ and FAM™ dye-labeled probes fluorescence	Allele 1-Allele 2 heterozygosity

About the example experiment

To illustrate how to perform genotyping experiments, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with a QuantStudio™ 12K Flex Real–Time PCR System.

The objective of the example genotyping experiment is to investigate SNP rs8039, where possible genotypes are AA, AC, and CC. In the example, 19 unknown genomic DNA (gDNA) samples were genotyped using TaqMan™ Drug Metabolism Genotyping Assay ID C___1240647_1_ and C___1213693_10. The reactions were set up so that the PCR primers and probes that target both alleles of SNP rs8039 were present in the same well. The PCR was performed using the TaqMan™ Genotyping Master Mix.

22 Design the experiment

This chapter explains how to design the example experiment from the **Setup** menu in the **Experiment Menu** pane.

Note: To automatically export the analyzed data to a specified location, select the **Auto Export** checkbox in the **Export** screen, before running the experiment. For more information on Auto Export, see the part "Getting started" on page 17.

Define the experiment properties

In the **Experiment Menu** pane, click **Setup** ▸ **Experiment Properties**.

Enter or select the following information.

Field or selection	Entry
Experiment Name field	Enter ***96-Well Genotyping Example***.
Barcode field	Leave the **Barcode** field empty.
User Name field	Enter ***Example User*** or enter a user name.
Comments field	Enter ***Genotyping example***.
Block	Select **96-Well (0.2 mL)**.
Experiment Type	Select **Genotyping**.
Reagents	Select **TaqMan Reagents**.
Ramp speed	Select **Standard**.

Select all three data collection check boxes.

- **Pre-PCR Read** checkbox
- **Amplification** checkbox
- **Post-PCR Read** checkbox

Save the experiment.

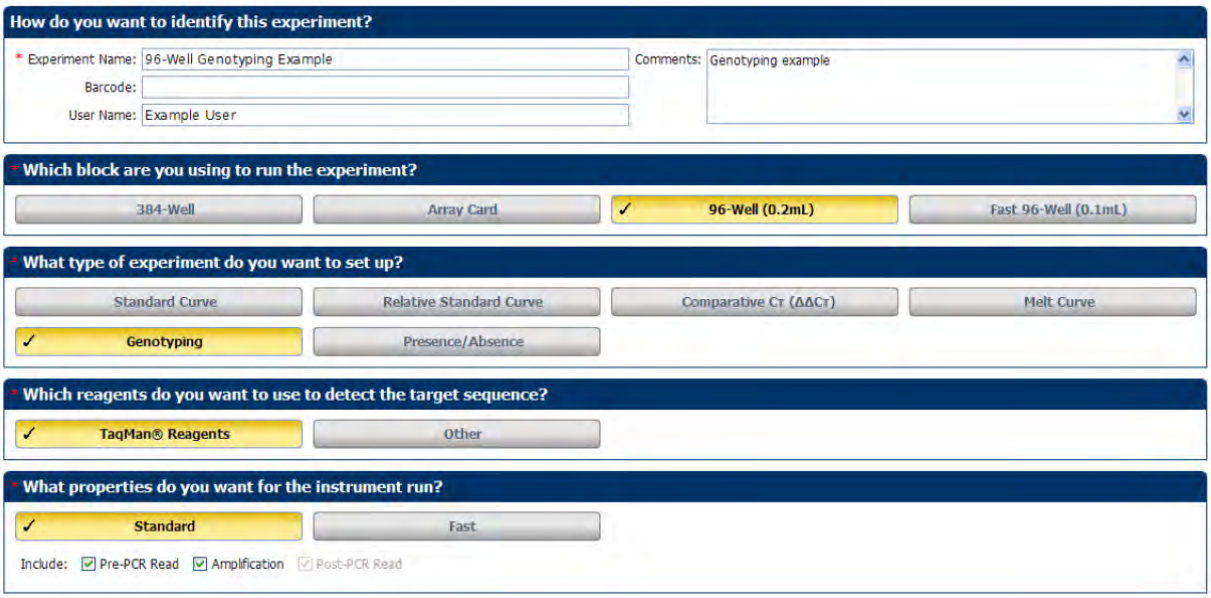


Figure 75 Experiment Properties screen

Define SNPs and samples

1. Click **Define** to access the **Define** screen.
2. Enter the following SNP assay information.

SNP assay name	NCBI SNP reference	Context sequence	Allele 1	Reporter	Quencher	Allele 2	Reporter	Quencher	Color
SNP Assay 1	—[1]	—[1]	*Allele 1*	VIC	NFQ-MGB	*Allele2*	FAM	NFQ-MGB	
SNP Assay 2	—[1]	—[1]	*Allele 1*	VIC	NFQ-MGB	*Allele2*	FAM	NFQ-MGB	

[1] The NCBI SNP reference and Context sequence fields are optional fields and are used for reference. They are not required to run an experiment.

3. Enter the following sample information.

Sample name	Color	Sample name	Color
Sample 1		*Sample 11*	
Sample 2		*Sample 12*	

(continued)

Sample name	Color	Sample name	Color
Sample 3		*Sample 13*	
Sample 4		*Sample 14*	
Sample 5		*Sample 15*	
Sample 6		*Sample 16*	
Sample 7		*Sample 17*	
Sample 8		*Sample 18*	
Sample 9		*Sample 19*	
Sample 10		—	—

4. In the **Passive Reference** dropdown list, select **ROX** .

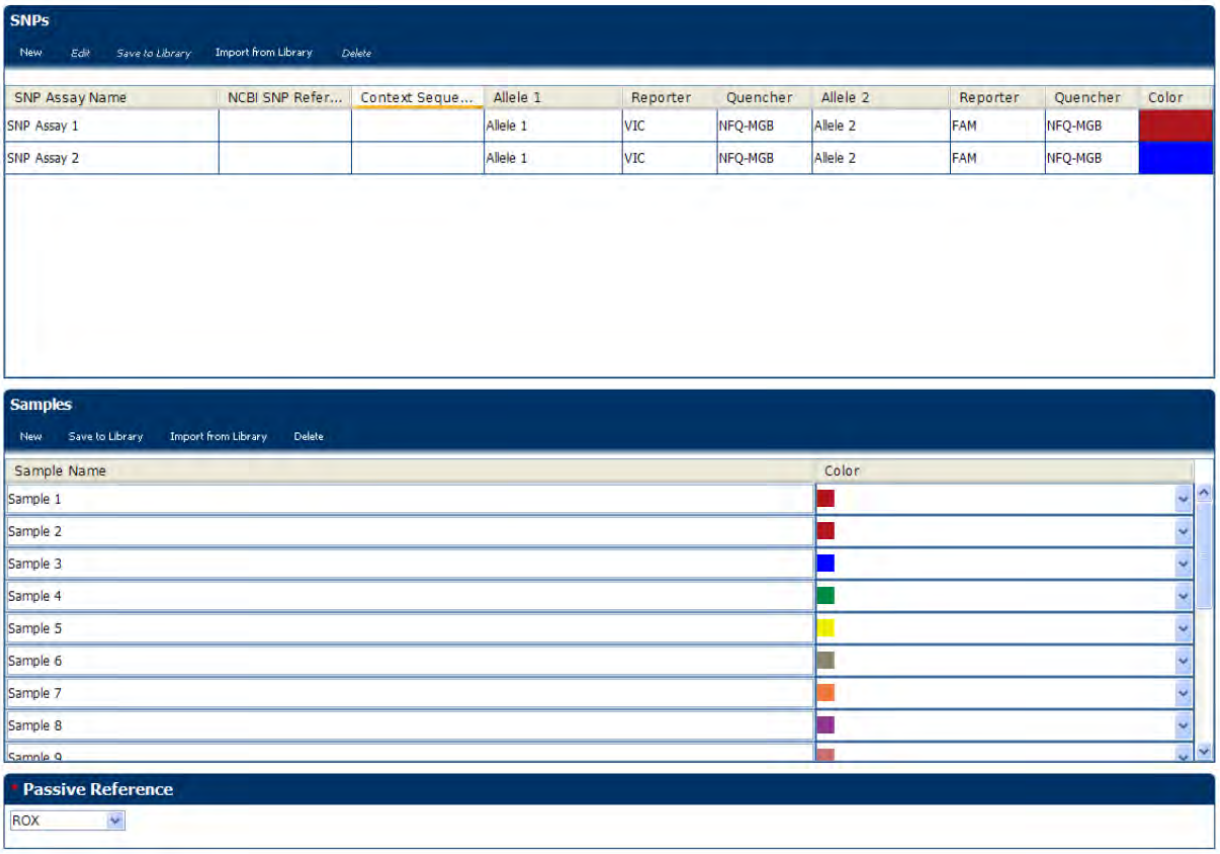


Figure 76 Define screen

Note: This example experiment does not define biological replicate groups. Leave the **Biological Replicate Groups** pane blank.

Assign markers and samples

Click **Assign** to access the **Assign** screen.

Enter the following SNPs and samples.

Table 3 SNP Assay 1

Target name	Well number	Task	Sample
SNP Assay 1	A1, E1	Unknown	Sample 1
	A2, E2		Sample 2
	A3, E3		Sample 3
	A4, E4		Sample 4
	A5, E5		Sample 5

Table 3 SNP Assay 1 *(continued)*

Target name	Well number	Task	Sample
SNP Assay 1	A6, E6	Unknown	Sample 6
	B1, F1		Sample 7
	B2, F2		Sample 8
	B3, F3		Sample 9
	B4, F4		Sample 10
	B5, F5		Sample 11
	B6, F6		Sample 12
	C1, G1		Sample 13
	C2, G2		Sample 14
	C3, G3		Sample 15
	C4, G4		Sample 16
	C5, G5		Sample 17
	C6, G6		Sample 18
	D1, H1		Sample 19
SNP Assay 1	D2 - D6	No Template Control	—
	H2 - H6		

Table 4 SNP Assay 2

Target name	Well number	Task	Sample
SNP Assay 2	A7, E7	Unknown	Sample 1
	A8, E8		Sample 2
	A9, E9		Sample 3
	A10, E10		Sample 4
	A11, E11		Sample 5
	A12, E12		Sample 6
	B7, F7		Sample 7
	B8, F8		Sample 8
	B9, F9		Sample 9
	B10, F10		Sample 10

Table 4 SNP Assay 2 *(continued)*

Target name	Well number	Task	Sample
SNP Assay 2	B11, F11	Unknown	Sample 11
	B12, F12		Sample 12
	C7, G7		Sample 13
	C8, G8		Sample 14
	C9, G9		Sample 15
	C10, G10		Sample 16
	C11, G11		Sample 17
	C12, H12		Sample 18
	D7, H7		Sample 19
SNP Assay 2	D8 - D12	No Template Control	—
	H8 - H12		

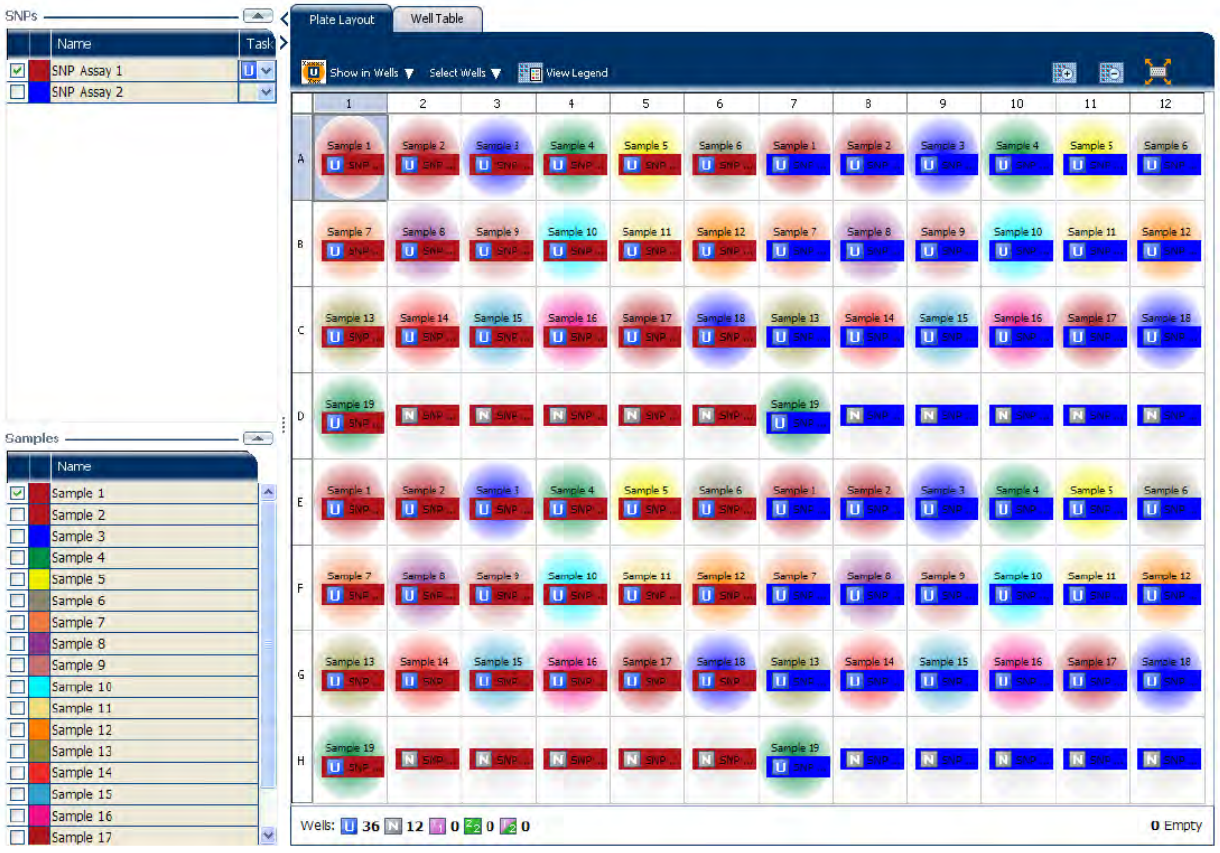


Figure 77 Assign screen

Set up the run method

Click **Run Method** to access the **Run Method** screen. Set the thermal profile under the **Graphical View** tab. Enter the following information:

- Reaction Volume Per Well: ***50 µL***
- Thermal profile

Stage	Step	Ramp rate	Temperature	Time
Pre-Read Stage	Step 1	1.6°C/s	60°C	30 seconds
Hold Stage	Step 1	1.6°C/s	95°C	10 minutes
PCR Stage • Number of Cycles: 40 (default) • Enable AutoDelta: Unchecked (default) • Starting Cycle: Disabled when Enable AutoDelta is unchecked	Step 1	1.6°C/s	95°C	15 seconds
	Step 2	1.6°C/s	60°C	1 minute
Post-Read Stage	Step 1	1.6°C/s	60°C	30 seconds

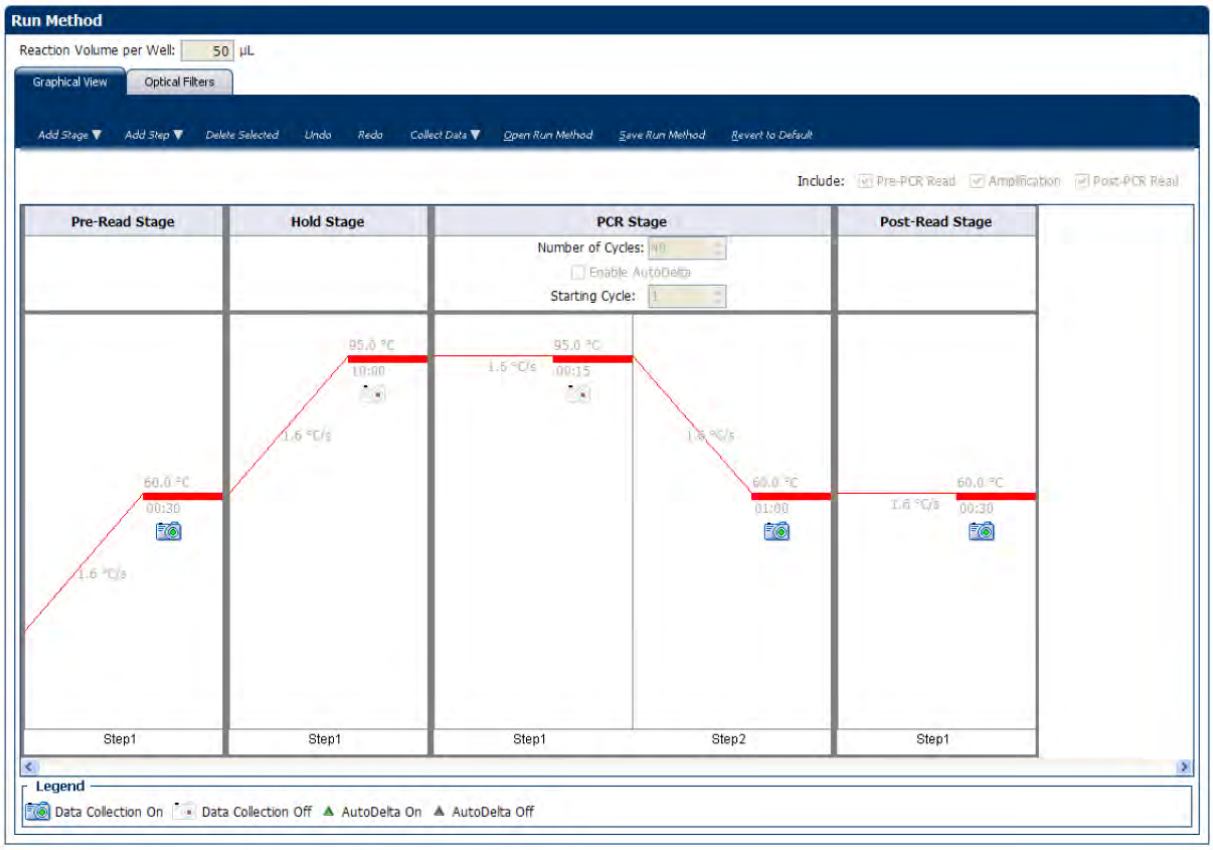


Figure 78 Run Method screen

For more information

Information	Reference
Consumables	Chapter 1, “General information and instructions”
Using alternative setup	Chapter 2, “Experiment shortcuts”

23 Prepare the reactions

This chapter explains how to prepare the PCR reactions for the Genotyping example experiment.

Assemble required materials

- Items listed in the part "Getting started" on page 17
- Samples—Sample 1 to Sample 19
- Example experiment reaction mix components:
  - TaqMan™ Genotyping Master Mix (2X)
  - SNP 1 Assay Mix (20X)
  - SNP 2 Assay Mix (20X)

Prepare the sample dilutions

For the example experiment, two targets are assigned to 38 wells each. Each well contains 20 ng of Coriell DNA. The stock concentration is 10 ng/µL.

1. Label a separate microcentrifuge tube for each sample to be diluted.

   Note: You can also use a MicroAmp™ Optical 96-Well Reaction Plate to prepare the sample dilutions.

2. Add 2 µL of sample stock to each empty tube.
3. Add 48 µL of sterile water (diluent) to each tube, such that each working stock tube has a final concentration of 10 ng/µL
4. Vortex each diluted sample for 3 to 5 seconds, then centrifuge the tubes briefly.
5. Place the diluted samples on ice until you prepare the reaction plate.

Prepare the reaction mix

1. Label an appropriately sized tube for each reaction mix.
   - SNP 1 Reaction Mix
   - SNP 2 Reaction Mix

2. For SNP Assay 1, prepare a reaction mix by adding the required volumes of each component to the SNP 1 reaction tube.

Reaction component	Reaction volume			
	Per well		38 Reactions + 10% overage	
	Dry	Wet	Dry	Wet
TaqMan™ Genotyping Master Mix (2✕)	10.0 µL	10.0 µL	420.0 µL	420.0 µL
SNP Assay Mix (20✕)	1.0 µL	1.0 µL	42.0 µL	42.0 µL
Water, DNase-free	39.0 µL	37.0 µL	1,638.0 µL	1,554.0 µL
Total reaction mix volume	**50.00 µL**	**48.00 µL**	**2,100.0 µL**	**2,016.0 µL**

3. Gently pipette the reaction mix up and down, then cap the tube.
4. Centrifuge the tube briefly.
5. Place the reaction mixes on ice until you prepare the reaction plate.
6. Repeat step 2 to step 5 for the SNP 2 assay.

Note: Do not add the sample at this time.

Prepare the reaction plate

Example experiment reaction plate components

The reaction plate for the Genotyping example experiment contains:

- A MicroAmp™ Optical 96-Well Reaction Plate
- Reaction volume: 50 µL/well
- 76 Unknown wells

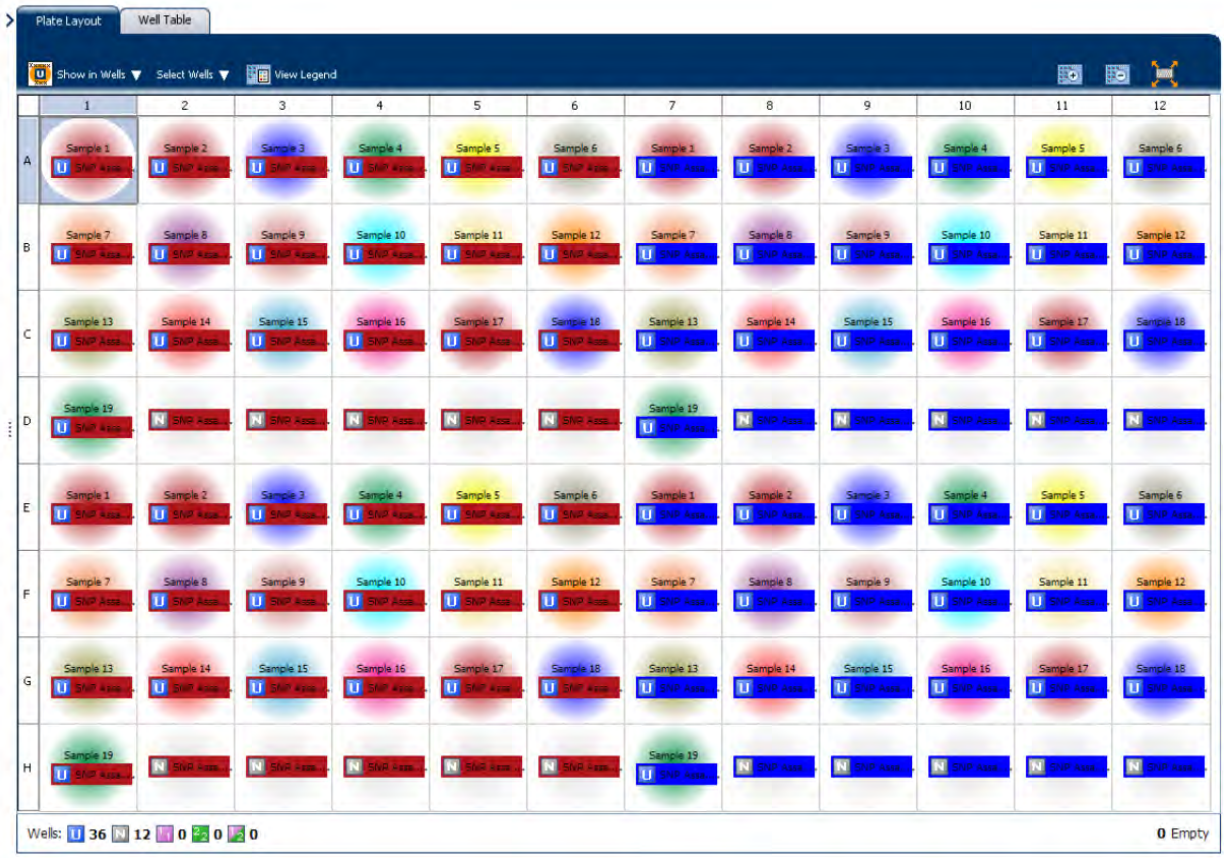


Figure 79 Plate layout for example experiment

To prepare the reaction plate: dried gDNA

1. Pipette 2.0 µL of the appropriate sample (20 ng of purified genomic DNA) into each well of the reaction plate.

   All wells belonging to the same Genotyping assay must contain approximately the same quantity of sample or control.

   Note: While preparing the reaction plate for your own Genotyping experiment, add between 1 ng and 20 ng of purified DNA per reaction.

2. Dry down the samples by evaporation at room temperature in a dark, amplicon-free location.

   Cover the reaction plate with a lint-free tissue while drying.

3. Transfer 48 µL of reaction mix to each well.

   IMPORTANT! Ensure sure that no cross-contamination occurs from well to well.

4. Seal the reaction plate with adhesive film.

5. Vortex the reaction plate for 3–5 seconds.

6. Briefly centrifuge the reaction plate.
7. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the plate again at a higher speed and for a longer period of time.

To prepare the reaction plate: wet gDNA

1. Add 2 µL of DNA to the appropriate wells.
2. Add 2 µL of water to wells containing the NTCs.
3. Transfer 48 µL of reaction mix to the appropriate wells.
4. Seal the reaction plate with optical adhesive film.
5. Vortex the reaction plate for 3–5 seconds, then briefly centrifuge it.
6. Centrifuge the reaction plate briefly.
7. Until you are ready to perform the run, place the reaction plate at 4°C, in the dark.

Tips for preparing reactions for your own experiments

Tips for preparing samples

When you prepare the samples for your own experiment, we recommend the following items:

- Use DNase-free water to dilute the samples.
- Use the same quantity of DNA per well for each experiment.

Tips for preparing the reaction mix

When you prepare the reaction mix for your own experiment, prepare the reactions for each SNP separately.

- Mix the master mix thoroughly by swirling the bottle.
- Resuspend the assay mix by vortexing, then centrifuge the tube briefly.
- Thaw frozen samples by placing them on ice. When thawed, resuspend the samples by vortexing, then centrifuge the tubes briefly.

Tips for preparing the reaction plate

- Ensure sure the reaction locations match the plate layout in the QuantStudio™ 12K Flex Software.
- Load 1–20 ng of purified genomic DNA per reaction
- All wells belonging to the same Genotyping assay must contain approximately the same quantity of sample or control.
- Multiple assays may be run on one reaction plate, but must be analyzed separately.

For more information

Information	Reference
Assigning the reaction plate components	Chapter 1, “General information and instructions”
Sealing the reaction plate	Chapter 1, “General information and instructions”

24 Run the experiment

This chapter explains how to run the example experiment on the QuantStudio™ 12K Flex Real-Time PCR Instrument.

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the instrument is in operation.

Start the run

1. Open the Genotyping example file that you created.
   See Chapter 22, “Design the experiment”.
2. Load the reaction plate into the instrument.
3. Start the run.

   Note: To collect real-time data during a run, click on the **Run Method** screen in the **Setup** menu.

Monitor the run

You can monitor an experiment run in three ways:

- From the **Run** screen of the QuantStudio™ 12K Flex Software, while the experiment is in progress
- From the **Instrument Console** screen of the QuantStudio™ 12K Flex Software, to monitor an experiment started from another computer or from the instrument touchscreen (see “Monitor a run from the software Instrument Console screen” on page 274)
- From the instrument touchscreen (see “Monitor a run from the instrument touchscreen” on page 277)

Monitor a run from the software Instrument Console screen

1. In the **Instrument Console** screen, select the icon of the instrument that you are using to run the experiment.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. In the **Instrument Manager** screen, click **Monitor Running Experiment** to access the **Run** screen.

View the amplification plot

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the QuantStudio™ 12K Flex Software for potential problems.

Click **Amplification Plot** from the **Run Experiment** menu, select the **Plate Layout** tab, then select the wells to view.

Note: The Amplification Plot is not available for experiments that do not include the PCR process.

Figure 80 The Amplification Plot screen as it appears at the end of the example experiment

View the temperature plot

Click **Temperature Plot** from the **Run Experiment** menu.

Figure 81 The Temperature Plot screen as it appears during the example experiment

Note: The sample temperature displayed in the **Current Temperatures** group is an estimated value.

View the run method

Click **Run Method** from the **Run Experiment** menu.

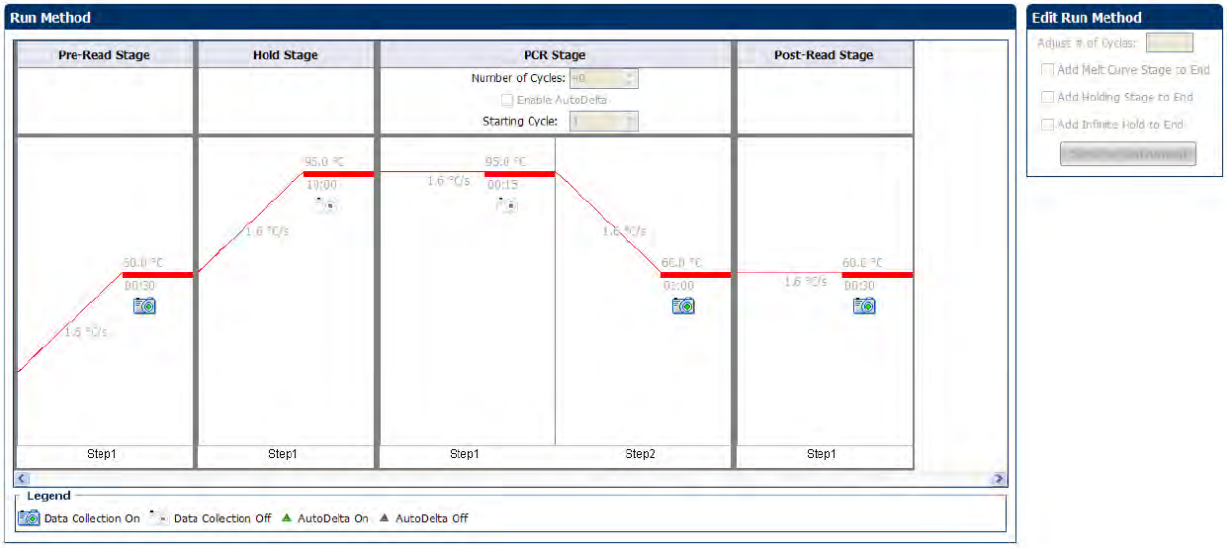


Figure 82 The Run Method screen as it appears in the example experiment

View run data

Click **View Run Data** from the **Run Experiment** menu.

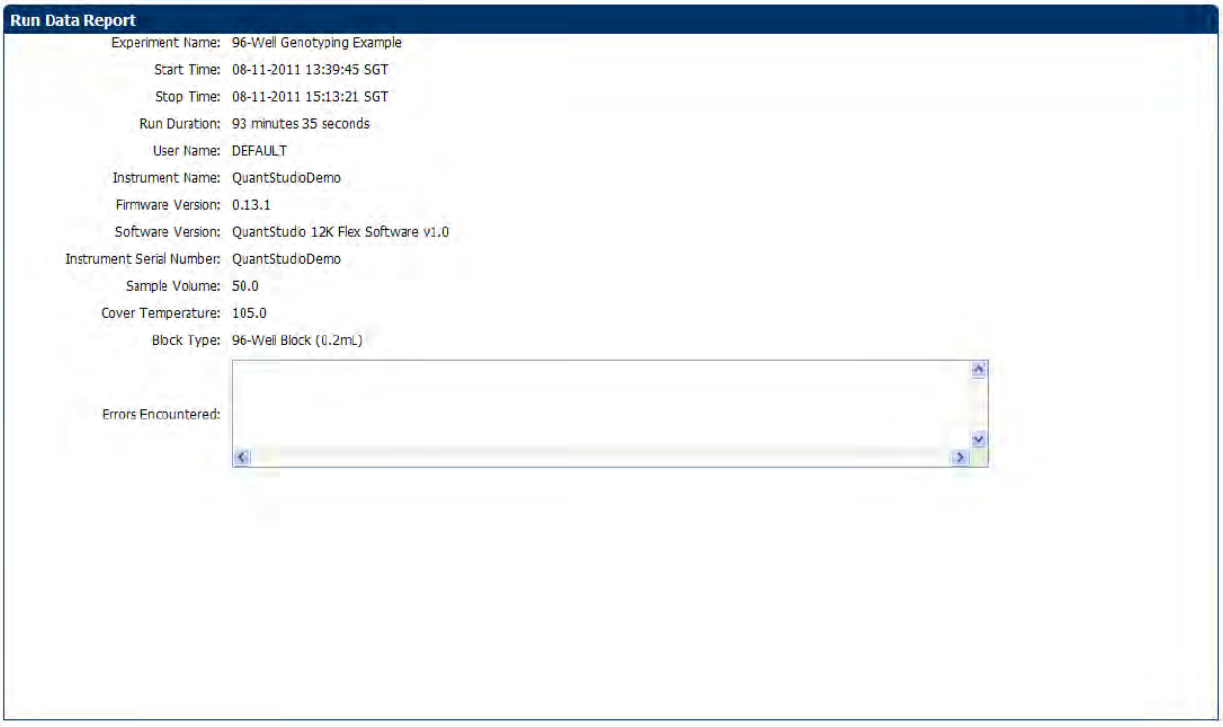


Figure 83 The View Run Data screen as it appears in the example experiment

Monitor a run from the instrument touchscreen

You can view the progress of the run from the instrument touchscreen.

The following figures are for visual representation only. Actual results vary with the experiment.

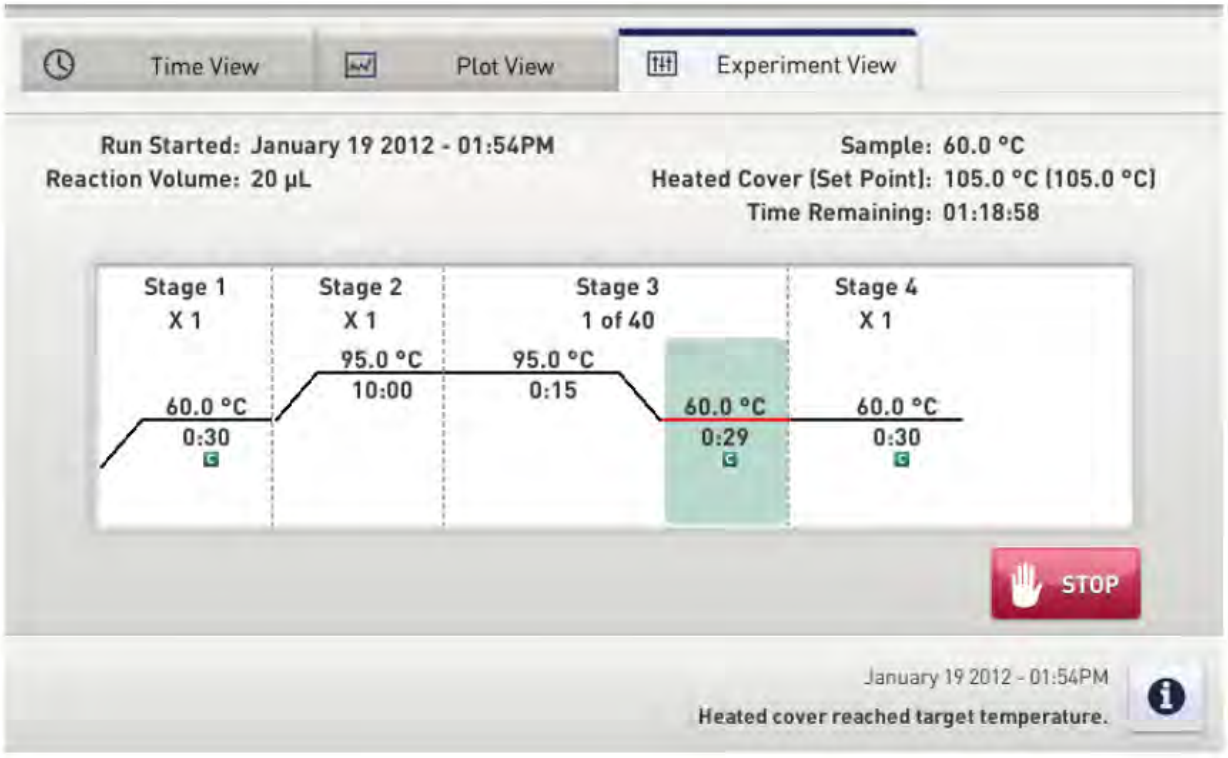


Figure 84 Experiment View tab

Figure 85 Time View tab

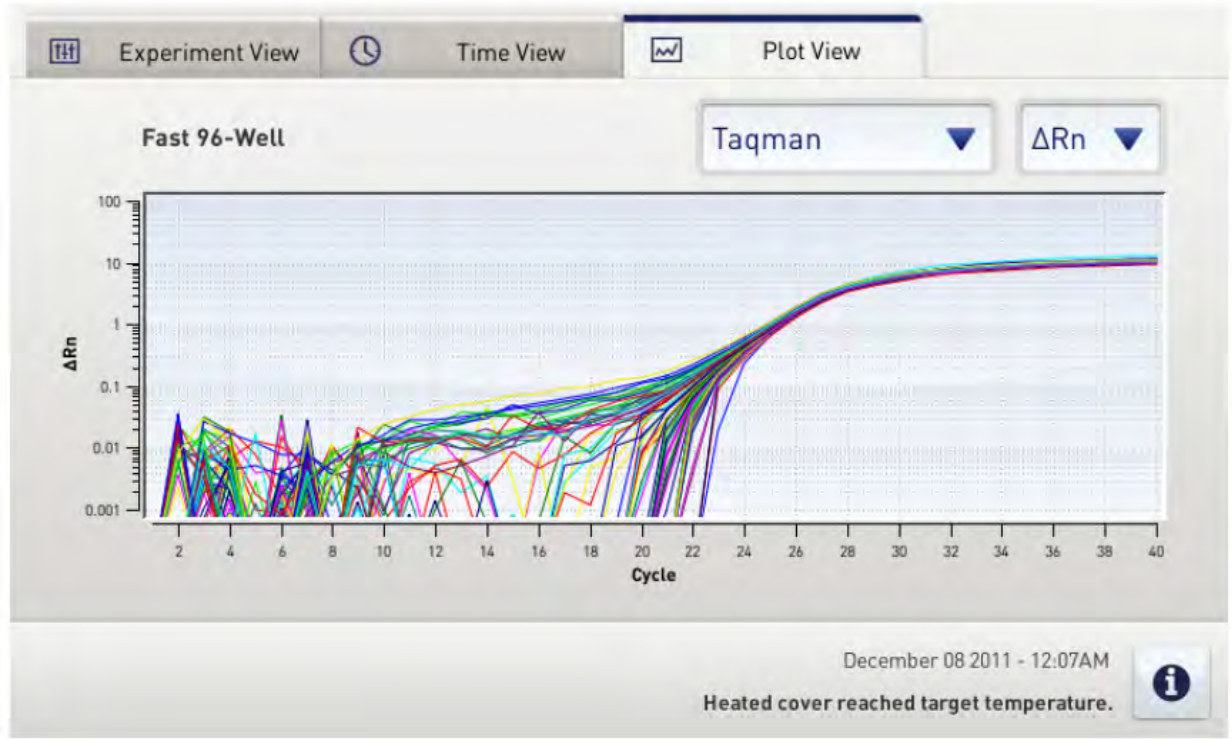


Figure 86 Plot View tab

Note: The plot view is available only if your experiment includes the PCR process.

25 Review results and adjust experiment parameters

The following topics are covered in this chapter:

- Review the analyzed data using several of the analysis screens and publish the data (see “Review results” on page 279)
- Modify experiment parameters to troubleshoot problems with experiment results before rerunning an experiment (see “Adjust parameters for re-analysis of your own experiments” on page 301)

Note: Additional analysis can be performed with TaqMan™ Genotyper Software.

Review results

Analyze the example experiment

1. Open the example experiment file.

   See Chapter 24, “Run the experiment”.

2. Click **Analyze**.

   Note: You can also access the experiment to analyze from the **Home** screen.

The software analyzes the data using the default analysis settings.

View clusters in the allelic discrimination plot

The Allelic Discrimination Plot contrasts the normalized reporter dye fluorescence (Rn) for the allele-specific probes of the SNP assay.

View the allelic discrimination plot to identify the following items:

- Clusters for the three possible genotypes (Allele 1 homozygous, Allele 2 homozygous, and Allele 1/2 heterozygous)
- A cluster for the no template controls

View and assess the allelic discrimination plot

1. From the **Experiment Menu** pane, select **Analysis** ▸ **Allelic Discrimination Plot**.
2. Click the **Plate Layout** tab, then click any empty well to select it.

   Note: In the Allelic Discrimination Plot, the software highlights all wells that are selected in the **Plate Layout** tab. If the plot displays a single color for all wells, then all wells in the plate layout are selected.

3. In the allelic discrimination plot, select **SNP Assay 1** from the **SNP Assay** dropdown list.
4. Ensure that the autocaller is enabled.
   a. Click **Analysis Settings**.
   b. In the **Analysis Settings** dialog box, select the **Call Settings** tab.
   c. In the **Default Call Settings** section, see the **Autocaller Enabled** field.
      The **Autocaller Enabled** field should display **Yes**.
   d. To enable the autocaller if it is not enabled, click **Edit Default Settings**.
   e. In the **Edit Default Call Settings** dialog box, select the **Autocaller Enabled** checkbox.
   f. Click **Save Changes**.
   g. In the **Analysis Settings** dialog box, click **Apply Analysis Settings**.

   If the autocaller is not enabled, the Allelic Discrimination Plot displays a crossmark (X – Undetermined) for each sample.
   The Allelic Discrimination Plot displays allele symbols for each sample evaluated for the selected SNP. The samples are grouped on the plot as follows:

Genotype	Symbol	Location
Homozygous for Allele 1 of the selected SNP assay	● (red)	X-axis of the plot
Homozygous for Allele 2 of the selected SNP assay	● (blue)	Y-axis of the plot
Heterozygous for both alleles of the selected SNP assay (Allele 1 and Allele 2)	● (green)	Midway between the homozygote clusters
No Template Control	■ (black)	Bottom-left corner of the plot
Undetermined	✷ (black)	Anywhere on plot

5. Review each cluster in the plot.
   a. Click and drag a box around the cluster to select the associated wells in the plate layout and well table.

b. Confirm that the expected wells are selected in the well table.

   For example, if you select the cluster at the bottom-left corner of the plot, only the no template controls should be selected. The presence of an unknown among the no template controls may indicate that the sample failed to amplify.

c. Repeat substep 5a and substep 5b for all other clusters in the plot.

d. The table below describes the elements of the Allelic Discrimination Plot.

Element	Description
SNP Assay dropdown list	Determines the SNP assay data that the QuantStudio™ 12K Flex Software displays in the plot.
Plot Type dropdown list	Determines the type of plot (Cartesian or Polar) that the QuantStudio™ 12K Flex Software uses to display the data.
Apply Call dropdown list	When a datapoint is selected, this menu allows you to assign an allele call to the datapoint within the scatterplot.
Toolbar	Contains tools for viewing the scatterplot: •  —Selection tool •  —Selection tool •  —Repositioning tool •  —Zooms in •  —Zooms out
Legend	Explains the symbols in the scatterplot.
Options	The **Reveal Traces** option allows you to trace the clusters throughout the PCR process. This option is not activated for the example experiment. To activate the feature, see “Adjust analysis settings” on page 303.

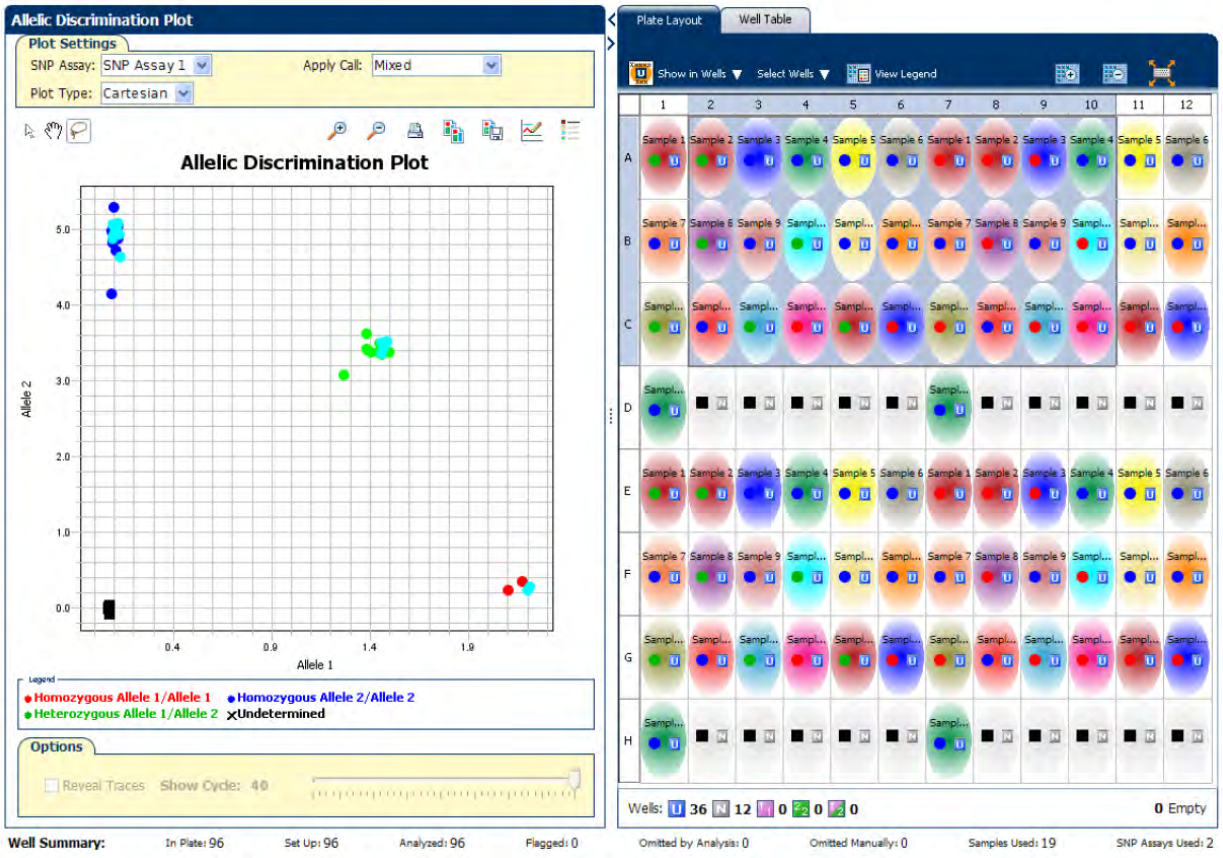


Figure 87 The Allelic Discrimination plot for the example experiment

Troubleshoot clustering on the allelic discrimination plot

Do all controls have the correct genotype?

In the example experiment and in your own experiments, confirm that data points cluster as expected.

Clustering in positive controls

1. From the well table, select the wells containing a positive control to highlight the corresponding data points (symbols) in the Allelic Discrimination Plot.
2. Check that the data points for the positive controls cluster along the expected axis of the plot. For example, if you select the Positive Control Allele 1/Allele 1, then the controls should cluster along the X-axis.
3. Repeat steps 1 on page 283 and 2 on page 283 for the wells containing the other positive controls.

Failed amplification in the unknown samples

1. Select the data points of the cluster in the lower left corner of the Allelic Discrimination Plot to select the corresponding wells in the well table.
2. Check that the selected wells in the well table are the no template controls, and not unknown samples.

Samples clustered with the no template controls

Samples that clustered with the no template controls may:

- Contain no DNA
- Contain PCR inhibitors
- Be homozygous for a sequence deletion

Confirm the results of these samples by retesting them.

Are outliers present?

If the Allelic Discrimination Plot contains clusters other than the three representative genotype clusters (heterozygous, homozygous allele 1, and homozygous allele 2), then those can be classified as outliers.

Confirm the results of the associated samples by retesting them.

Note: The results displays are synchronized. For example, selecting a well in the plate layout selects the corresponding data in the well table and Allelic Discrimination Plot.

Confirm setup accuracy using plate layout

Review the experiment results in the **Plate Layout** tab. The plate layout displays the assay-specific setup and analysis properties for the experiment in a well format corresponding to the type of reaction plate used for the run.

Example experiment plate layout values

For the example experiment, confirm that the software made the following calls:

- 24 samples as Allele 1 homozygous ( )
- 38 samples as Allele 2 homozygous ( )
- 14 samples as heterozygous ( )
- 0 samples as undetermined (**X**)

Confirm that no wells of the reaction plate triggered QC flags ( ). The example experiment does not display any flags.

View the layout

1. Click ❮ beside the Allelic Discrimination Plot to maximize the plate layout.
2. Click **Show in Wells**, then select or deselect a parameter that you want the wells to display. Repeat this step until the plate layout contains all of the desired parameters.

Parameter	Description
Sample Name	The name of the sample applied to the well.
Task	The task assigned to the well: U —Unknown N —No Template Control 1 1 —Positive Control - Allele 1 2 2 —Positive Control - Allele 2 1 2 —Positive Control - Allele 1/2
SNP Assay Name	The name of the SNP evaluated by the well.
Assay ID	The Assay ID number of the SNP evaluated by the well.
Allele 1 / Allele 2	The name of the associated allele for the SNP evaluated by the well
Allele 1 Dyes / Allele 2 Dyes	The name of the reporter and quencher dyes of the associated allele for the SNP evaluated by the well
SNP Assay Color	The color of the SNP evaluated by the well.
Sample Color / Task Color	The color of the sample or task applied to the well.
Genotype Call	The allele call assigned to the sample: • ● —Homozygous 1/1 • ● —Homozygous 2/2 • ● —Heterozygous 1/2 • ■ —No Template Control • **X**—Undetermined
Flag	The number of QC flags the well triggered as listed in the ▲ symbol.

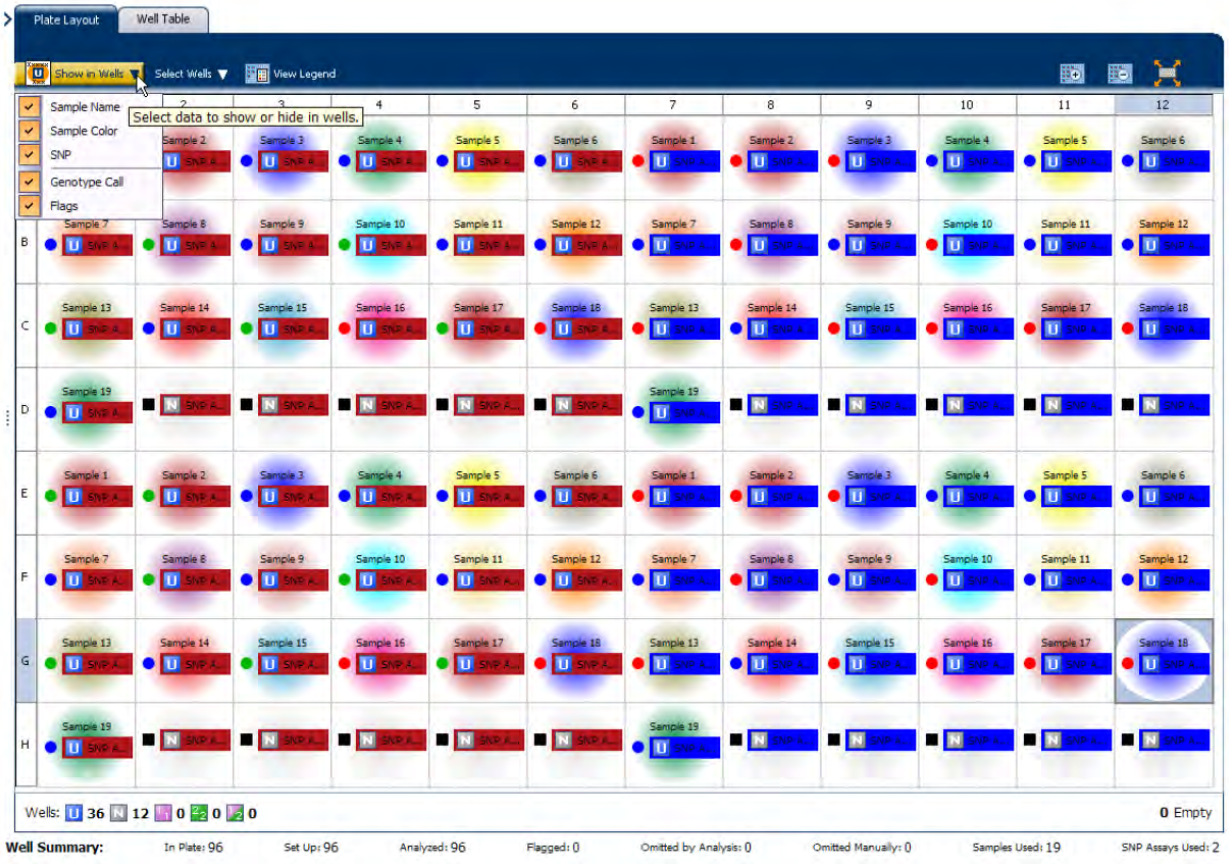


Figure 88 Plate layout of the example genotyping experiment

Tips for troubleshooting plate setup in your own experiment

Adjust your view of the plate layout.

- Note the location of any samples that trigger QC flags (▲). Understanding the position of errors can aid in diagnosing any failures that may occur.
- You can select the entire reaction plate, areas of the reaction plate, or specific wells:
  - Click the upper left corner of the reaction plate to select all 96 wells.
  - Left-click the mouse and drag across the area to select it.
  - Select **Sample**, **SNP Assay**, or **Task** from the **Select Wells** dropdown list in the **Plate Layout** tab to select wells of a specific type using the well-selection criteria.
- Use the **(Zoom In)** button, the **(Zoom Out)** button, and the **(Fit Plate)** button to magnify or compress the view of the wells shown.
- Use the ‹ button to expand the plate layout to cover the entire screen.

Assess amplification results using the amplification plot

IMPORTANT! Amplification plots are not used to make SNP calls. Examine the plots to help with troubleshooting and quality control.

If you collected real-time data for your experiment, review the amplification data to further understand the flags triggered by the experiment data.

About amplification plots

The **Amplification Plot** screen displays amplification of all samples in the selected wells. Use the amplification plots to confirm the results of the experiment:

- **ΔRn vs. Cycle**—ΔRn is the difference in normalized fluorescence signal generated by the reporter between the pre-PCR read and the post-PCR read. This plot displays ΔRn as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.

  Note: Viewing the ΔRn vs. Cycle plot is discussed in this booklet as an example of how to view the plot.

- **Rn vs. Cycle**—Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays Rn as a function of cycle number. You can use this plot to identify and examine irregular amplification.
- **C_T vs. Well**—C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. You can use this plot to locate outlying amplification (outliers).

Each plot can be viewed as a linear or log10 graph type.

View the ΔRn vs. cycle plot

1. In the **Experiment Menu** pane, click **Analysis** ▸ **Amplification Plot**.

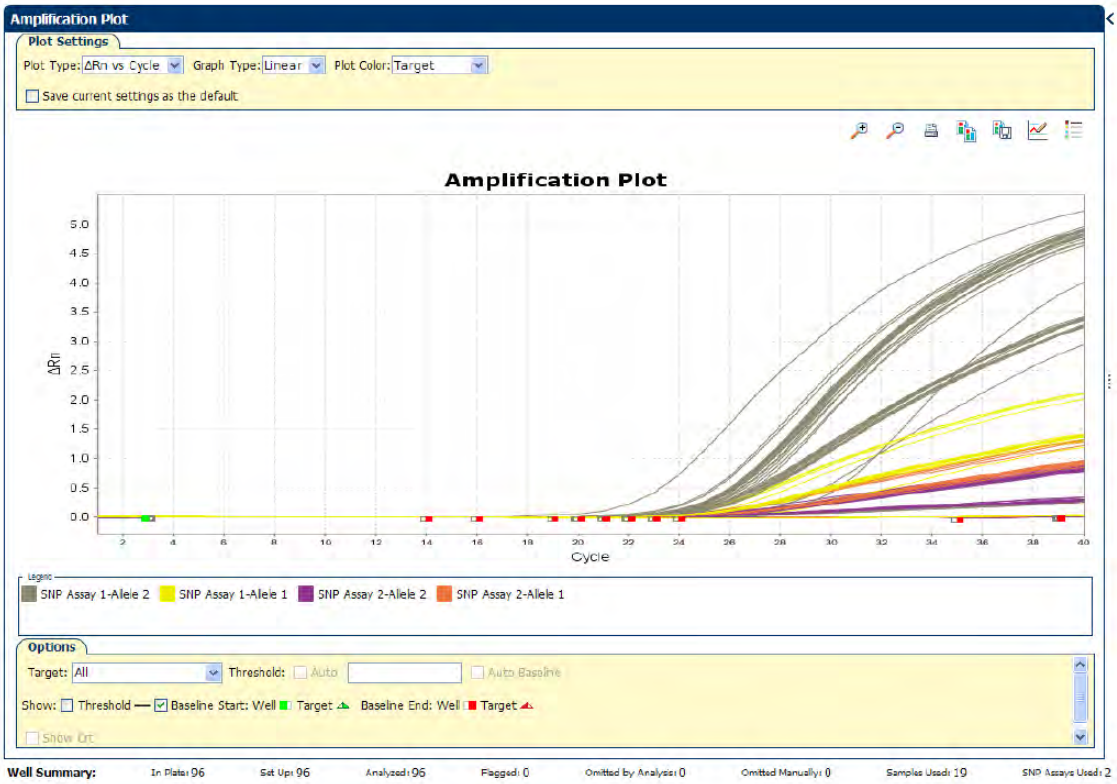
   Note: If no data are displayed, click **Analyze**.

2. Select the plot type and format.

Item	Select
Plot Type dropdown list	**ΔRn vs Cycle**
Plot Color dropdown list	**Target**
 (This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**).	Check (default)

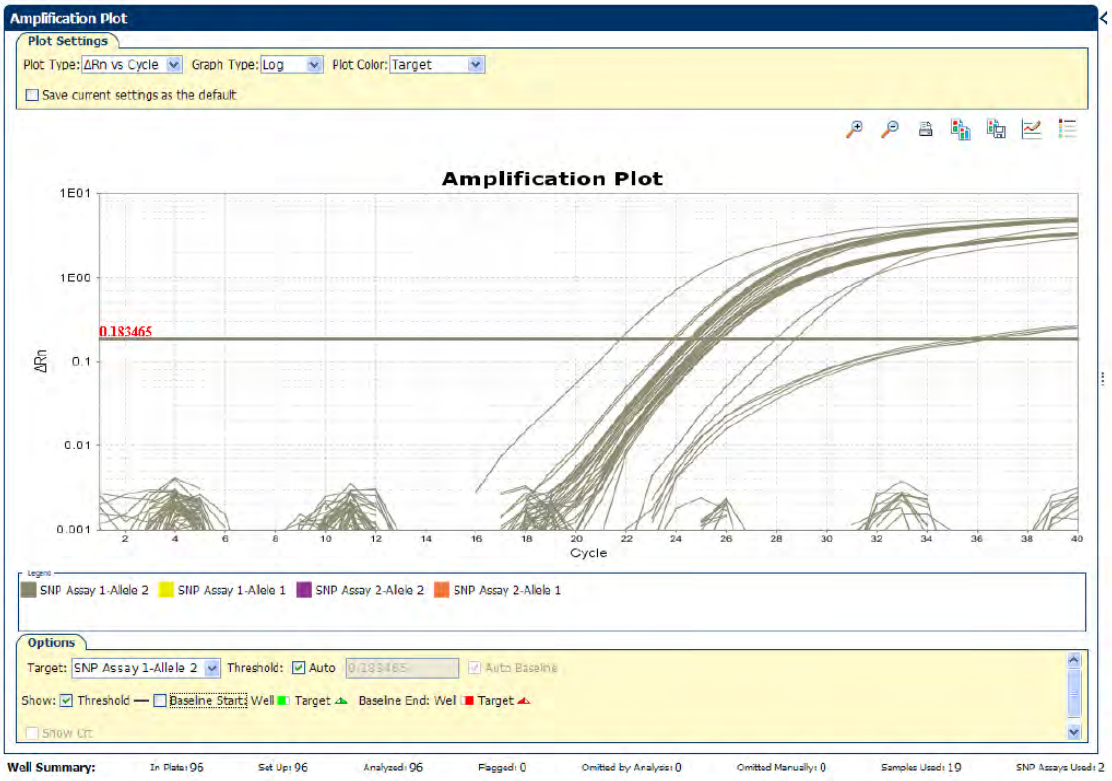
3. View the baseline values.
   a. From the **Graph Type** dropdown list, select **Linear**.

b. Select the **Baseline** checkbox to show the start cycle and end cycle.

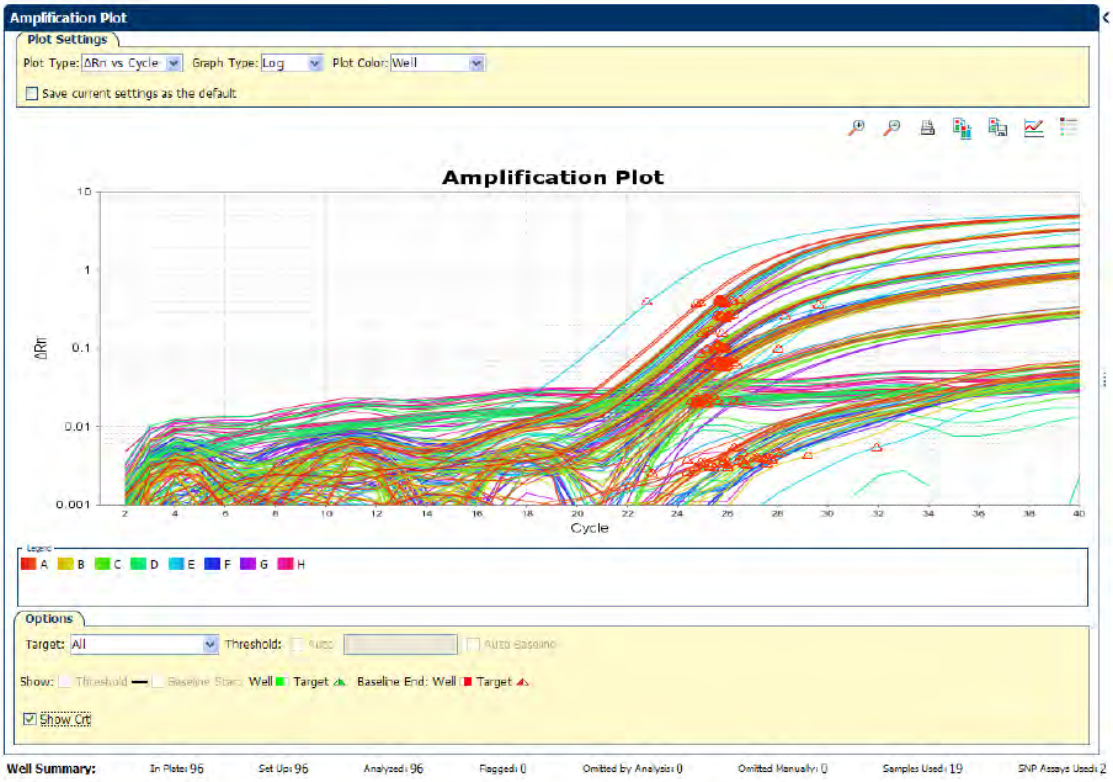


4. View the threshold values.
   a. From the **Graph Type** dropdown list, select **Log**.
   b. From the **Target** dropdown list, select **SNP Assay 1-Allele 2**.

c. Select the **Threshold** checkbox to show the threshold.



Note: If you use the Relative Threshold algorithm to analyze an experiment that includes amplification, select to view the analysis results using the ΔRn vs Cycle, Rn vs Cycle, or C_{RT} vs Well plot type and Linear or Log graph type. Also select the **Show Crt** checkbox to view the derived fractional cycle on the amplification plot.



Identify well problems using the well table

Review the details of the experiment results in the Well Table and identify any flagged wells. The Well Table displays the assay-specific setup and analysis properties for the experiment in a tabular format.

Example experiment values and flags

For the example experiment, look for wells that triggered QC flags (▲). The example experiment has no flags.

View the well table

1. Select the **Well Table** tab.
2. Click the **Flag** column header to sort the data so that the wells that triggered flags appear at the top of the table.
3. Confirm the integrity of the controls.
   a. In the **Group By** dropdown list, select **Task** to organize the table rows by their function on the reaction plate.
   b. Confirm that each of the controls does not display a flag (▲).

c. Click ⊟ to collapse the negative and positive controls.

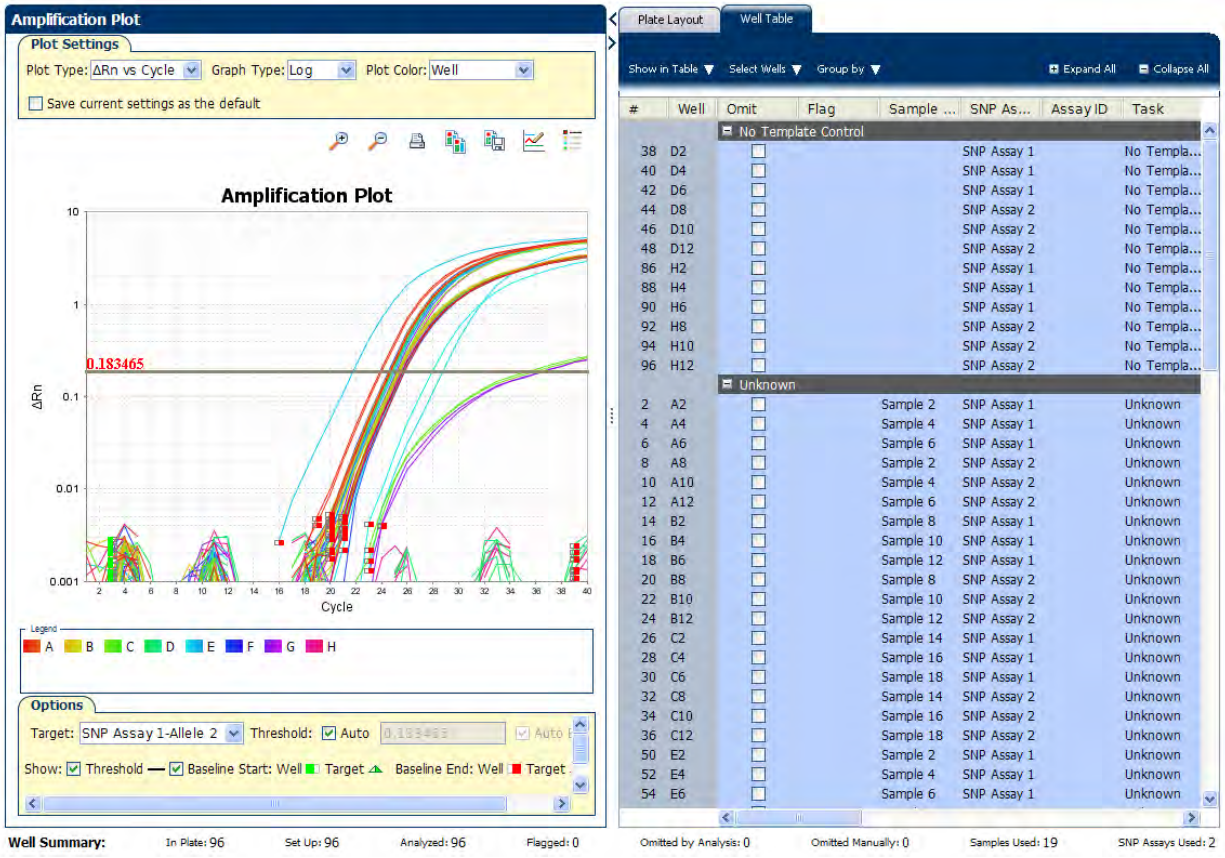


Figure 89 The well table of the example Genotyping experiment

The following table gives the names and description of the columns in the well table.

Column	Description
Well column	The position of the well on the reaction plate.
Omit column	A check mark indicates that the well has been removed from the analysis.
Flag column	A ▲ symbol indicates that the well triggered the number of flags listed inside the symbol.
Sample Name column	The name of the sample.
SNP Assay Name column	The name of the SNP assay evaluated by the well.
Assay ID	The Assay ID number of the SNP evaluated by the well.
Task column	The task assigned to the well (Unknown, No Template Control, or Positive Control).
Allele 1 column and **Allele 2** column	The name of the associated allele for the SNP evaluated by the well.

(continued)

Column	Description
Allele 1 Dye column and **Allele 2 Dye** column	The name of the reporter and quencher dyes of the associated allele for the SNP evaluated by the well.
Allele 1 R_n column and **Allele 2 R_n** column	Normalized signal (R_n) of the reporter dye of the associated allele for the SNP evaluated by the well.
Passive Reference column	The signal of the passive reference dye for the well.
Call column	The allele call assigned to the sample, where possible calls are: • ● **Homozygous 1/1**—Homozygous for allele 1 • ● **Homozygous 2/2**—Homozygous for allele 2 • ● **Heterozygous 1/2**—Heterozygous • ■ **Negative Control (NC)** • × **Undetermined**
Quality (%) column	The quality value calculated for the genotype call.
Method column	The method used to assign the call to the sample (Auto if assigned by the software, or Manual if applied by a user).
Comments column	Comments entered for the associated sample well.
Allele 1 C_T column and **Allele 2 C_T** column	Threshold cycle (C_T) of the sample for the associated allele for the SNP evaluated by the well.

Identify quality control (QC) problems

The Well Table displays columns for QC flags that are triggered by the experimental data. If the experiment data does not trigger a QC flag, then the software does not display a corresponding column for the flag.

A ▲ symbol in one of the following columns indicates that the associated well triggered the flag.

Flag	Description
BADROX	The well produced a passive reference signal greater than the limit defined in the analysis settings.
OFFSCALE	The well produced a level of fluorescence greater than the QuantStudio™ 12K Flex Real–Time PCR System can measure.
NOSIGNAL	The well did not produce a detectable level of fluorescence.
CLUSTER#	For the SNP evaluated by the well, the number of clusters generated from the experiment data is greater than the limit defined in the analysis settings.
PCFAIL	The positive control did not produce an R_n for the associated allele greater than the limit defined in the analysis settings indicating that the control may have failed to amplify.

(continued)

Flag	Description
SMCLUSTER	The number of data points in the associated cluster is less than the limit defined in the analysis settings.
AMPNC	The negative control has produced an R_n greater than the limit defined in the analysis settings indicating possible amplification.
NOAMP	The well did not produce an R_n for either allele that is greater than the limit defined in the analysis settings indicating that the well may have failed to amplify.
NOISE	The background fluorescence (noise) produced by the well is greater than the other wells on the reaction plate by a factor greater than the limit **defined in the analysis settings.**
SPIKE	The amplification plot for the well contains one or more data points inconsistent with the other points in the plot.
EXPFAIL	The software cannot identify the exponential region of the amplification plot for the well.
BLFAIL	The software cannot calculate the best fit baseline for the data for the well.
THOLDFAIL	The software cannot calculate a threshold for the associated well.
CTFAIL	The software cannot calculate a threshold cycle (C_T) for the associated well.
AMPSCORE	Amplification in the linear region is below a certain threshold, corresponding to the score set in the analysis settings

Tips for analyzing your own experiments

Confirm the integrity of positive controls

When you analyze the example experiment or your own experiment, if you are using positive controls, confirm the integrity of the positive controls.

1. In the **Group By** dropdown list, select **Task** to organize the table rows by their function on the reaction plate

2. Confirm that the positive controls do not display flags (▲) and that their normalized reporter dye fluorescence (R_n) is appropriate for the genotype (for example, if evaluating the Positive Control Allele 1/Allele 1, you would expect to see significant increase in R_n for the Allele 1 probe and very little for the Allele 2 probe).

Adjust the well table

- Review the data for the Unknown samples. For each row that displays the ▲ symbol in the **Flag** column, note the data and the flag or the flags triggered by the associated well.
- Select areas of the table or wells of a specified type by the following parameters:
  - Left-clicking the mouse and dragging across the area you want to select an area of the table.
  - Selecting **Sample**, **SNP Assay**, or **Task** from the **Select Wells** dropdown list in the **Well Table** tab to select wells of a specific type using the well-selection tool.

- Group the rows of the plate layout by selecting an option from the **Group By** dropdown list. You can then collapse or expand the lists either by clicking the **+** button or the **–** next to individual lists, or by clicking **Collapse All** or **Expand All**.
- Omit a well from the analysis by selecting the **Omit** checkbox for that well. To include the well in the analysis, deselect the **Omit** checkbox.

Note: You must reanalyze the experiment each time you omit or include a well.

Confirm accurate dye signal using the multicomponent plot

The **Multicomponent Plot** screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the example experiment, you review the **Multicomponent Plot** screen for:

- ROX™ dye (passive reference)
- FAM™ dye (reporter)
- VIC™ dye (reporter)
- Spikes, dips, and/or sudden changes
- Amplification in the no template control wells

View the multicomponent plot

1. In the **Experiment Menu** pane, select **Analysis ▸ Multicomponent Plot.**

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

2. Display the unknown wells in the plate layout to display the corresponding data in the **Multicomponent Plot** screen.
   a. Click the **Plate Layout** tab.
   b. Select one well in the plate layout. The well is shown in the Multicomponent Plot screen.

      Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

3. In the **Plot Color** dropdown list, select **Dye**.
4. Click **Show a legend for the plot** (default).

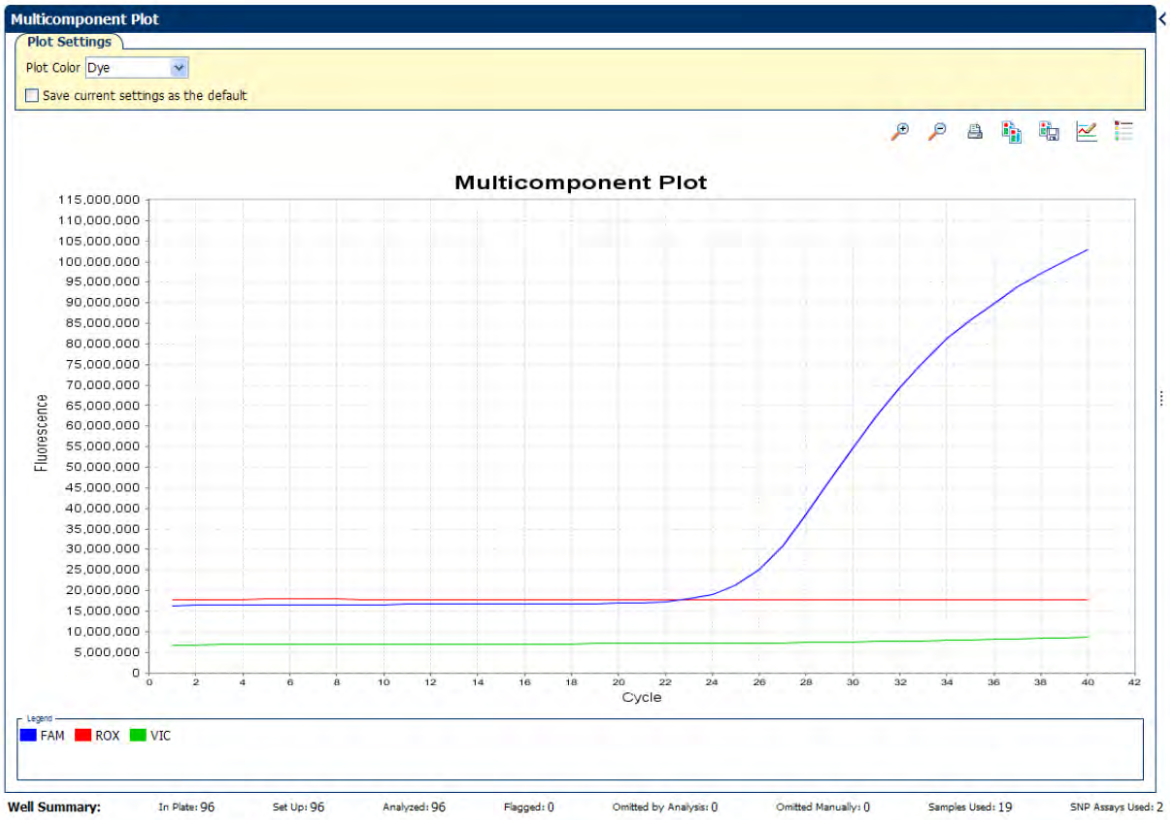
   Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

5. Check the ROX™ dye signal.

   In the example experiment, the ROX™ dye signal remains constant throughout the PCR process. A constant ROX™ dye signal indicates typical data.

6. Check the FAM™ dye signal.

   In the example experiment, the FAM™ dye signal increases throughout the PCR process. The increase in FAM™ dye signal indicates normal amplification.



7. Select the no template control wells one at time and check for amplification.

   Wells with the no template control should not show amplification. In the example experiment the wells with no template controls do not show any amplification.

Tips for confirming dye accuracy in your own experiment

When you analyze your own Genotyping experiment, look for the following items:

1. **Passive Reference**—The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
2. **Reporter Dye**—The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
3. **Irregularities in the signal**—There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
4. **No Template Control wells**—There should not be any amplification in the no template control wells.

Determine signal accuracy using the raw data plot

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

Purpose

In the Genotyping example experiment, you review the **Raw Data Plot** screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the raw data plot

1. In the **Experiment Menu** pane, select **Analysis** ▸ **Raw Data Plot**.

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

2. Display all 96 wells in the **Raw Data Plot** screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.

3. Click **Show a legend for the plot** (default).
   The legend displays the color code for each row of the reaction plate (see Figure 90 on page 298).

4. Click and drag the **Show Cycle** pointer from cycle 1 to cycle 42.
   In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.

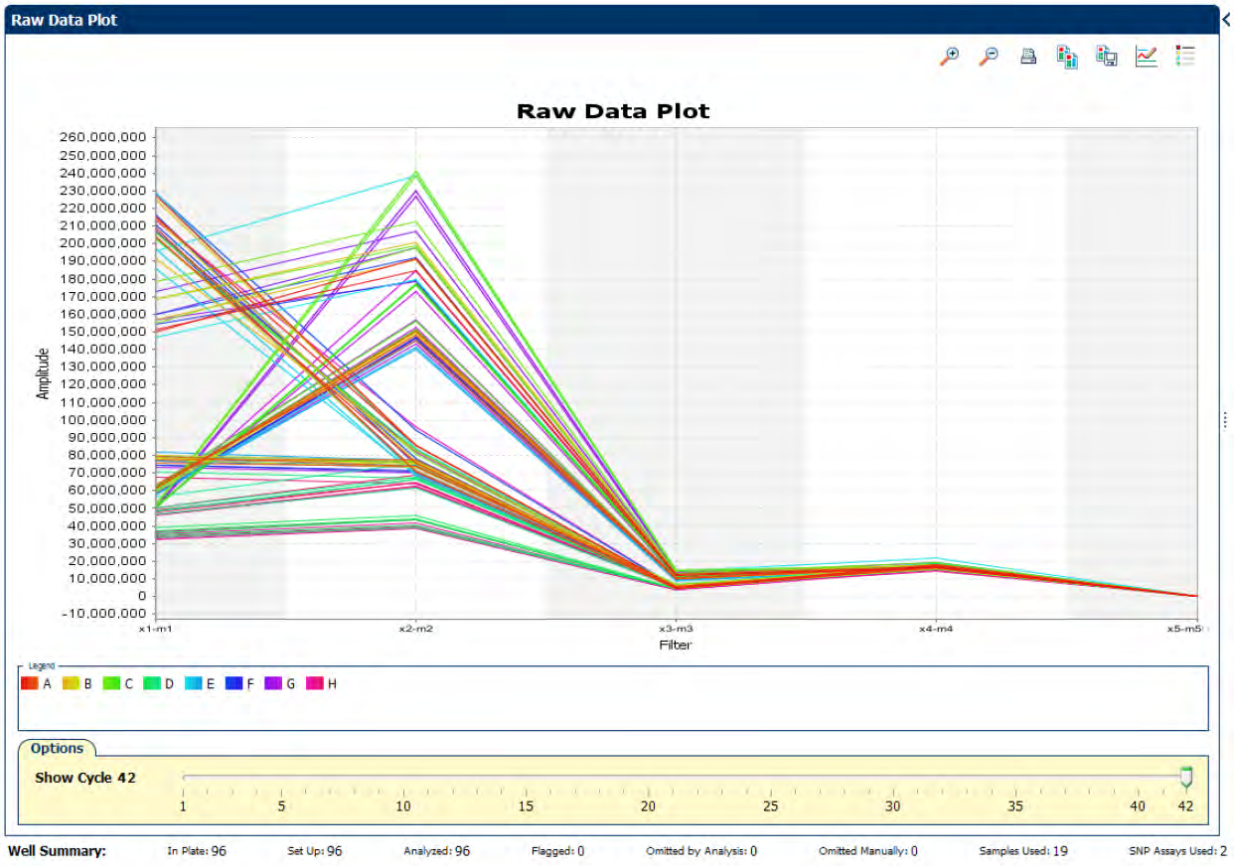


Figure 90 Raw data plot

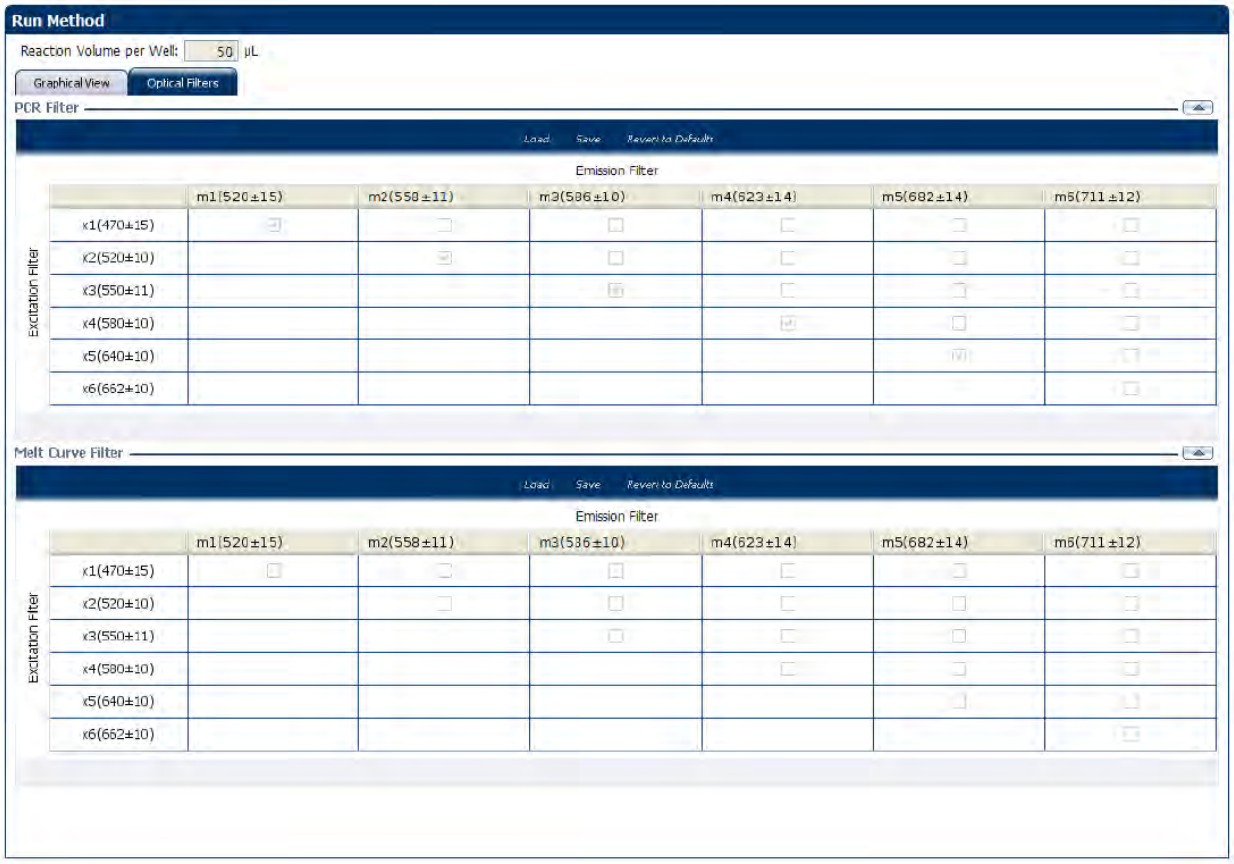


Figure 91 Filters

Tips for determining signal accuracy in your own experiment

When you analyze your own Genotyping experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

Review the flags in the QC summary

The **QC Summary** screen displays a list of the QuantStudio™ 12K Flex Software flags, including the flag frequency and location for the open experiment.

For Genotyping experiments, flag appearance is triggered by experiment data or the assay. If a flag has been triggered by the assay, the Plate Layout does not display the ⚠ icon. The flag details appear in the QC Summary.

In the example experiment, there are no flags.

View the QC summary

1. In the **Experiment Menu** pane, select **Analysis** ▸ **QC Summary**.

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

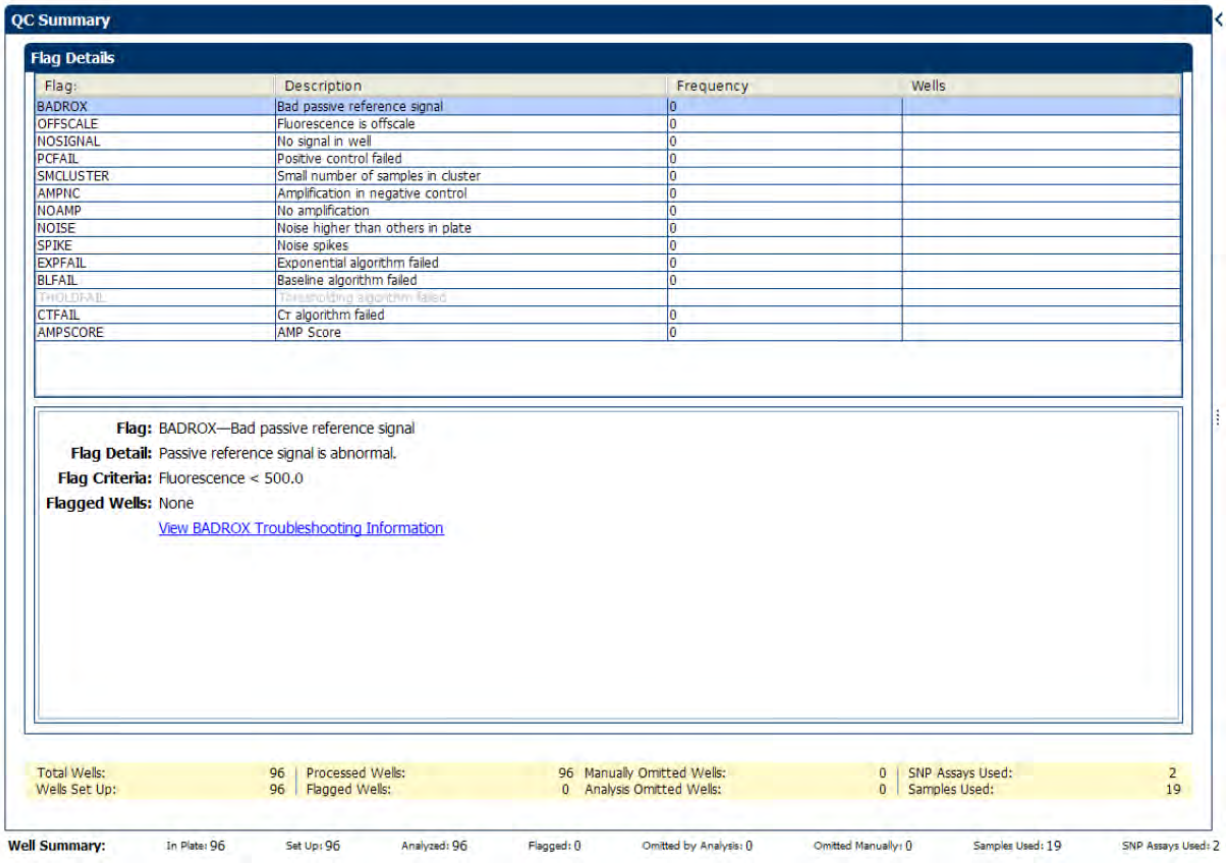
2. Review the Flags Summary.

Note: A 0 displayed in the **Frequency** column indicates that the flag does not appear in the experiment. If the frequency is greater than 0, the flag appears somewhere in the experiment. The well position is listed in the **Wells** column.

---

3. In the **Flag Details** table, click each flag with a frequency greater than 0 to display detailed information about the flag.

4. *(Optional)* For those flags with frequency greater than 0, click each flag with a frequency greater than 0 to display detailed information about the flag.



Possible flags

The flags listed below may be triggered by the experiment data or the assay.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal

(continued)

Flag	Description
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
AMPSCORE	Amplification in the linear region is below a certain threshold, corresponding to the score set in the analysis settings
Secondary analysis flags	
AMPNC	Amplification in negative control
PCFAIL	Positive Control failed
SMCLUSTER#	Small number of samples in clusters

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the **Frequency** column for each flag).

For more information

Information	Reference
Publishing data	Chapter 1, “General information and instructions”

Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

The **Analysis Settings** dialog box displays the analysis settings for the call, threshold cycle (C_T), flags, and advanced options.

You can change the settings in the **Analysis Settings** dialog box, then reanalyze your experiment.

View the analysis settings

1. In the **Experiment Menu** pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the **Analysis Settings** dialog box.
   In the example experiment, the default analysis settings are used for each tab:
   - Call Settings
   - C_T Settings
   - Flag Settings
   - Advanced Settings

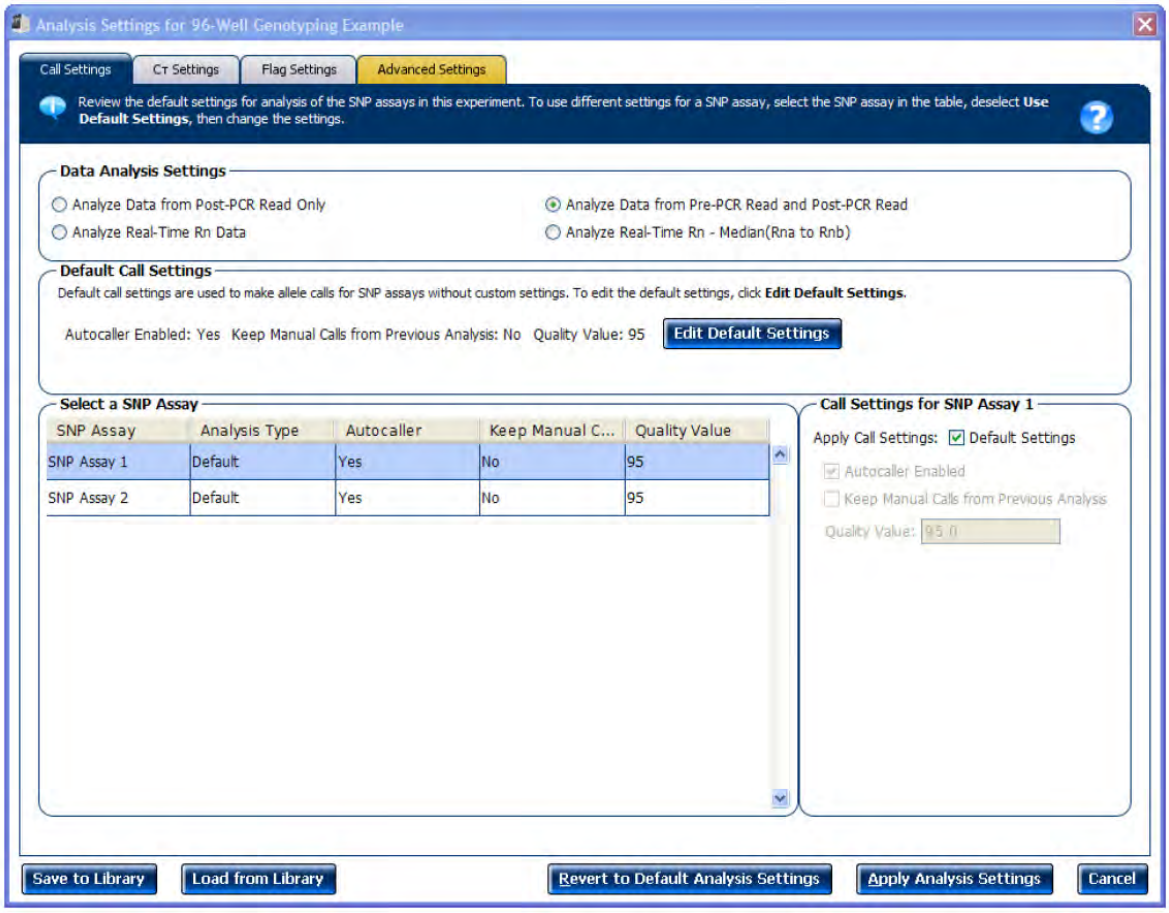


Figure 92 Analysis Settings dialog box for a Genotyping experiment

3. View and, if necessary, change the analysis (see “Adjust analysis settings” on page 303).

Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see “About the analysis settings library” on page 62.

---

4. Click **Apply Analysis Settings** to apply the current analysis settings.

Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

Call settings

- Change the default data analysis settings. You can select from the following items:
  - **Analyze Data from Post-PCR Read Only** radio button—Select if you do not want to use data from the pre-PCR read to determine genotype calls.
  - **Analyze Data from Pre-PCR Read and Post-PCR Read** radio button—If you included the pre-PCR read in the run, select if you want to use data from the pre-PCR read to determine genotype calls.
  - **Analyze Real-Time Rn Data** radio button—If you included amplification in the run, select if you want to use the normalized reporter (Rn) data from the cycling stage to determine genotype calls.
  - **Analyze Real-Time Rn - Median (Rna to Rnb)** radio button—If you included amplification in the run, select if you want to use the subtracted median of the normalized reporter (Rn) data from the cycling stage to determine genotype calls, where Rna to Rnb refers to all the cycles from the Start Cycle Number to the End Cycle Number. The average subtraction provides improved data accuracy.

    Note: To activate the Reveal Traces feature on the **Allelic Discrimination Plot** screen, select the **Analyze Real-Time Rn Data** radio button or the **Analyze Real-Time Rn - Median (Rna to Rnb)** radio button.

- Edit the default call settings. Click **Edit Default Settings**, then specify the default settings in the **Edit Default Call Settings** dialog box.
  - **Autocaller Enabled** checkbox—Select for the software to make genotype calls using the autocaller algorithm.
  - **Keep Manual Calls from Previous Analysis** checkbox—If the autocaller is enabled, select to maintain manual calls after reanalysis
  - **Quality Value** field—Enter a value to use to make genotype calls. If the confidence value is less than the call setting, the call is undetermined.
  - **Call Cycle** field—The cycle number at which genotyping calls are evaluated.
- Use custom call settings for a SNP assay.
  - Select one or more SNP assays in the table, then deselect the **Default Settings** checkbox.

    The checkboxes and fields in the right pane are editable when the **Default Settings** checkbox is deselected.
  - In the right pane, specify the settings for each SNP assay. The settings are the same settings that are available in the **Edit Default Call Settings** dialog box.

C_T settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method.

- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

  The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for quantification.

  The Relative Threshold algorithm is a well-based analysis based on the PCR reaction efficiency and fitted to the Amplification curve. This setting is ideal for a single sample across genes with no dependence on targets, thereby reducing variability. It is not necessary to set either a baseline or a threshold when you use the Relative Threshold algorithm, so any settings for baseline or threshold will not affect the analysis.

- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the alleles that do not have custom settings. To edit the default settings, click **Edit Default Settings**.

- **C_T Settings for Target**

  When you manually set the threshold and baseline, we recommend the following settings:

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

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag settings

Use the **Flag Settings** tab to perform the following tasks:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the QuantStudio™ 12K Flex Software.

1. In the **Use** column, select the check boxes for flags to apply during analysis.

2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

3. In the **Reject Well** column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations may change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.

4. Click **Apply Analysis Settings** in the **Analysis Settings** dialog box.

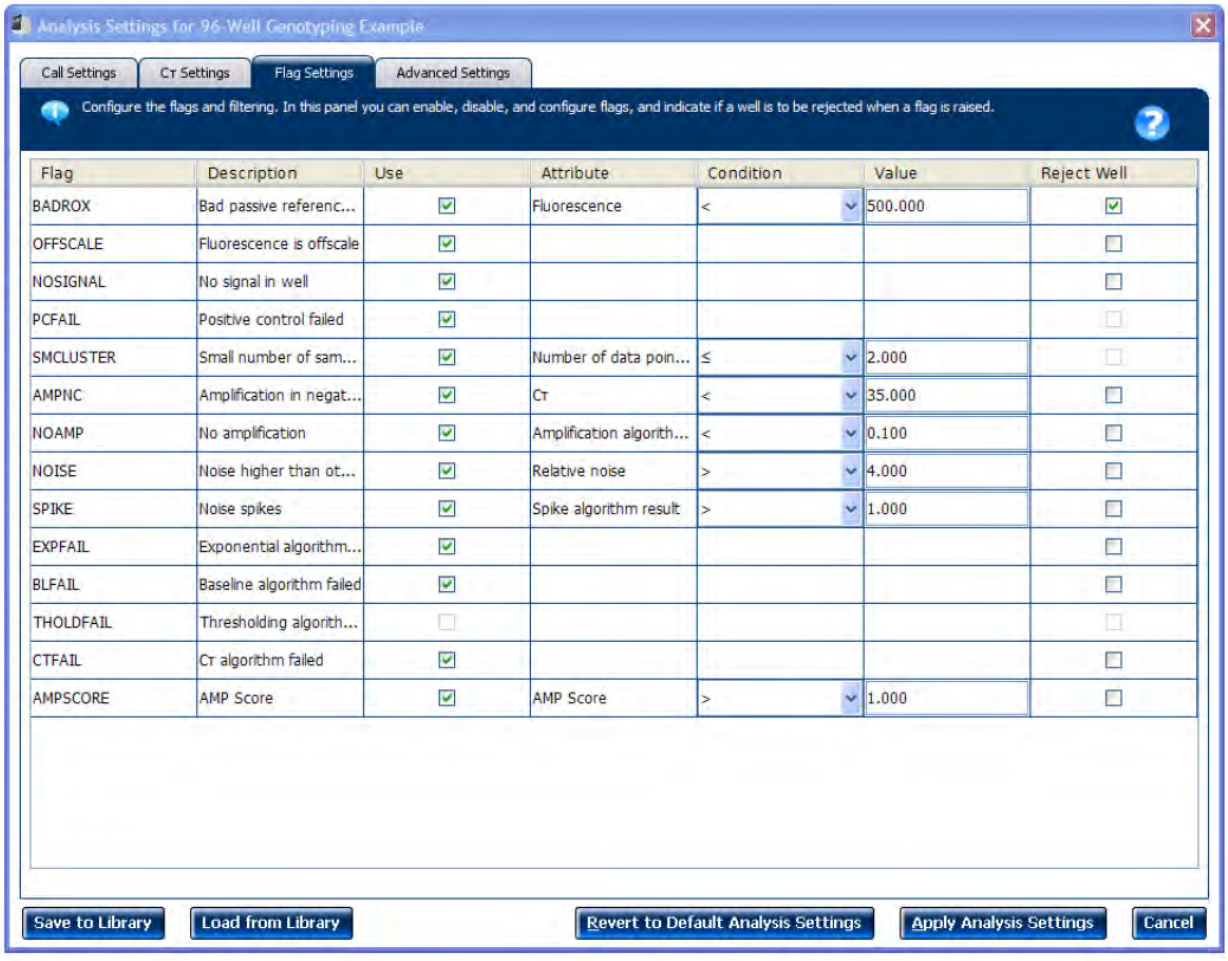


Figure 93 Flag Settings tab

Advanced settings

Use the **Advanced Settings** tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold setting.

The following steps describe the use of custom baseline settings for a well-target combination.

1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** checkbox.
3. Define the custom baseline settings
   - For automatic baseline calculations, select the **Automatic Baseline** checkbox.
   - To define the baseline, deselect the **Automatic Baseline** checkbox, then enter the baseline start cycle and baseline end cycle.

26 Export analysis results

1. Open the Genotyping example experiment file has been analyzed.

   For information about analysis, see Chapter 25, “Review results and adjust experiment parameters”.

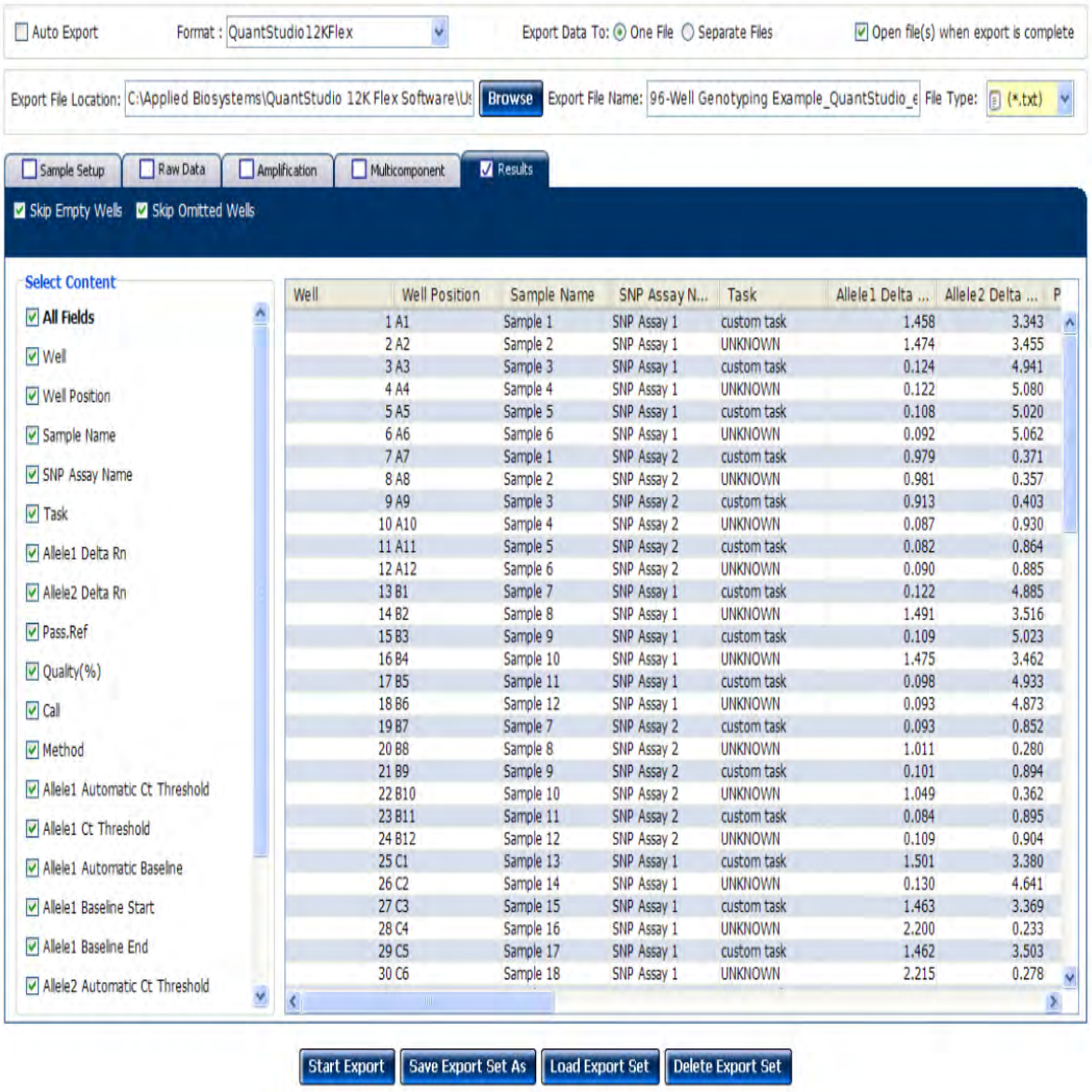
2. In the **Experiment Menu** pane, click **Export**.

   Note: To export data automatically after analysis, select the **Auto Export** checkbox during experiment setup or before running the experiment. The **Auto Export** checkbox is unchecked for the example experiment.

3. In the **Format** dropdown list, select **QuantStudio 12K Flex format**.

4. Complete the **Export** dialog box as shown below.

Field or Selection	Entry
Select Data to export/Select Content	Results
Export Data To options	**One File** radio button
Export File Name field	***96-Well Genotyping Example_QuantStudio_export***
File Type dropdown list	***.txt**
Export File Location field	Use the default file location or click **Browse** to select a different location



Well	Well Position	Sample Name	SNP Assay N...	Task	Allele1 Delta ...	Allele2 Delta ...	P
1	A1	Sample 1	SNP Assay 1	custom task	1.458	3.343	
2	A2	Sample 2	SNP Assay 1	UNKNOWN	1.474	3.455	
3	A3	Sample 3	SNP Assay 1	custom task	0.124	4.941	
4	A4	Sample 4	SNP Assay 1	UNKNOWN	0.122	5.080	
5	A5	Sample 5	SNP Assay 1	custom task	0.108	5.020	
6	A6	Sample 6	SNP Assay 1	UNKNOWN	0.092	5.062	
7	A7	Sample 1	SNP Assay 2	custom task	0.979	0.371	
8	A8	Sample 2	SNP Assay 2	UNKNOWN	0.981	0.357	
9	A9	Sample 3	SNP Assay 2	custom task	0.913	0.403	
10	A10	Sample 4	SNP Assay 2	UNKNOWN	0.087	0.930	
11	A11	Sample 5	SNP Assay 2	custom task	0.082	0.864	
12	A12	Sample 6	SNP Assay 2	UNKNOWN	0.090	0.885	
13	B1	Sample 7	SNP Assay 1	custom task	0.122	4.885	
14	B2	Sample 8	SNP Assay 1	UNKNOWN	1.491	3.516	
15	B3	Sample 9	SNP Assay 1	custom task	0.109	5.023	
16	B4	Sample 10	SNP Assay 1	UNKNOWN	1.475	3.462	
17	B5	Sample 11	SNP Assay 1	custom task	0.098	4.933	
18	B6	Sample 12	SNP Assay 1	UNKNOWN	0.093	4.873	
19	B7	Sample 7	SNP Assay 2	custom task	0.093	0.852	
20	B8	Sample 8	SNP Assay 2	UNKNOWN	1.011	0.280	
21	B9	Sample 9	SNP Assay 2	custom task	0.101	0.894	
22	B10	Sample 10	SNP Assay 2	UNKNOWN	1.049	0.362	
23	B11	Sample 11	SNP Assay 2	custom task	0.084	0.895	
24	B12	Sample 12	SNP Assay 2	UNKNOWN	0.109	0.904	
25	C1	Sample 13	SNP Assay 1	custom task	1.501	3.380	
26	C2	Sample 14	SNP Assay 1	UNKNOWN	0.130	4.641	
27	C3	Sample 15	SNP Assay 1	custom task	1.463	3.369	
28	C4	Sample 16	SNP Assay 1	UNKNOWN	2.200	0.233	
29	C5	Sample 17	SNP Assay 1	custom task	1.462	3.503	
30	C6	Sample 18	SNP Assay 1	UNKNOWN	2.215	0.278	

Figure 94 Export screen

Your exported file when opened in Notepad should look like this:

96-Well Genotyping Example_QuantStudio_export.txt - Notepad

File Edit Format View Help

```
* Barcode = NA
* Block Type = 96-Well Block (0.2mL)
* Calibration Background is expired  = No
* Calibration Background performed on  = 2011-08-08 01:15:53 AM SGT
* Calibration FAM is expired  = No
* Calibration FAM performed on  = 2011-08-08 01:39:58 AM SGT
* Calibration ROI is expired  = No
* Calibration ROI performed on  = 2011-08-08 01:05:24 AM SGT
* Calibration ROX is expired  = No
* Calibration ROX performed on  = 2011-08-08 02:07:15 AM SGT
* Calibration SYBR is expired  = No
* Calibration SYBR performed on  = 2011-08-08 01:58:11 AM SGT
* Calibration TAMRA is expired  = No
* Calibration TAMRA performed on  = 2011-08-08 02:16:10 AM SGT
* Calibration Uniformity is expired  = No
* Calibration Uniformity performed on  = 2011-08-08 01:24:47 AM SGT
* Calibration VIC is expired  = No
* Calibration VIC performed on  = 2011-08-08 01:49:09 AM SGT
* Chemistry = TAQMAN
* Comment = NA
* Date Created = 1970-01-01 07:30:00 AM SGT
* Experiment File Name = C:\Program Files\Applied Biosystems\QuantStudio12KFlex\examples\Genotyping\96-Well Genotyping Example.eds
* Experiment Name = 96-Well Genotyping Example
* Experiment Run End Time = Not Started
* Experiment Type = Genotyping
* Instrument Name = QuantStudioDemo
* Instrument Serial Number = QuantStudioDemo
* Instrument Type = QuantStudio 12K Flex
* Passive Reference = ROX
* Quantification Cycle Method = Ct
* Signal Smoothing On = true
* Stage/ Cycle where Analysis is performed = Stage 3, Step 2
* User Name = NA

[Results]
Well    Well Position   Sample Name     SNP Assay Name  Task    Allele1 Delta Rn        Allele2 Delta Rn        Pass.Ref        Quality(%)
Call    Method  Allele1 Automatic Ct Threshold  Allele1 Ct Threshold    Allele1 Automatic Baseline      Allele1 Baseline Start  Allele1
Baseline End    Allele2 Automatic Ct Threshold  Allele2 Ct Threshold    Allele2 Automatic Baseline      Allele2 Baseline Start  Allele2
Baseline End
1       A1      Sample 1        SNP Assay 1     custom task     1.458   3.343           98.416  Heterozygous Allele 1/Allele 2  Auto
true    0.068   true    3       21      true    0.183   true    3       20
2       A2      Sample 2        SNP Assay 1     UNKNOWN 1.474   3.455           98.416  Heterozygous Allele 1/Allele 2  Auto    true
0.068   true    3       22      true    0.183   true    3       21
3       A3      Sample 3        SNP Assay 1     custom task     0.124   4.941           98.416  Homozygous Allele 2/Allele 2    Auto
true    0.068   true    3       39      true    0.183   true    3       20
4       A4      Sample 4        SNP Assay 1     UNKNOWN 0.122   5.080           98.416  Homozygous Allele 2/Allele 2    Auto    true
0.068   true    3       39      true    0.183   true    3       19
5       A5      Sample 5        SNP Assay 1     custom task     0.108   5.020           98.416  Homozygous Allele 2/Allele 2    Auto
true    0.068   true    3       39      true    0.183   true    3       20
6       A6      Sample 6        SNP Assay 1     UNKNOWN 0.092   5.062           98.416  Homozygous Allele 2/Allele 2    Auto    true
0.068   true    3       39      true    0.183   true    3       19
7       A7      Sample 1        SNP Assay 2     custom task     0.979   0.371           98.416  Homozygous Allele 1/Allele 1    Auto
true    0.040   true    3       22      true    0.040   true    3       23
```

Part VI

Running presence absence experiments

27 About presence/absence experiments

IMPORTANT! First-time users of the QuantStudio™ 12K Flex Real–Time PCR System, see the part "Getting started" on page 17 and Appendix A, "Documentation and support". The sections provide information and general instructions that are applicable to all the experiments described in this document.

Note: For more information about any of the topics discussed in this guide, access the Help from within QuantStudio™ 12K Flex Software by pressing **F1,** clicking in the toolbar, or selecting **Help ▸ QuantStudio™ 12K Flex Software** Help.

About data collection

Presence/Absence experiments are end-point experiments that are performed to detect a target nucleic acid sequence in a sample.

You can collect the experiment data at the end of the run or continuously in real time.

End-point PCR data

The QuantStudio™ 12K Flex Real–Time PCR System collects data at an end-point, that is after the process has completed.

The data collected is the normalized intensity of the reporter dye, or Rn.

Note: Some end-point experiments also include pre-PCR (data collected before the amplification process) datapoints. If so, the system calculates the delta Rn (ΔRn) value per the following formula:

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

Real-Time PCR data

The QuantStudio™ 12K Flex Real–Time PCR System provides the option of collecting real-time data, during the PCR process.

Note: Real-time data collection is used only for troubleshooting, and not for Presence/Absence analysis.

Setting up PCR reactions

With Presence/Absence experiments, you prepare PCR reactions that contain primers and probes to amplify the target and a reagent to detect amplification of the target. You can set up the PCR reactions for the Presence/Absence experiments two different ways.

Note: The example experiment uses IPC (internal positive control) setup for setting up the PCR reactions.

IPC setup

Use an internal positive control (IPC) to monitor the PCR progress and ensure that a negative result is not caused by failed PCR in the sample. The PCR contains two primer/probe sets:

- One to detect the unknown target (unknown target primer set and TaqMan™ probe to detect the unknown target)
- One to detect the IPC (IPC primer set and a VIC™ dye-labeled TaqMan™ probe to detect the IPC template)

There are three well types with this setup:

1. **Unknown-IPC wells** contain sample template and IPC template. The presence of the target is not known.
2. **Negative control-IPC wells** contain IPC template and water or buffer instead of sample template in the PCR reaction. Only the IPC template should amplify in negative control-IPC wells because the reaction contains no sample template. This is also called **IPC+.**
3. **Negative control-blocked IPC wells** do not contain sample template in the PCR reaction. Amplification is prevented by a blocking agent. As a result, no amplification should occur in negative control-blocked IPC wells because the reaction contains no sample template and amplification of the IPC is blocked. The negative control-blocked IPC is called *no amplification control (NAC).*

   If the run method includes amplification, real-time data are plotted in an amplification plot.

No IPC, singleplex setup

Omit the IPC from your Presence/Absence experiment. PCR reactions contain one primer/probe set. PCR reactions do not contain the IPC. With this setup, there are two well types:

- **Unknown wells**—Wells contain sample template. The presence of the target is not known.
- **Negative controls**—Wells contain water or buffer instead of sample template.

About the instrument run

With Presence/Absence experiments, the instrument runs can include:

- **Pre-PCR read**—Perform the pre-PCR read on the QuantStudio™ 12K Flex Software before PCR amplification to collect baseline fluorescence data.
- **Amplification**—Perform amplification on the QuantStudio™ 12K Flex Software to collect fluorescence data during PCR amplification. If you do not include amplification in the run method, perform amplification on another instrument.
- **Post-PCR read**—To determine the results for Presence/Absence experiments, perform the post-PCR read on the instrument after PCR amplification to collect endpoint fluorescence data.

  Fluorescence data collected during the instrument run are stored in an experiment data file (EDS).

About the analysis

Data from the instrument run are used to determine Presence/Absence calls. Results are plotted in a Presence/Absence plot. If the experiment includes amplification, results are plotted in an amplification plot.

- **Pre-PCR read**—If included, the data collected from the pre-PCR read can be used to normalize data collected from the post-PCR read.
- **Amplification**—If included, the data collected from the amplification can be used to troubleshoot.
- **Post-PCR read**—The data collected from the post-PCR read are used to make Presence/Absence calls:
  - **Presence**—The target amplified above the target's threshold. The target is present in the sample.
  - **Absence**—The target did not amplify above the target's threshold. The target is absent in the sample.
  - **Unconfirmed**—The data collected is below the target threshold, and the intensity of IPC is below the IPC threshold.

With the IPC setup, the data collected form the post-PCR read are used to make the following calls:

- **IPC Failed**—The IPC target did not amplify in the IPC wells and/or the IPC target amplified in the blocked IPC wells.
- **IPC Succeeded**—The IPC target amplified in the IPC wells and the IPC target did not amplify in the blocked IPC wells.

About the example experiment

To illustrate how to perform Presence/Absence experiments, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with a QuantStudio™ 12K Flex Real–Time PCR System.

The objective of the Presence/Absence example experiment is to determine if a pathogen is present or absent in each batch of ground beef.

- DNA is extracted from samples using the PrepMan™ Ultra Sample Preparation Reagent (Cat. No. 4318930). The DNA is extracted from each of the four samples of ground beef or from the bacteria found in the ground beef.
- The target is a pathogen.
- The experiment is designed for duplex PCR, where each reaction contains two primer/probe sets. One set detects the pathogen sequence, TGFB (primer set and FAM™ dye-labeled probe to detect the TGFB sequence). The other primer/probe set detects the IPC primer set and VIC™ dye-labeled TaqMan™ probe detects the IPC template.

28 Design the experiment

This chapter explains how to design the example experiment from the **Setup** menu in the **Experiment Menu** pane.

Note: To automatically export the analyzed data to a specified location, select the **Auto Export** checkbox in the **Export** screen, before running the experiment. For more information on Auto Export, see the part "Getting started" on page 17.

Define the experiment properties

In the **Experiment Menu** pane, click **Setup** ▸ **Experiment Properties**.

Enter or select the following information.

Field or selection	Entry
Experiment Name field	Enter ***96-Well Presence Absence Example***.
Barcode field	Leave the **Barcode** field empty.
User Name field	Enter ***Example User*** or enter a user name.
Comments field	Enter ***Presence/Absence example***.
Block	Select **96-Well (0.2 mL)**.
Experiment Type	Select **Presence/Absence**.
Reagents	Select **TaqMan Reagents**.
Ramp speed	Select **Standard**.

Select all three data collection check boxes.

- **Pre-PCR Read** checkbox
- **Amplification** checkbox
- **Post-PCR Read** checkbox

Save the experiment.

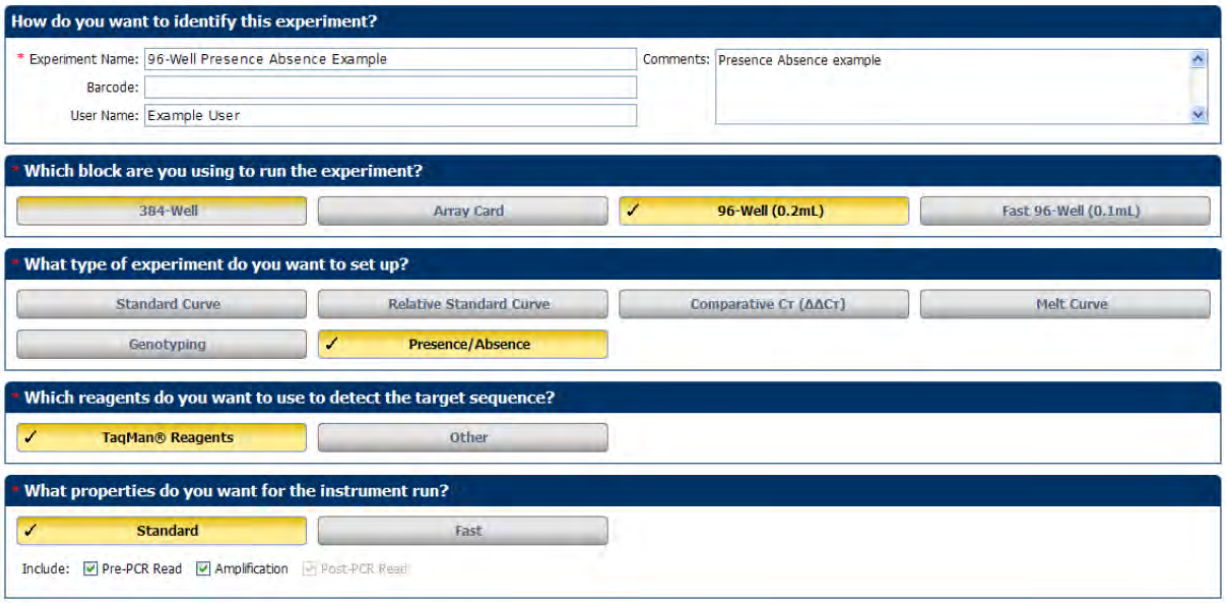


Figure 95 Experiment Properties screen

Define targets and samples

1. Click **Define** to access the **Define** screen.
2. Enter the following target information.

Target Name	Reporter	Quencher	Color
TGFB	FAM	NFQ-MGB	
IPC	VIC	TAMRA	

3. Enter the following sample information.

Sample Name	Color
(+)	
(-)	
NAC	
NTC	

4. In the **Passive Reference** dropdown list, select **ROX** .

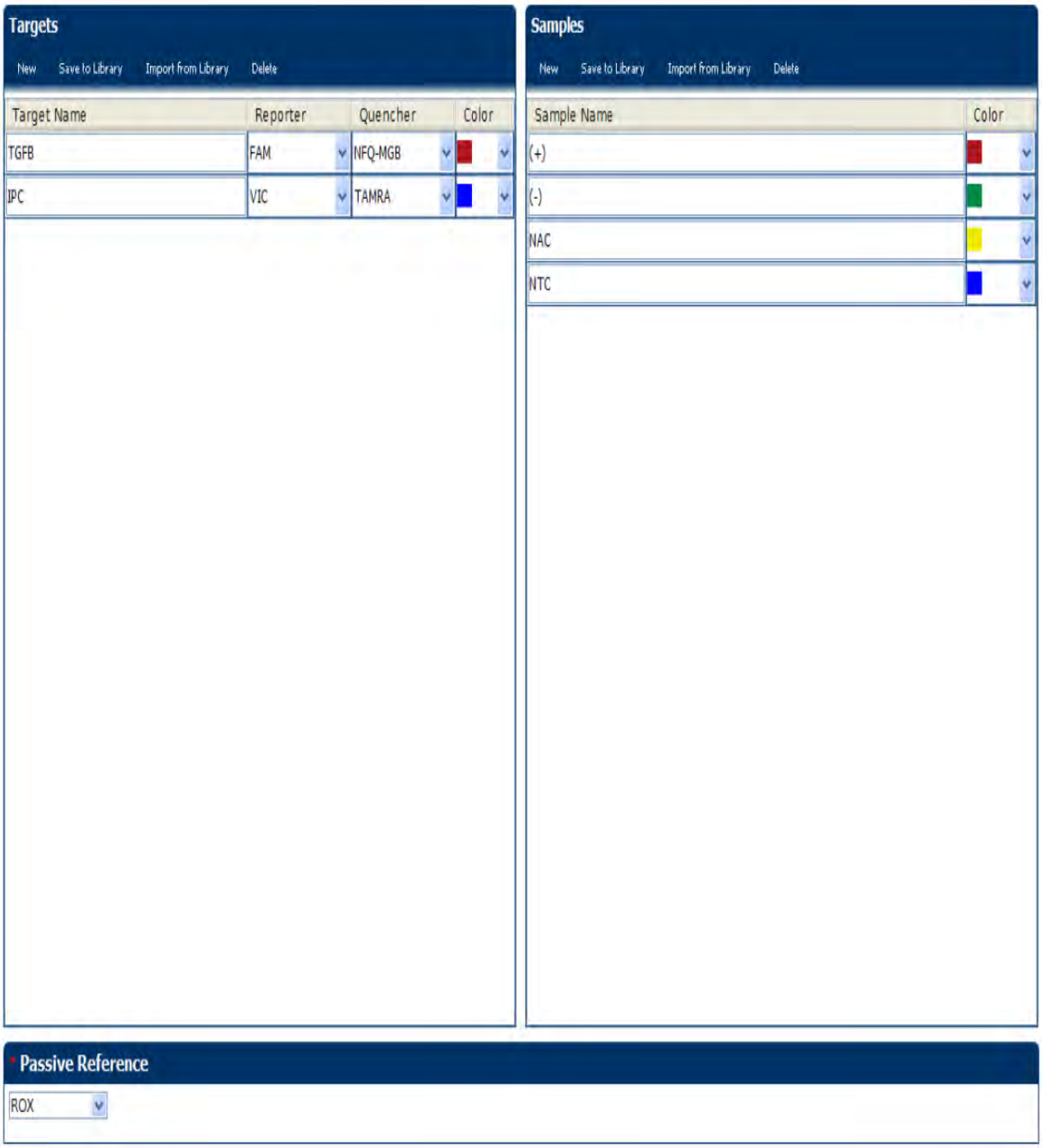


Figure 96 Define screen

Assign targets and samples

Click **Assign** to access the **Assign** screen.

Enter the following targets and samples.

Target Name	Well Number	Task	Sample
TGFB IPC	A1 - A4 (Columns 1 - 4)	Negative No IPC	NAC
TGFB IPC	A5 - A8 (Columns 5 - 8)	IPC Negative	NTC
TGFB IPC	B1 - B10 (Columns 1 - 10)	Unknown IPC	(+)
TGFB IPC	C1 - C10 (Columns 1 - 10)	Unknown IPC	(-)

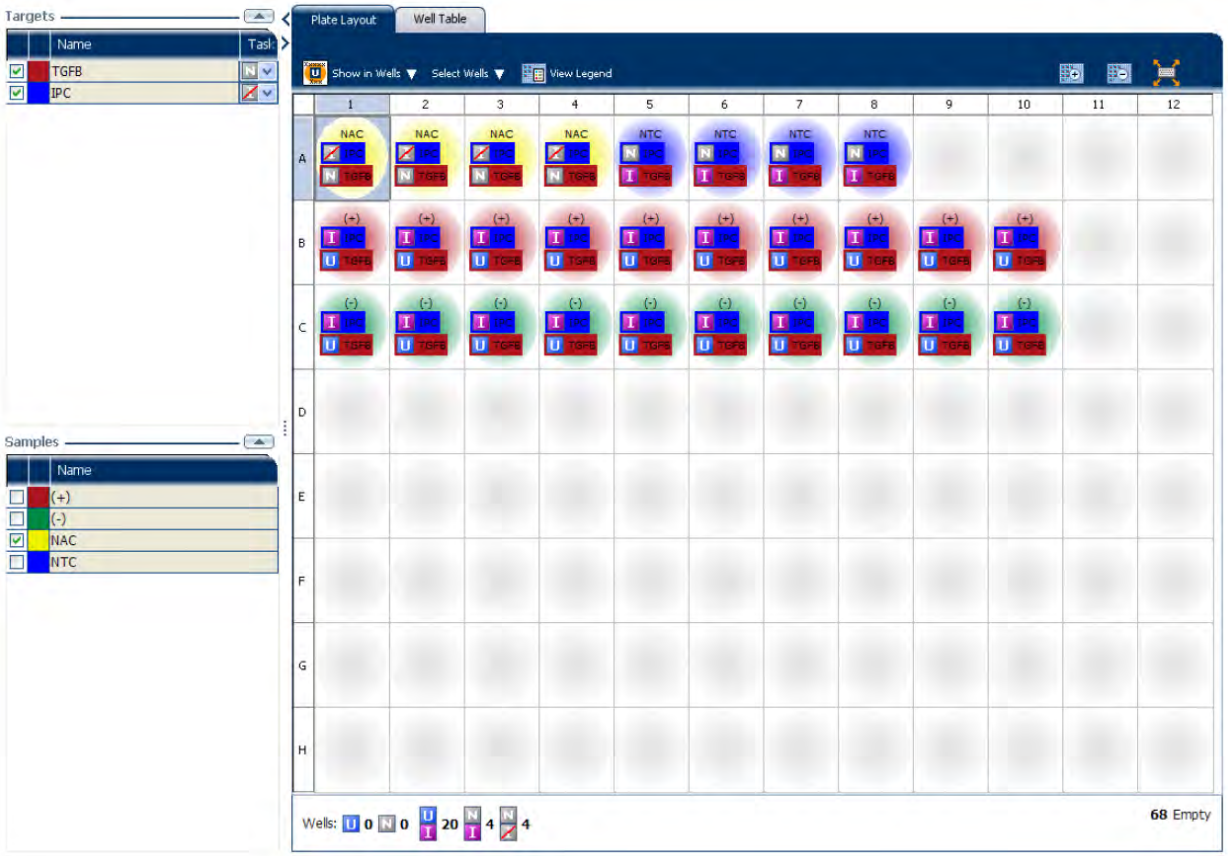


Figure 97 Assign screen

Set up the run method

Click **Run Method** to access the **Run Method** screen. Set the thermal profile under the **Graphical View** tab. Enter the following information:

- Reaction Volume Per Well: ***25 µL***
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Pre-Read Stage	Step 1	1.6°C/s	60°C	30 seconds
Hold Stage	Step 1	1.6°C/s	95°C	10 minutes
PCR Stage • Number of Cycles: 40 (default) • Enable AutoDelta: Unchecked (default) • Starting Cycle: Disabled when Enable AutoDelta is unchecked	Step 1	1.6°C/s	95°C	15 seconds
	Step2	1.6°C/s	60°C	1 minute
Post-Read Stage	Step 1	1.6°C/s	60°C	30 seconds

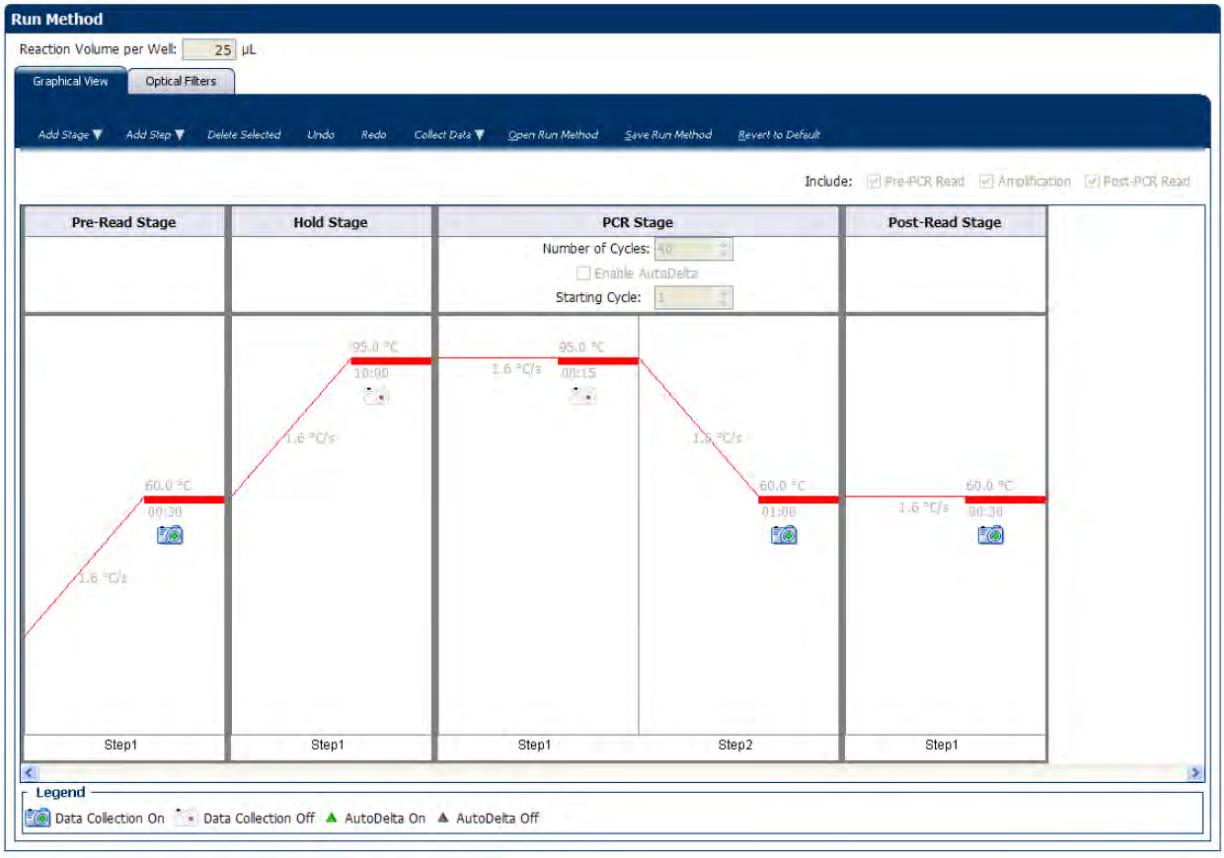


Figure 98 Run Method screen

For more information

Information	Reference
Consumables	Chapter 1, “General information and instructions”
Using alternative setup	Chapter 2, “Experiment shortcuts”

29 Prepare the reactions

This chapter explains how to prepare the PCR reactions for the Presence/Absence example experiment.

Assemble required materials

- Items listed in the part "Getting started" on page 17
- Samples—DNA extracted from ground beef (100 ng/µL)
- Example experiment reaction mix components:
  - TaqMan™ Universal PCR Master Mix
  - 10× IPC Mix
  - 50× IPC DNA
  - 20× Primer/Probe Mix

Prepare the reaction mix

For the Presence/Absence example experiment, four reaction mixes are used.

- (+)
- (-)
- NTC/IPC+
- NAC/IPC-

The following tables list the universal assay conditions (volume and final concentration) for using the TaqMan™ Universal PCR Master Mix for the four reaction mixes.

Reaction mix	Reaction component	Volume for 1 reaction	Volume for 11 reactions (10 wells + 10% overage)
Reaction mix 1 for Sample (+)	TaqMan™ Universal PCR Master Mix (2.0×)	12.50 µL	137.50 µL
	10× IPC Mix	2.50 µL	27.5 µL
	50× IPC DNA	0.50 µL	5.5 µL
	20× Primer/Probe Mix	1.25 µL	13.75 µL
	Water/Buffer	5.75 µL	63.25 µL
	Diluted unknown 1	2.5 µL	27.5 µL

(continued)

Reaction mix	Reaction component	Volume for 1 reaction	Volume for 11 reactions (10 wells + 10% overage)
Reaction mix 1 for Sample (+)	**Total reaction mix volume**	**25.0 µL**	**275 µL**
Reaction mix 2 for Sample (-)	TaqMan™ Universal PCR Master Mix (2.0×)	12.50 µL	137.50 µL
	10× IPC Mix	2.50 µL	27.5 µL
	50× IPC DNA	0.50 µL	5.5 µL
	20× Primer/Probe Mix	1.25 µL	13.75 µL
	Water/Buffer	5.75 µL	63.25 µL
	Diluted unknown 2	2.5 µL	27.5 µL
	Total reaction mix volume	**25.0 µL**	**275 µL**

Reaction mix	Reaction component	Volume for 1 reaction	Volume for 5 reactions (4 wells + 10% overage)
Reaction mix 3 for NTC/IPC+	TaqMan™ Universal PCR Master Mix (2.0×)	12.50 µL	62.5 µL
	10× IPC Mix	2.50 µL	12.5 µL
	50× IPC DNA	0.50 µL	2.5 µL
	20× Primer/Probe Mix	1.25 µL	6.25 µL
	Water/Buffer	8.25 µL	41.25 µL
	Total reaction mix volume	**25.0 µL**	**125.0 µL**
Reaction mix 4 for NAC/IPC-	TaqMan™ Universal PCR Master Mix (2.0×)	12.50 µL	62.5 µL
	10× IPC Mix	2.50 µL	12.5 µL
	50× IPC DNA	0.50 µL	2.5 µL
	20× Primer/Probe Mix	1.25 µL	6.25 µL
	IPC Block	2.5 µL	12.5 µL
	Water/Buffer	5.75 µL	28.75 µL
	Total reaction mix volume	**25.0 µL**	**125.0 µL**

1. Label four appropriately sized tubes for the reaction mixes.
   - Sample (+)
   - Sample (-)
   - NTC
   - NAC
2. Add the required volumes of each reaction mix component to the tube.
3. Mix the contents thoroughly by gently pipetting up and down several times, then cap the tube.
4. Centrifuge the tube briefly to remove air bubbles.
5. Place the reaction mix on ice until you prepare the reaction plate.

Note: You can separately add the sample to the reaction plate, as opposed to preparing individual reaction mixes for each sample.

Prepare the reaction plate

The reaction plate for the Presence/Absence example experiment contains the following items:

- A MicroAmp™ Optical 96-Well Reaction Plate (0.2 mL)
- Reaction volume of 25 µL/well
- 10 (+) wells U I
- 10 Sample (-) wells U I
- 4 NTC/IPC+ N
- 4 NAC/IPC- N I

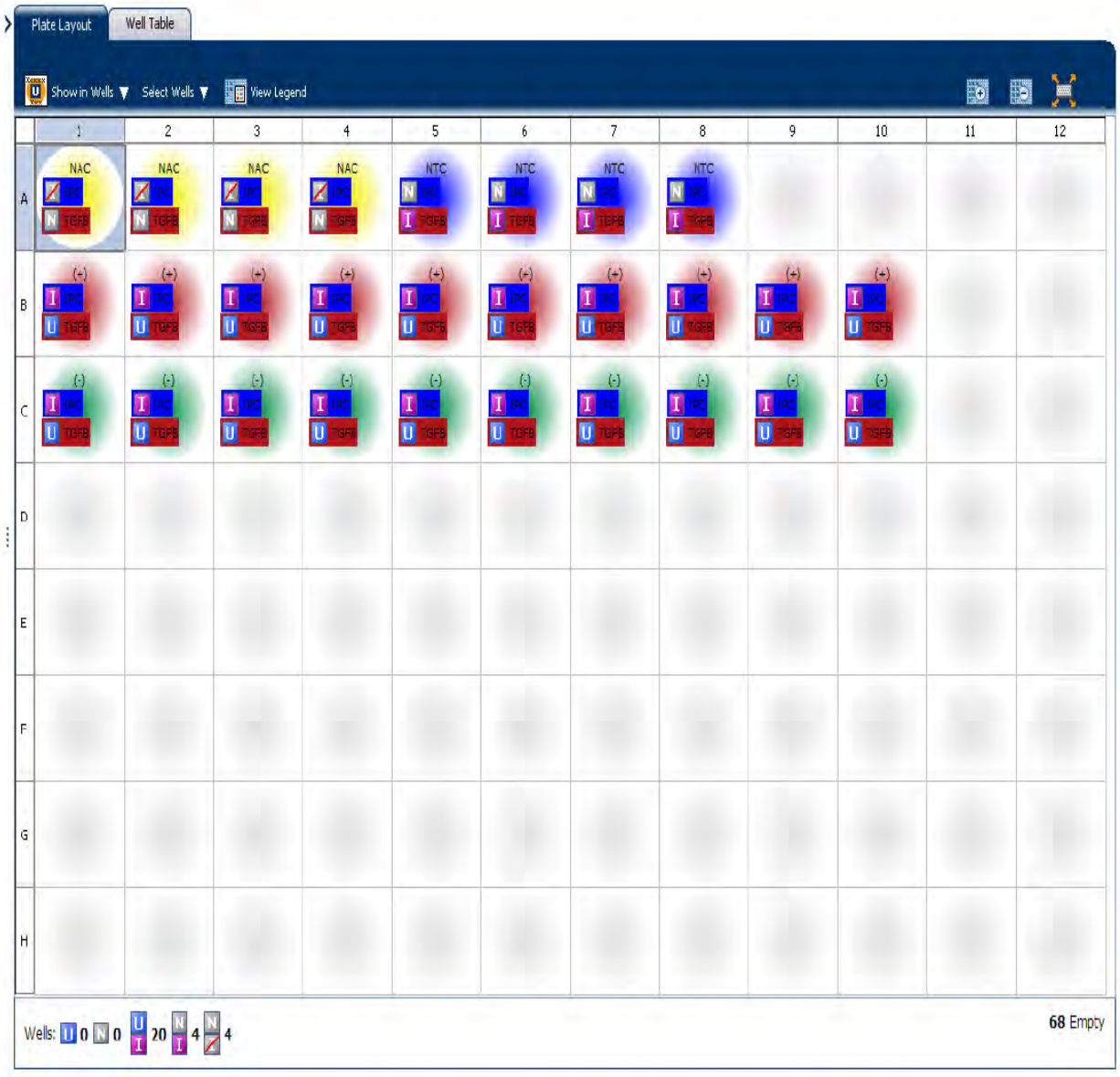


Figure 99 Plate layout

1. Add 25 µL of reaction mix 1 to wells B1 - B10.
2. Add 25 µL of reaction mix 2 to wells C1 - C10.
3. Add 25 µL of reaction mix 3 to wells A5 - A8.
4. Add 25 µL of reaction mix 4 to wells A1 - A4.
5. Seal the reaction plate with optical adhesive film.
6. Centrifuge the reaction plate briefly to remove air bubbles.

7. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.

8. Until you are ready to perform the run, place the reaction plate at 4°C, in the dark.

For more information

Information	Reference
Assigning the reaction plate components	Chapter 1, “General information and instructions”
Sealing the reaction plate	Chapter 1, “General information and instructions”

30 Run the experiment

This chapter explains how to run the example experiment on the QuantStudio™ 12K Flex Real-Time PCR Instrument.

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the instrument is in operation.

Start the run

1. Open the Presence/Absence example file that you created.
   See Chapter 28, “Design the experiment”.
2. Load the reaction plate into the instrument.
3. Start the run.

Monitor the run

You can monitor an experiment run in three ways:

- From the **Run** screen of the QuantStudio™ 12K Flex Software, while the experiment is in progress
- From the **Instrument Console** screen of the QuantStudio™ 12K Flex Software, to monitor an experiment started from another computer or from the instrument touchscreen (see “Monitor a run from the software Instrument Console screen” on page 327)
- From the instrument touchscreen (see “Monitor a run from the instrument touchscreen” on page 331)

Monitor a run from the software Instrument Console screen

1. In the **Instrument Console** screen, select the icon of the instrument that you are using to run the experiment.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. In the **Instrument Manager** screen, click **Monitor Running Experiment** to access the **Run** screen.

View the temperature plot

Click **Temperature Plot** from the **Run Experiment** menu.

Figure 100 The Temperature Plot screen as it appears during the example experiment

Note: The sample temperature displayed in the **Current Temperatures** group is an estimated value.

View the run method

Click **Run Method** from the **Run Experiment** menu.

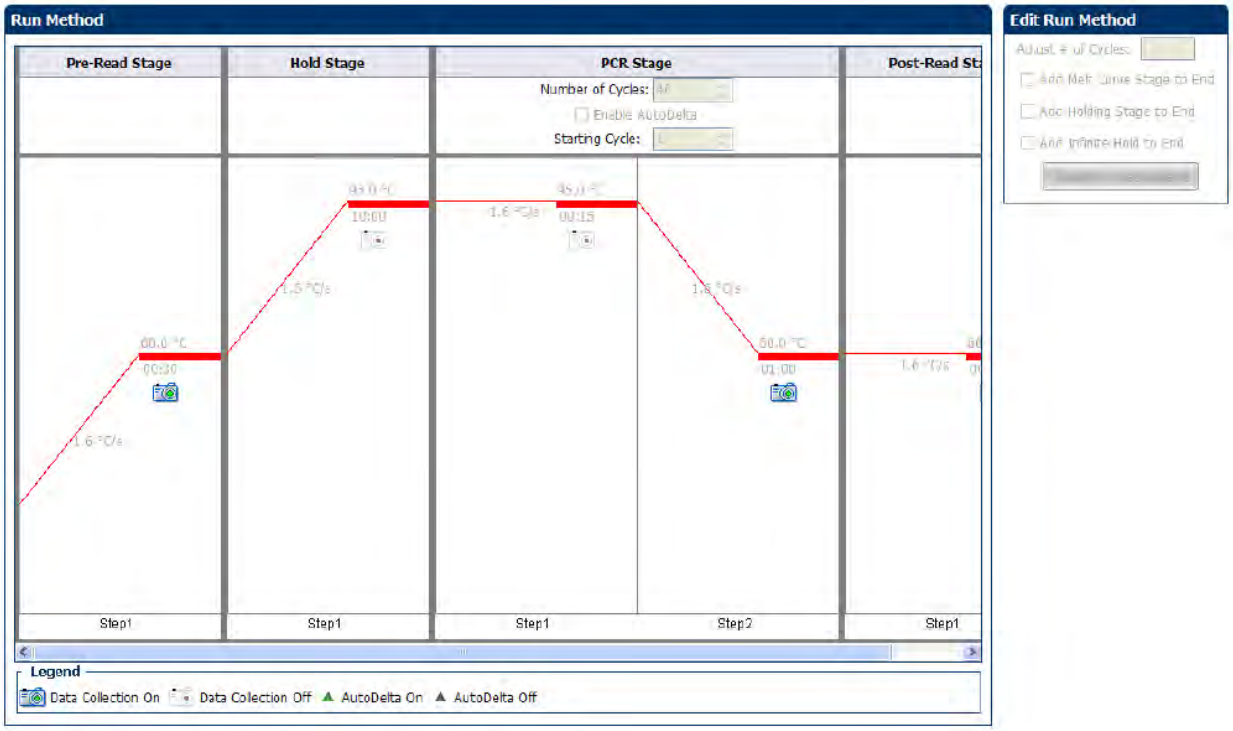


Figure 101 The Run Method screen as it appears in the example experiment

View run data

Click **View Run Data** from the **Run Experiment** menu.

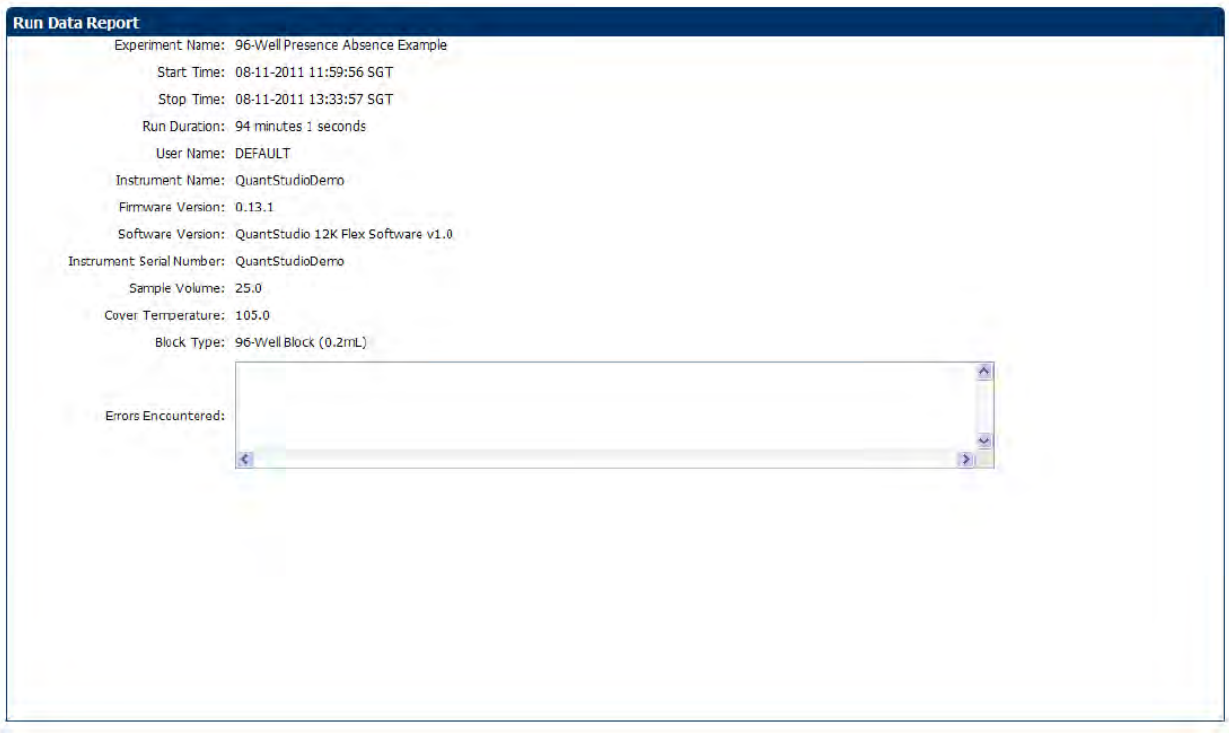


Figure 102 The View Run Data screen as it appears in the example experiment

Monitor a run from the instrument touchscreen

You can view the progress of the run from the instrument touchscreen.

The following figures are for visual representation only. Actual results vary with the experiment.

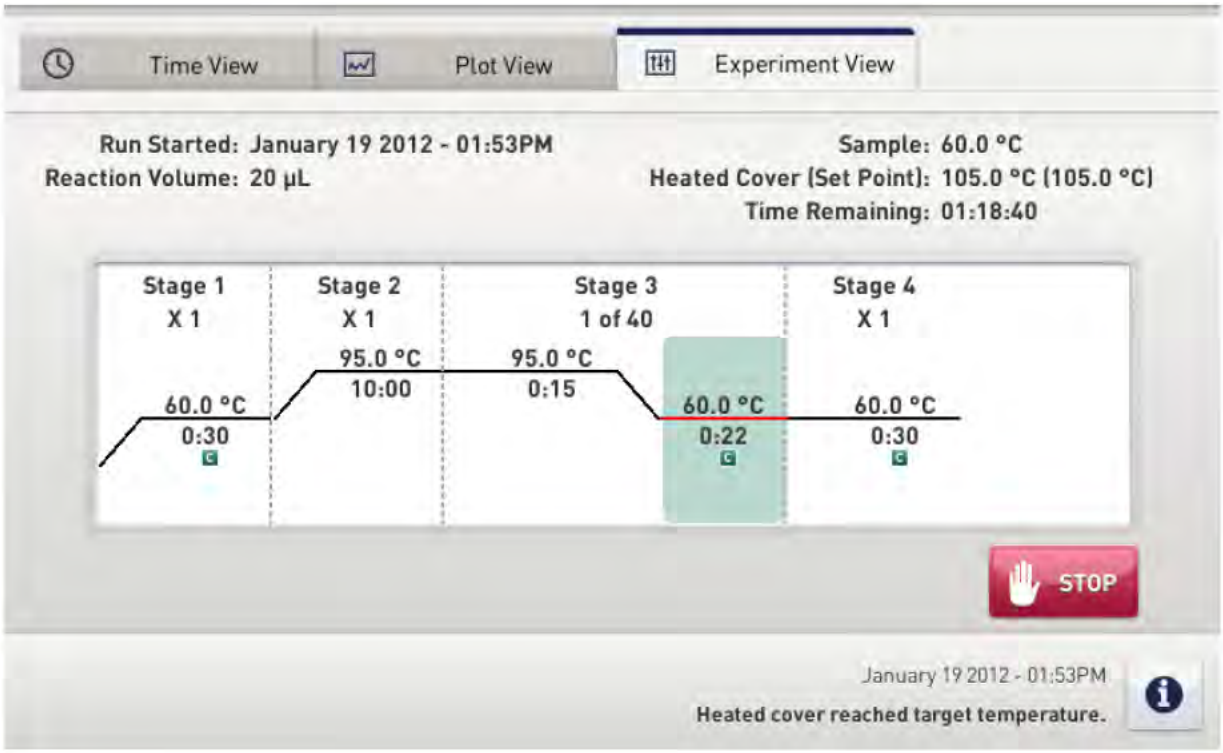


Figure 103 Experiment View tab

Figure 104 Time View tab

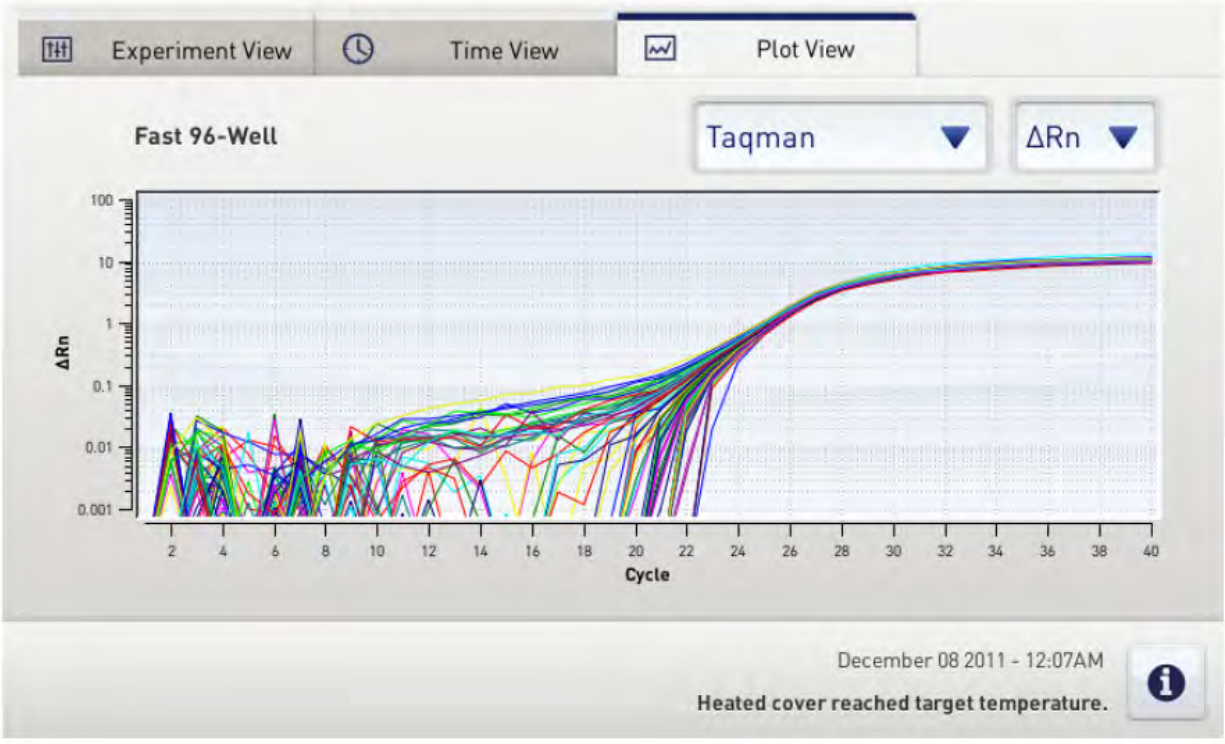


Figure 105 Plot View tab

Note: You will see the Plot View only if your experiment includes the PCR process.

31 Review results and adjust experiment parameters

The following topics are covered in this chapter:

- Review the analyzed data using several of the analysis screens and publish the data (see “Review results” on page 333)
- Modify experiment parameters to troubleshoot problems with experiment results before rerunning an experiment (see “Adjust parameters for re-analysis of your own experiments” on page 353)

Review results

Analyze the example experiment

1. Open the example experiment file.
   See Chapter 30, “Run the experiment”.
2. Click **Analyze**.

Note: You can also access the experiment to analyze from the **Home** screen.

The software analyzes the data using the default analysis settings.

View the presence/absence plot

The Presence/Absence Plot displays the intensity of the fluorescence for each well position. There are four Presence/Absence plot views available:

- All Calls
- Presence calls only
- Absence calls only
- Unconfirmed calls

For each view you can choose to perform the following tasks:

- Show IPC
- Show Controls

Purpose

The purpose of viewing the Presence/Absence Plot for the example experiment is to confirm the following items:

- The target is absent in samples NTC and Sample (-)
- The target is present in Sample (+)
- There are no unconfirmed wells
- The IPC succeeded in all wells
- There is no amplification in NAC wells

To view and assess the presence/absence plot

1. In the **Experiment Menu** pane, click **Analysis** ▸ **Presence/Absence Plot**.

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

2. Display all 96 wells in the **Presence/Absence Plot** screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.

3. Enter the following plot settings.

Menu	Selection
Target Reporter dropdown list	**TGFB**
Control Reporter dropdown list	**IPC**
Show Calls dropdown list	**All Calls**
Show a legend for the plot (This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.)	Check (default)

4. Select the **Show IPC** checkbox to view the fluorescence intensity of the IPC target in the Unknown-IPC wells.

5. Select the **Show Controls** checkbox to view the fluorescence intensity of the IPC target in the negative control-IPC wells and the negative control-Blocked IPC wells.

6. View the fluorescence intensity.
   - Presence calls—Select **Presence** from the **Show Calls** dropdown list.
   - Absence calls—Select **Absence** from the **Show Calls** dropdown list.
   - Unconfirmed calls—Select **Unconfirmed** from the **Show Calls** dropdown list.

Note: The Presence/Absence example experiment does not contain any unconfirmed calls.

Figure 106 The Presence/Absence Plot for the example experiment

Tips for viewing presence/absence plots in your own experiments

- The **IPC threshold** is calculated from the negative control-Blocked IPC reactions.
- **The Target Threshold** is calculated from the negative control-IPC reactions. If the intensity of the target is:
  - Above the target threshold, the call is present (regardless of the intensity of the IPC).
  - Below the target threshold, and the IPC's intensity is above the IPC threshold, the call is absent.
  - Below the target threshold, and the IPC's intensity is below the IPC threshold, the call is unconfirmed.
- **Target Calls:**
  - Presence
  - Absence
  - Unconfirmed
- **IPC Calls:**
  - IPC Succeeded
  - IPC Failed

- **Control Well Calls:**
  - Negative control - IPC
  - Negative control - Blocked IPC

Assess amplification results using the amplification plot

IMPORTANT! Amplification plots are not used to make Presence/Absence calls. Examine the plots to help with troubleshooting and quality control.

Amplification plots available for viewing

The **Amplification Plot** screen displays amplification of all samples in the selected wells. There are three plots available:

- **ΔRn vs Cycle**—ΔRn is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. This plot displays ΔRn as a function of cycle number. Use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle**—Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays Rn as a function of cycle number. Use this plot to identify and examine irregular amplification.
- **C_T vs Well**—C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. Use this plot to locate outlying amplification (outliers).

Each plot can be viewed as a linear or log10 graph type.

Purpose

The purpose of viewing the amplification plot for the example experiment is to review the target to identify:

- Correct baseline and threshold values
- Irregular amplification
- Outliers

View the amplification plot

1. In the **Experiment Menu** pane, click **Analysis** ▸ **Amplification Plot**.

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

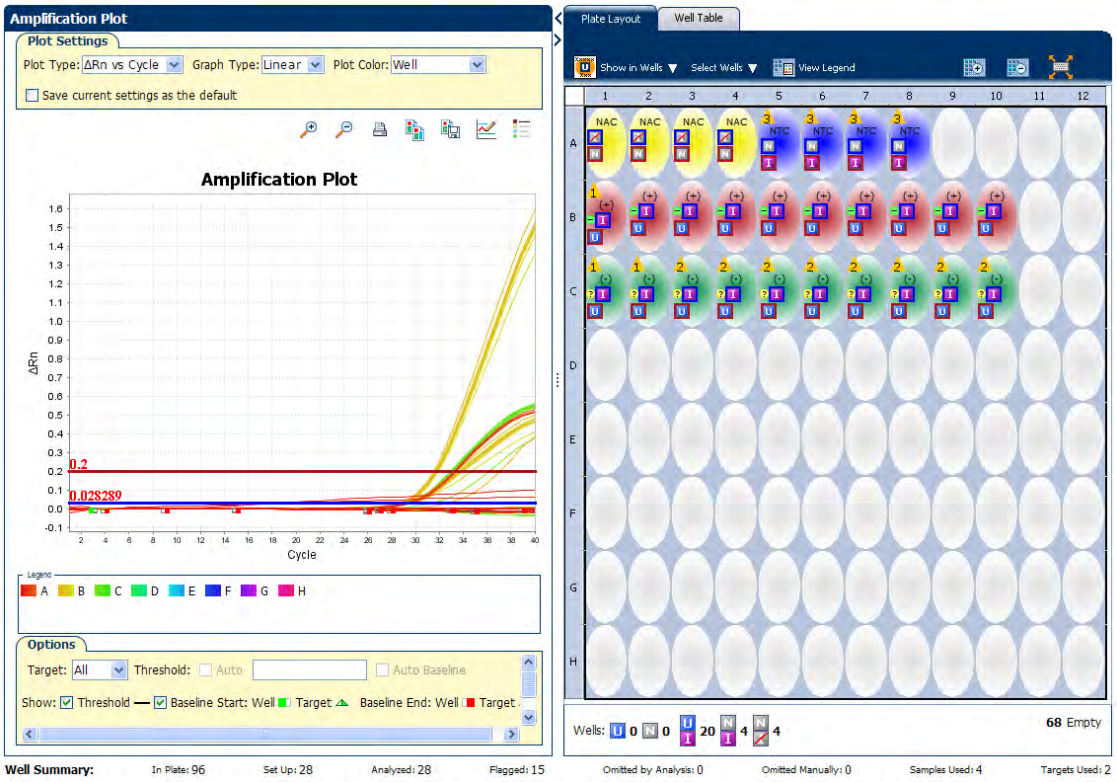
2. Display all 96 wells in the amplification plot by clicking the upper left corner of the plate layout in the **Plate Layout** tab.

3. Expand the Plate Layout tab by clicking the left facing arrow that is left of the tab.

4. In the **Amplification Plot** screen, enter the following information.

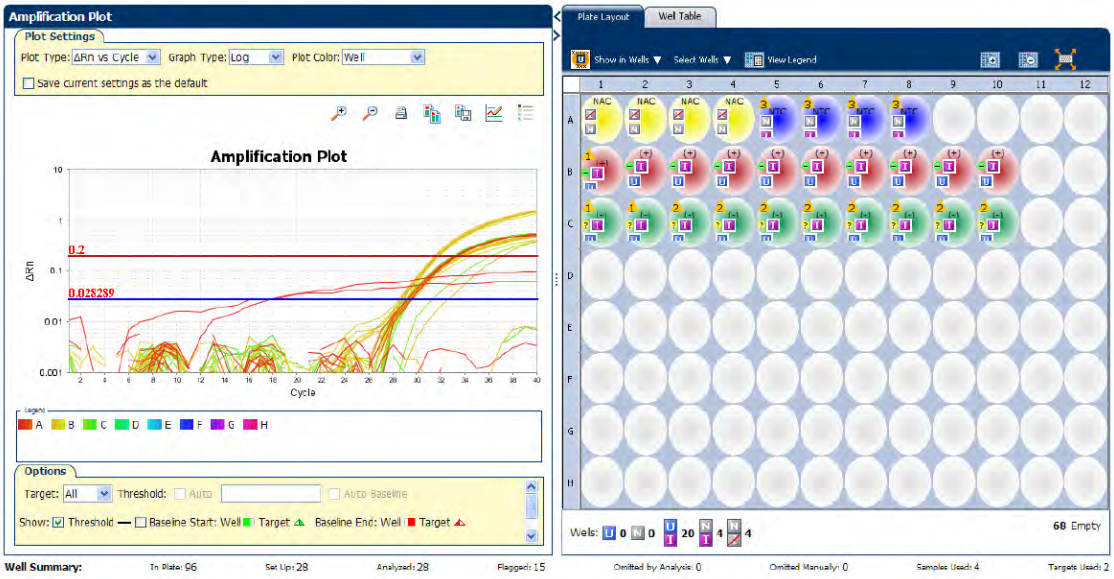
Item	Select
Plot Type dropdown list	**ΔRn vs Cycle**
Plot Color dropdown list	**Well** (default)
 (This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**).	Check (default)

5. View the baseline values.
   a. From the **Graph Type** dropdown list, select **Linear**.
   b. Select the **Baseline** checkbox to show the start cycle and end cycle.
   c. Confirm that the baseline is set correctly. The end cycle should be set a few cycles before the cycle number where significant fluorescent signal is detected. In the example experiment, the baseline is set correctly.



6. View the threshold values.
   a. From the **Graph Type** dropdown list, select **Log**.
   b. Select the **Threshold** checkbox to show the threshold.

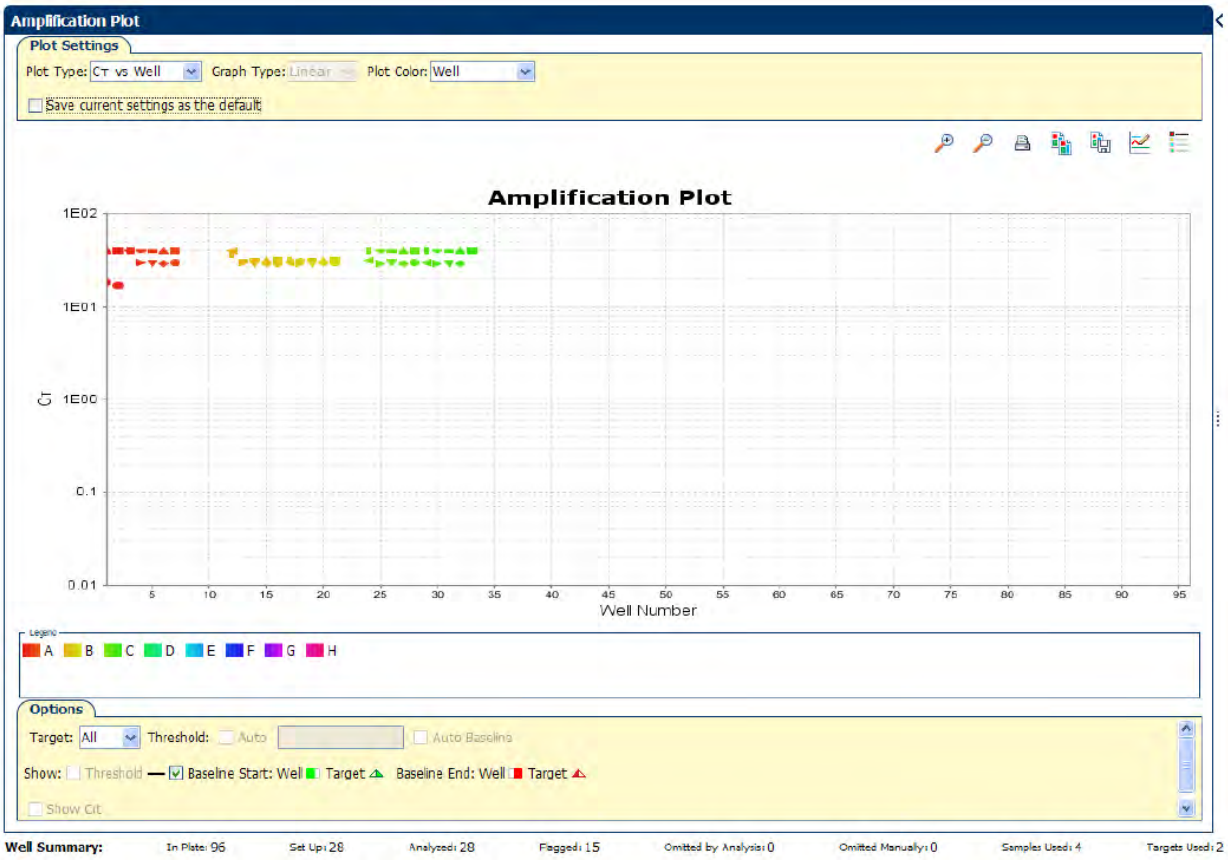
c. Confirm that the threshold is set correctly.



7. Locate any outliers.

a. From the **Plot Type** dropdown list, select **C_T vs. Well**.

b. Look for outliers from the amplification plot. In the example experiment, there are no outliers for IPC.



Tips for viewing amplification plots in your own experiments

When you analyze your own Presence/Absence experiment, look for the following items:

- **Outliers**
- **A typical amplification plot**—The QuantStudio™ 12K Flex Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear phase
  - Exponential (geometric phase)
  - Baseline

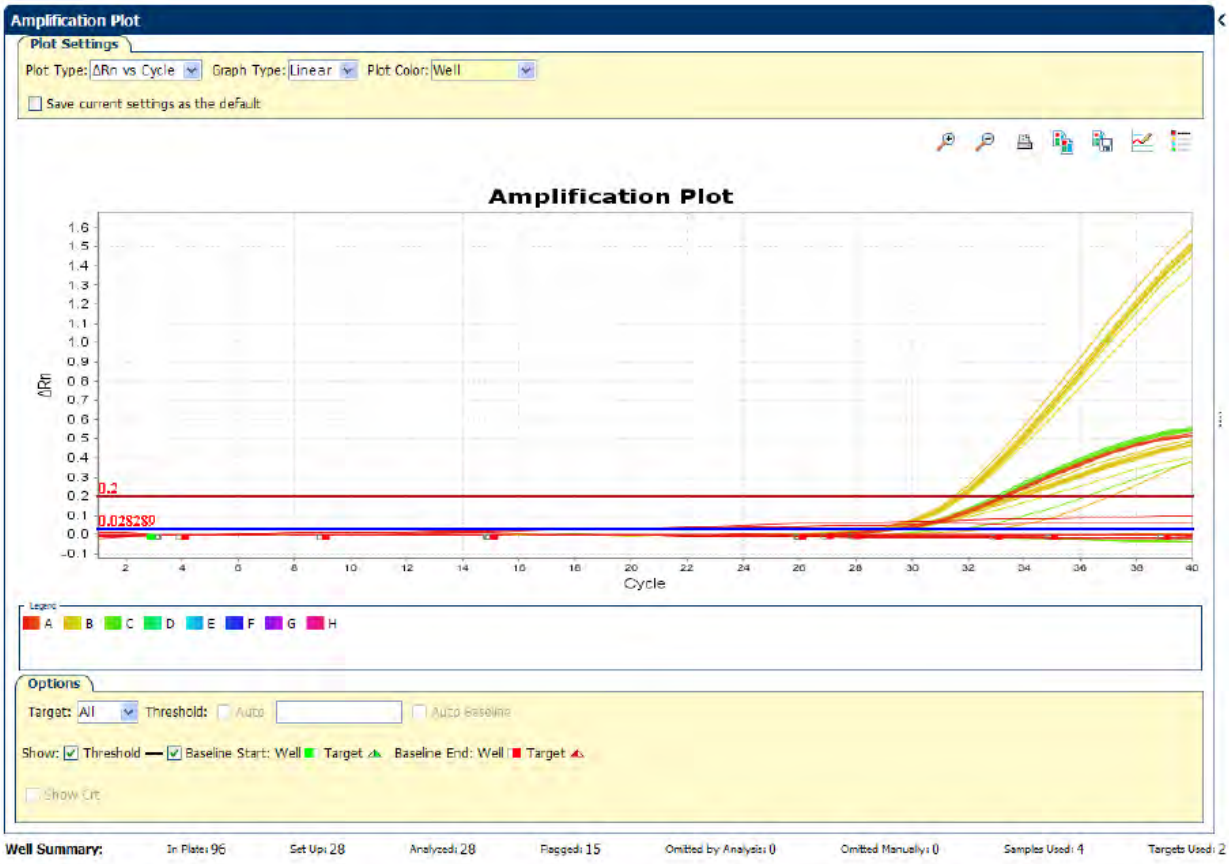


Figure 107 Typical amplification plot

IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudio™ 12K Flex Software. We recommend that you examine the **Amplification Plot** screen and review the assigned baseline and threshold values for each well after analysis completes.

Note: If you use the Relative Threshold algorithm to analyze an experiment that includes amplification, select to view the analysis results using the ΔRn vs Cycle, Rn vs Cycle, or C_{RT} vs Well plot type and Linear or Log graph type. Also select the **Show Crt** check box to view the derived fractional cycle on the amplification plot.

View the well table

The well table displays results data for each well in the reaction plate, including the following items:

- The well number, sample name, target name, task, and dyes
- The calculated values: ΔRn, ΔRn mean, and ΔRn SD

  Note: ΔRn, ΔRn mean, and ΔRn SD are calculated only when the analysis call settings specify to analyze data from the pre-PCR read and the post-PCR read.

- Target and IPC thresholds, Call, Comments
- Flags

Purpose

In the Presence/Absence example experiment, you review the well table for:

- Call
- ΔRn
- Flag

View the well table

1. In the **Experiment Menu** pane, select **Analysis ▸ Amplification Plot,**, then click the **Well Table** tab.

2. Use the **Group By** dropdown list to group wells by a specific category.

   For the example experiment, group the wells by flag, call, and ΔRn value. You can select only one category at a time.

   a. From the **Group By** dropdown list, select **Flag**.

   - 30 wells are listed under **Flagged Wells**.
   - 66 wells are listed under **Unflagged Wells**.

   b. From the **Group By** dropdown list, select **Call**.

   Wells are listed in the following order:

   - Absence
   - Blocked IPC Control
   - IPC Failed
   - IPC Succeeded
   - Negative Control

- Unconfirmed
- No Call

c. From the **Group By** dropdown list, select **None**, then click the column heading **ΔRn**.

Wells are listed in order of increasing ΔRn. Click the column heading again to reverse the sort order.

Tips for analyzing your own experiments

When you analyze your own Presence/Absence experiment, group the wells by the following items:

- **Flag**—The software groups the flagged and unflagged wells. A flag indicates that the software has found an error in the flagged well. For a description of the flags, see “Review the flags in the QC summary” on page 350.
- **Call**—The software groups the wells by call: Negative Control, Blocked-IPC, Presence, Absence, Unconfirmed, IPC Succeeded, and IPC Failed.

Confirm accurate dye signal using the multicomponent plot

The **Multicomponent Plot** screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the Presence/Absence example experiment, review the **Multicomponent Plot** screen for the following items:

- ROX™ dye (passive reference)
- FAM™ dye (reporter)
- VIC™ dye (reporter)
- TAMRA™ (reporter)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

View the multicomponent plot

1. In the **Experiment Menu** pane, select **Analysis** ▸ **Multicomponent Plot**.

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

2. Display the wells one at a time in the **Multicomponent Plot** screen:
   a. Click the **Plate Layout** tab.
   b. Select one well in the plate layout. The well is shown in the Multicomponent Plot.

      Note: If you select multiple wells, the Multicomponent Plot displays the data for all selected wells simultaneously.

3. In the **Plot Color** dropdown list, select **Dye**.

4. Click **Show a legend for the plot** (default).

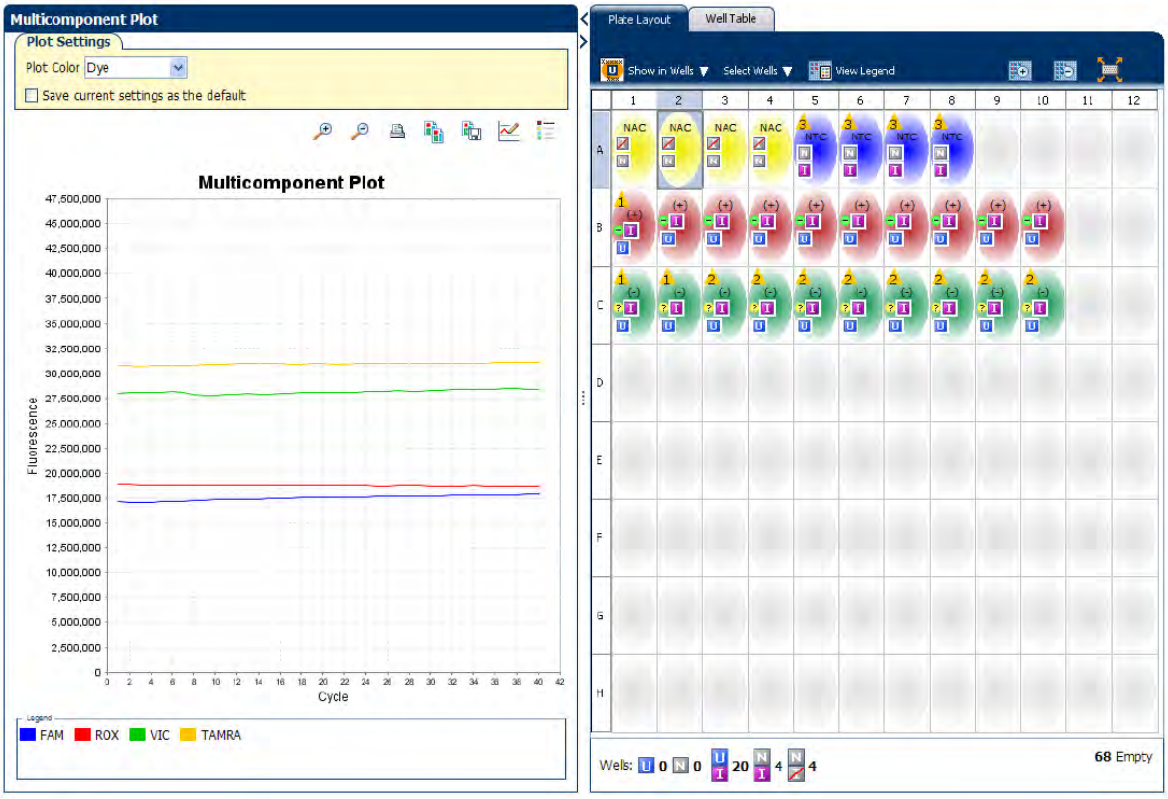
   Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

5. Check the ROX™ dye signal.

   In the example experiment, the ROX™ dye signal remains constant throughout the PCR process. A constant ROX™ dye signal indicates typical data.

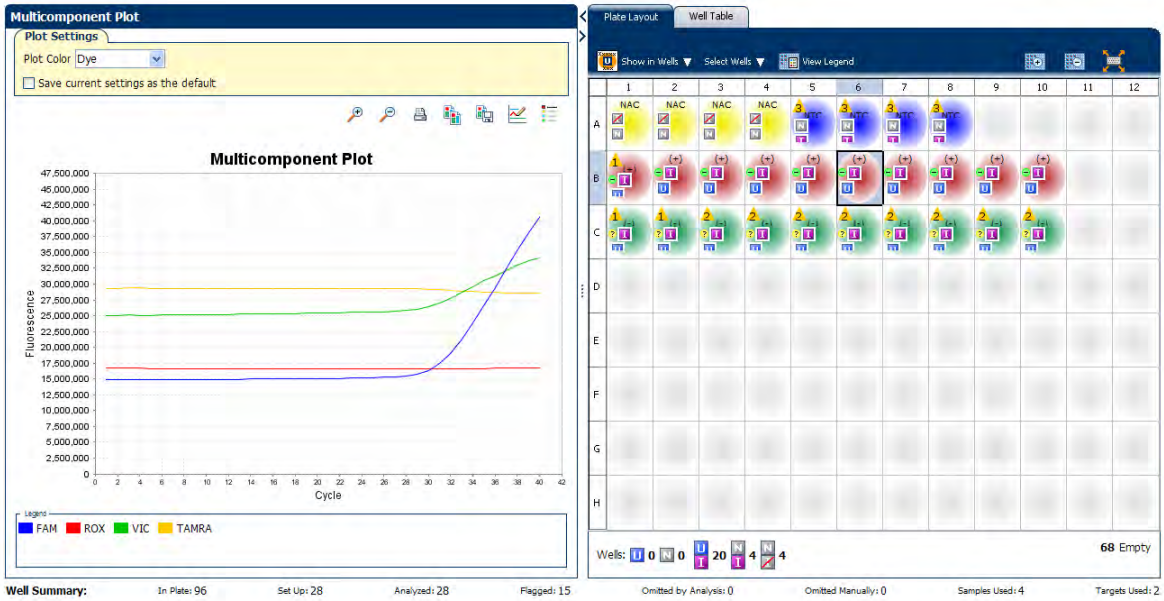
6. Check the VIC™ dye signal.

   In the example experiment the VIC™ dye signal should not amplify for NAC-Blocked IPC wells or if the IPC call for the Unknown-IPC well is IPC Failed.



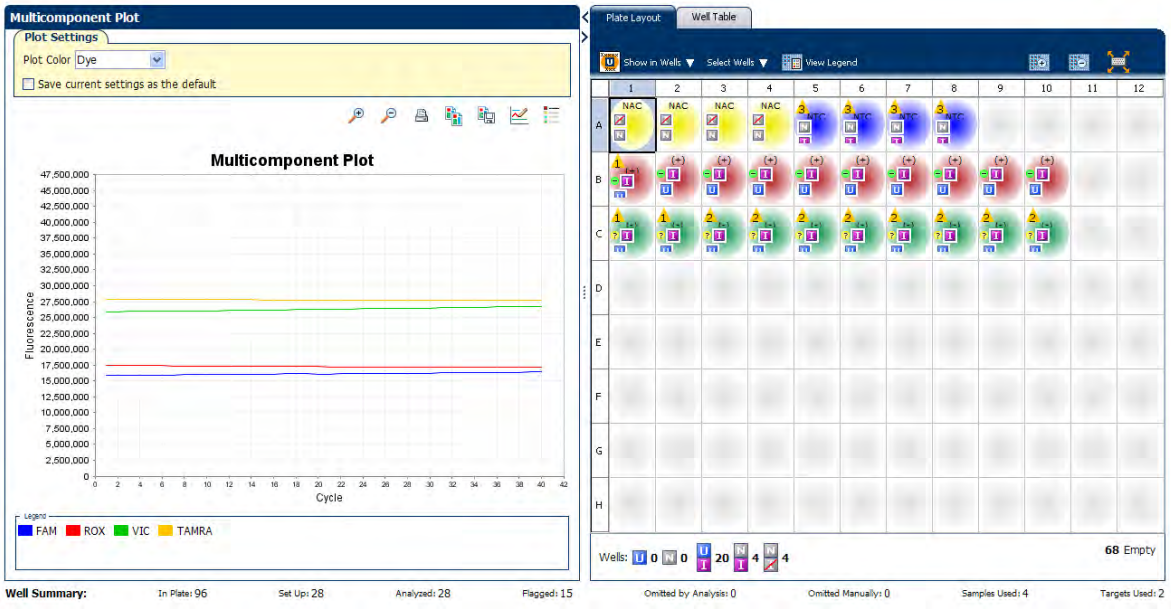
7. Check the FAM™ dye signal.

   In the example experiment, for the sample (+), the FAM™ dye signal increases throughout the PCR process. An increase in FAM™ dye signal indicates normal amplification.



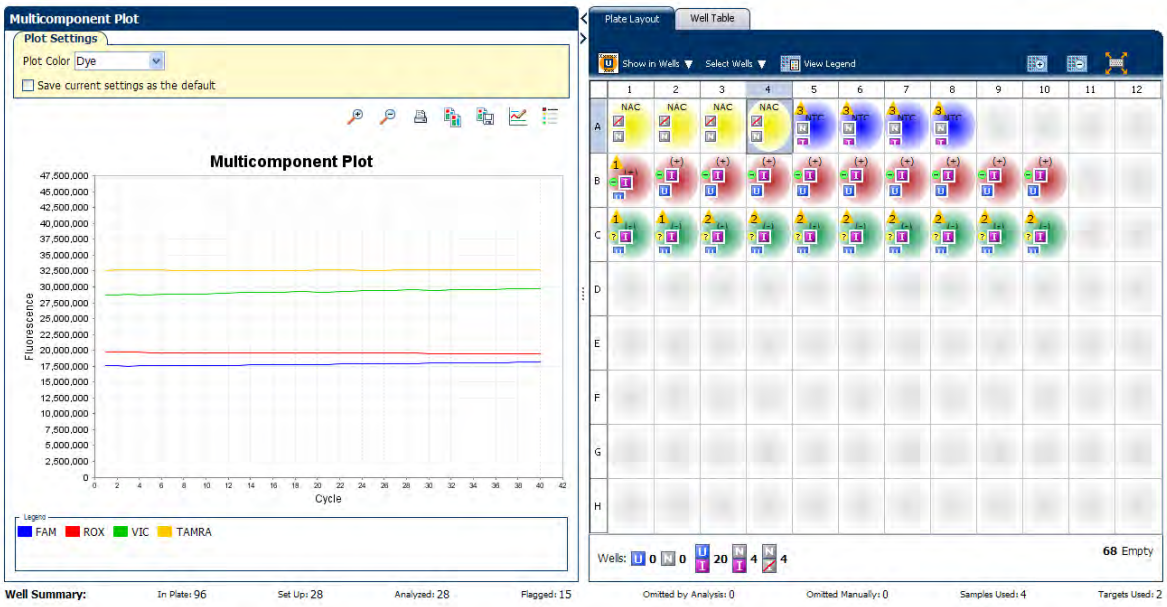
8. Check the TAMRA™ dye signal.

   In the example experiment the TAMRA™ dye signal should not amplify for NAC-Blocked IPC wells or if the IPC call for the Unknown-IPC well is IPC Failed.



9. Select the negative control (NTC) wells one at time and check for amplification.

   In the example experiment, there is no amplification in the negative control wells.



Tips for confirming dye accuracy in your own experiment

When you analyze your own Presence/Absence experiment, look for the following items:

- **Passive reference (ROX™ dye)**—The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye (FAM™ dye)**—The reporter dye fluorescence level should display a flat region corresponding to the baseline. If target is present in the sample (a Presence call is made), the baseline will be followed by a rapid rise in fluorescence as the amplification proceeds.
- **Irregularities in the signal**—There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative control wells**—There should not be any amplification in the negative control wells.

Determine signal accuracy using the raw data plot

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

Purpose

In the Presence/Absence example experiment, review the **Raw Data Plot** screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the raw data plot

1. In the **Experiment Menu** pane, select **Analysis** ▸ **Raw Data Plot**.

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

2. Click **Show a legend for the plot** (default).
   The legend displays the color code for each row of the reaction plate (see the legend in Figure 108 on page 349).

3. Display all 96 wells in the **Raw Data Plot** screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.

4. Select wells that correspond to a replicate group.
   - (-) wells: From the Select Wells with drop-down menus, select sample (-).
   - (+) wells: From the Select Wells with drop-down menus, select sample (+).
   - Negative control-IPC wells: Select wells A5-A8.
   - Negative control-blocked IPC wells: Select wells A1-A4.

5. Click and drag the **Show Cycle** pointer from cycle 1 to cycle 40. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.

Figure 108 Raw data plot

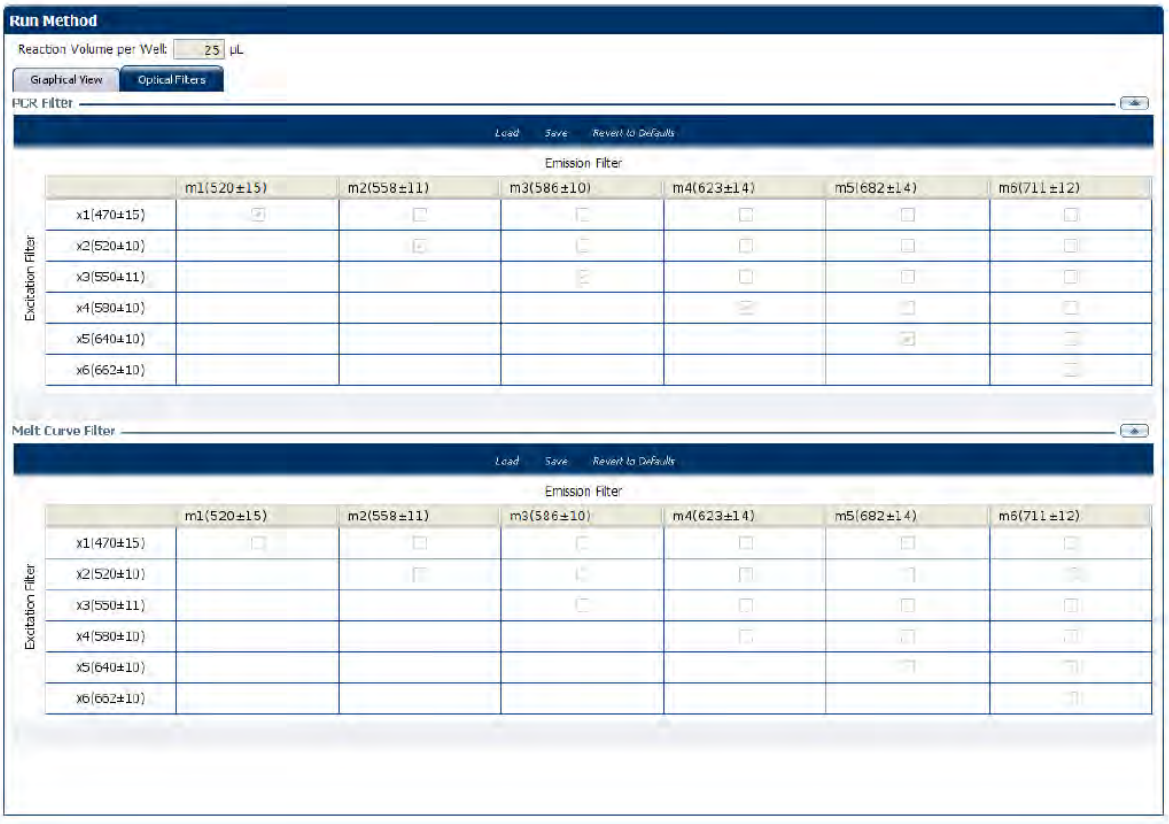


Figure 109 Filters used for the example experiment

Tips for determining signal accuracy in your own experiments

When you analyze your own Presence/Absence experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

Review the flags in the QC summary

The **QC Summary** screen displays a list of the QuantStudio™ 12K Flex Software flags, including the flag frequency and location for the open experiment. In the example experiment, 31 flags have been triggered.

Note: The flags triggered in the example experiment are seen in the (+) and (-) wells. The flag NOAMP indicates that the well containing the sample (+) did not amplify. The flags, NOAMP and EXPFAIL indicate that the wells containing the sample (-) did not amplify and that the software could not identify the exponential region of the amplification plot (as amplification did not take place). The occurrence of these flags in the (-) wells in the example experiment is valid because it indicates the absence of the target in the sample.

View the QC summary

1. In the **Experiment Menu** pane, select **Analysis ▸ QC Summary**.

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

2. Review the Flags Summary

   Note: A 0 displayed in the **Frequency** column indicates that the flag does not appear in the experiment. If the frequency is greater than 0, the flag appears somewhere in the experiment. The well position is listed in the **Wells** column.

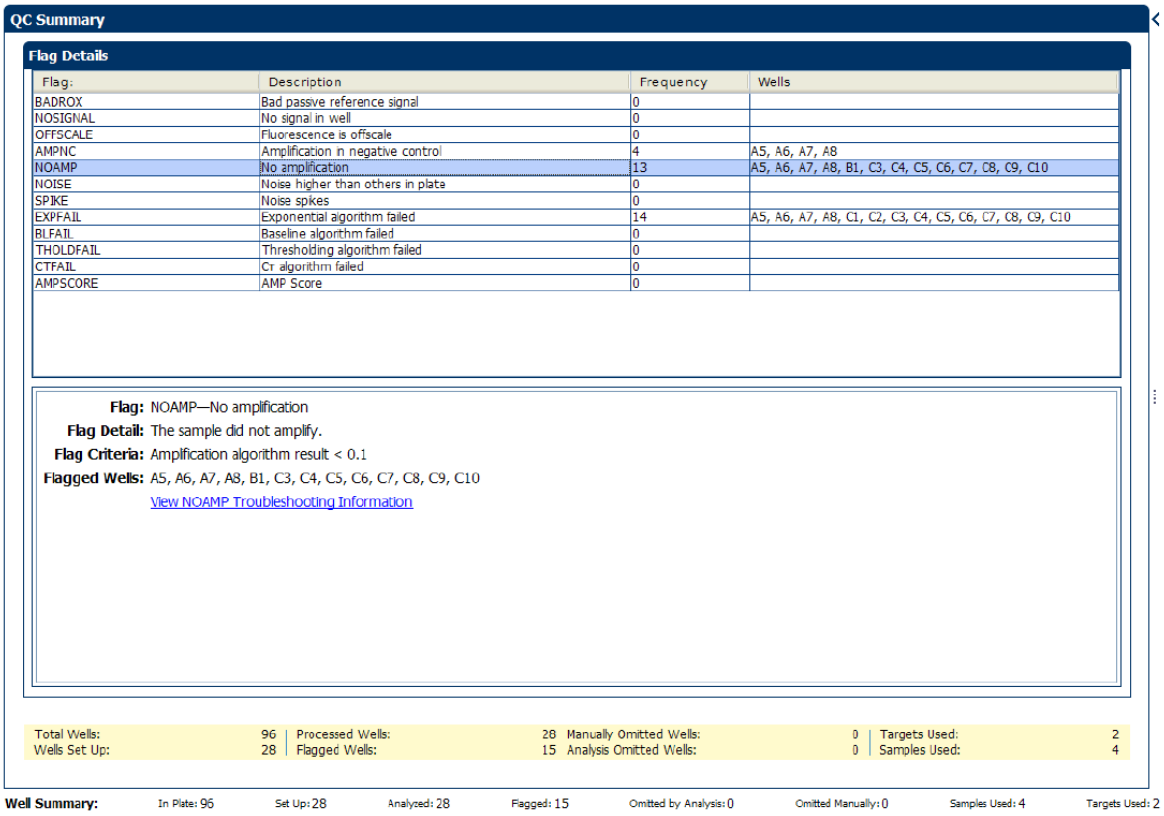
   In the example experiment, there are 15 flagged wells.

3. In the **Flag Details** table, click each flag with a frequency greater than 0 to display detailed information about the flag.

   The following details apply to the example experiment:

   - The NOAMP flag appears 13 times, in the wells A5 - A8, B1, C3 - C10.
   - The EXPFAIL flag appears 14 times, in the same wells as the NOAMP flag, that is, A5 - A8, B1, C3 - C10. In addition, the flag EXPFAIL also appears in the B1 well.
   - The AMPNC flag appears 4 times, in the wells A5-A8.

4. *(Optional)* For those flags with frequency greater than 0, click the troubleshooting link to view information on correcting the flag.



Possible flags

For Presence/Absence experiments, the flags listed below may be triggered by the experiment data.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed

(continued)

Flag	Description
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
AMPSCORE	Amplification in the linear region is below a certain threshold, corresponding to the score set in the analysis settings
Secondary analysis flags	
AMPNC	Amplification in negative control

Note: If the experiment does not include amplification, then the only flags are BADROX, NOSIGNAL, and OFFSCALE.

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the Frequency column for each flag).

For more information

Information	Reference
Publishing data	Chapter 1, "General information and instructions"

Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

The **Analysis Settings** dialog box displays the analysis settings for the call, threshold cycle (C_T), flags, and advanced options.

If the default analysis settings in the QuantStudio™ 12K Flex Software are not suitable for your own experiment, you can change the settings in the **Analysis Settings** dialog box, then reanalyze your experiment.

View the analysis settings

1. In the **Experiment Menu** pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the **Analysis Settings** dialog box.
   In the example experiment, the default analysis settings are used for each tab:
   - Call Settings
   - C_T Settings
   - Flag Settings
   - Advanced Settings

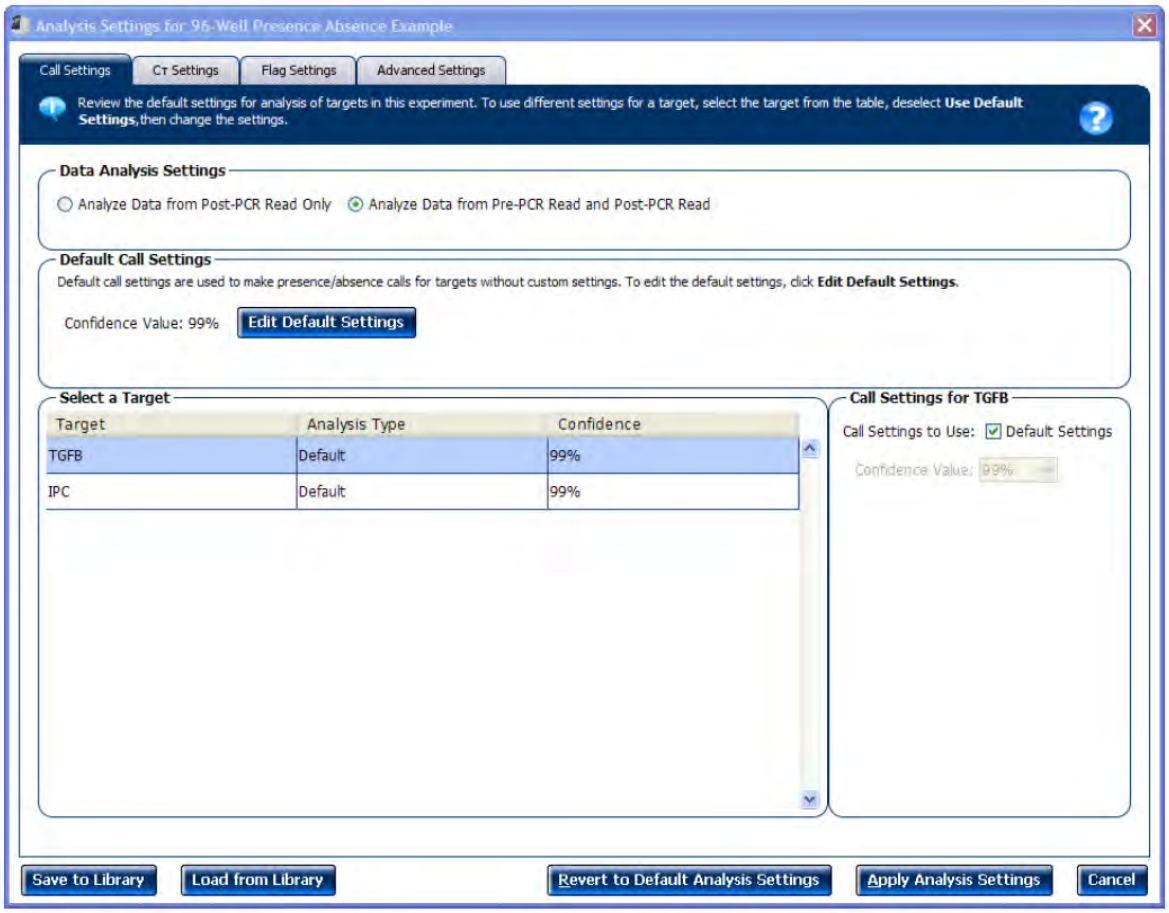


Figure 110 Analysis Settings dialog box for a Presence/Absence experiment

3. View and, if necessary, change the analysis settings (see "Adjust analysis settings" on page 355).

Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see "About the analysis settings library" on page 62.

4. Click **Apply Analysis Settings** to apply the current analysis settings.

Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

Call settings

Use the **Call Settings** tab to:

- Change the default data analysis settings. You can select from the following items:
  - **Analyze Data from Post-PCR Read Only** radio button
  - **Analyze Data from Pre-PCR Read and Post-PCR Read** radio button
- Edit the default call settings.
  - Click **Edit Default Settings**, then select the confidence value to use to make presence/absence calls in the **Edit Default Confidence Value** dialog box. If the confidence value is less than the call setting, the call is unconfirmed.
  - Click **Save Changes**.
- Use custom call settings for a target.
  - Select one or more targets in the table, then deselect the **Default Settings** checkbox.

    The **Confidence Value** field in the right pane is editable when the **Default Settings** checkbox is deselected.
  - Select the confidence value to use to make Presence/Absence calls for the selected target or targets.

C_T settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method.
- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

  The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for quantification.

  The Relative Threshold algorithm is a well-based analysis based on the PCR reaction efficiency and fitted to the Amplification curve. This setting is ideal for a single sample across genes with no dependence on targets, thereby reducing variability. It is not necessary to set either a baseline or a threshold when you use the Relative Threshold algorithm, so any settings for baseline or threshold will not affect the analysis.
- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the targets that do not have custom settings. To edit the default settings, click **Edit Default Settings**.
- **C_T Settings for Target**

When you manually set the threshold and baseline, we recommend the following settings:

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

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag settings

Use the **Flag Settings** tab to perform the following tasks:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the QuantStudio™ 12K Flex Software.

1. In the **Use** column, select the check boxes for flags to apply during analysis.
2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

3. In the **Reject Well** column, select the check boxes if you want the software to reject wells with the flag.

   Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations may change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.

4. Click **Apply Analysis Settings** in the **Analysis Settings** dialog box.

   If the run status is complete, the data are reanalyzed.

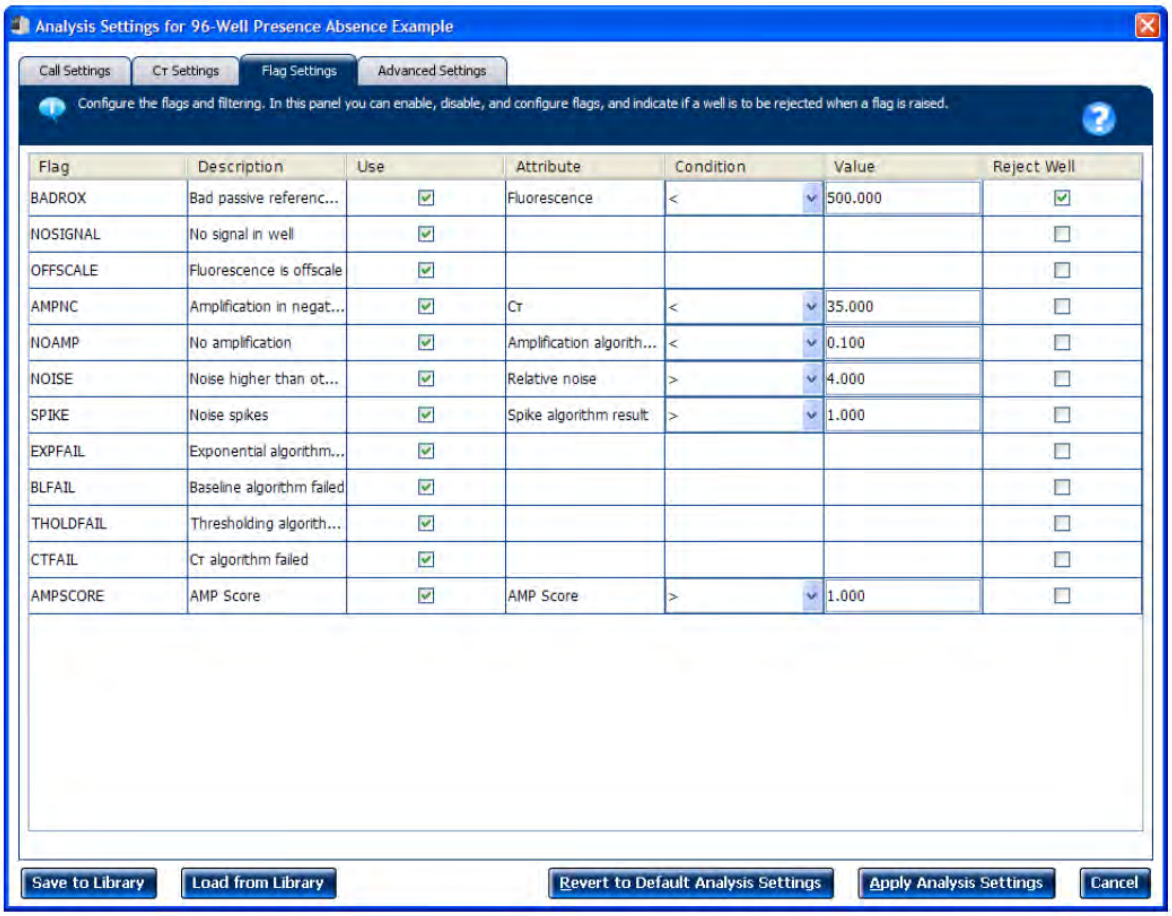


Figure 111 Flag Settings tab

Advanced settings

Use the **Advanced Settings** tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold setting.

The following steps describe the use of custom baseline settings for a well-target combination.

1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** checkbox.
3. Define the custom baseline settings.
   - For automatic baseline calculations, select the **Automatic Baseline** checkbox.
   - To define the baseline, deselect the **Automatic Baseline** checkbox, then enter the baseline start cycle and baseline end cycle.

32 Export analysis results

1. Open the Presence/Absence example experiment file that has been analyzed.

   For information about analysis, see Chapter 31, “Review results and adjust experiment parameters”.

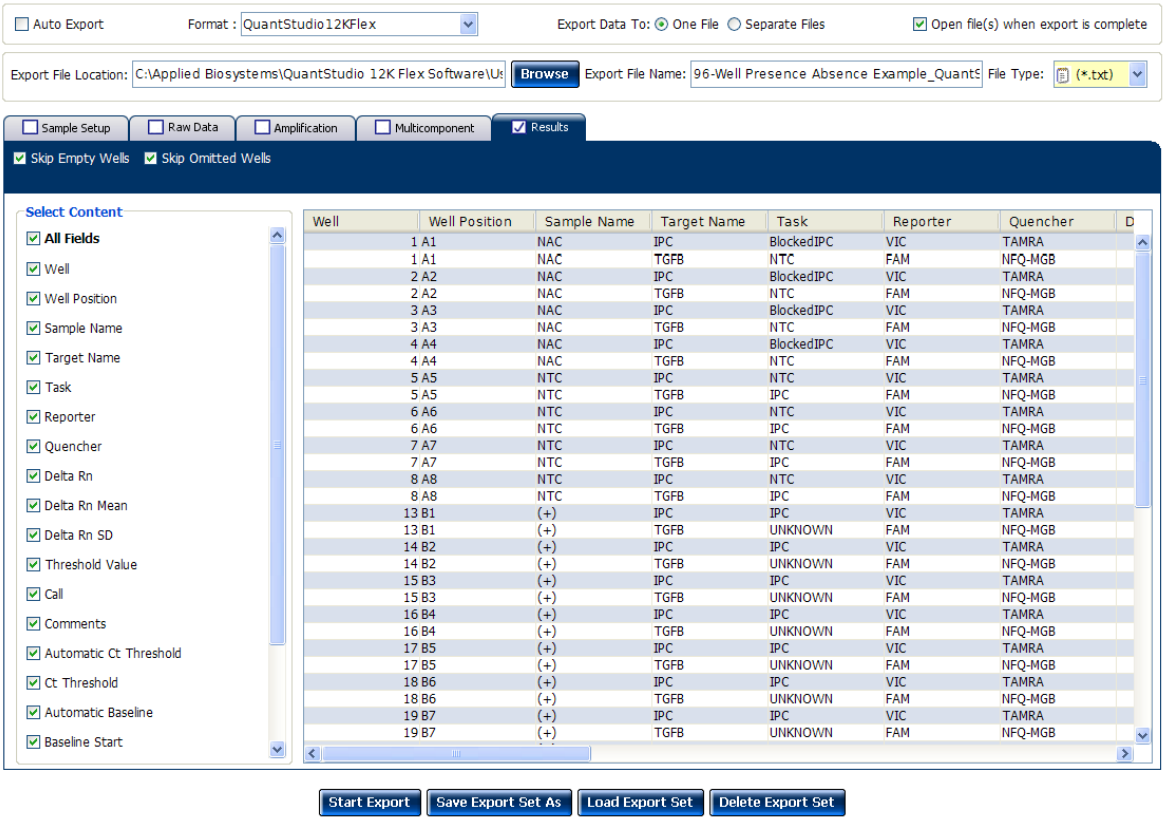
2. In the **Experiment Menu** pane, click **Export**.

   Note: To export data automatically after analysis, select the **Auto Export** checkbox during experiment setup or before running the experiment. The **Auto Export** checkbox is unchecked for the example experiment.

3. In the **Format** dropdown list, select **QuantStudio 12K Flex format**.

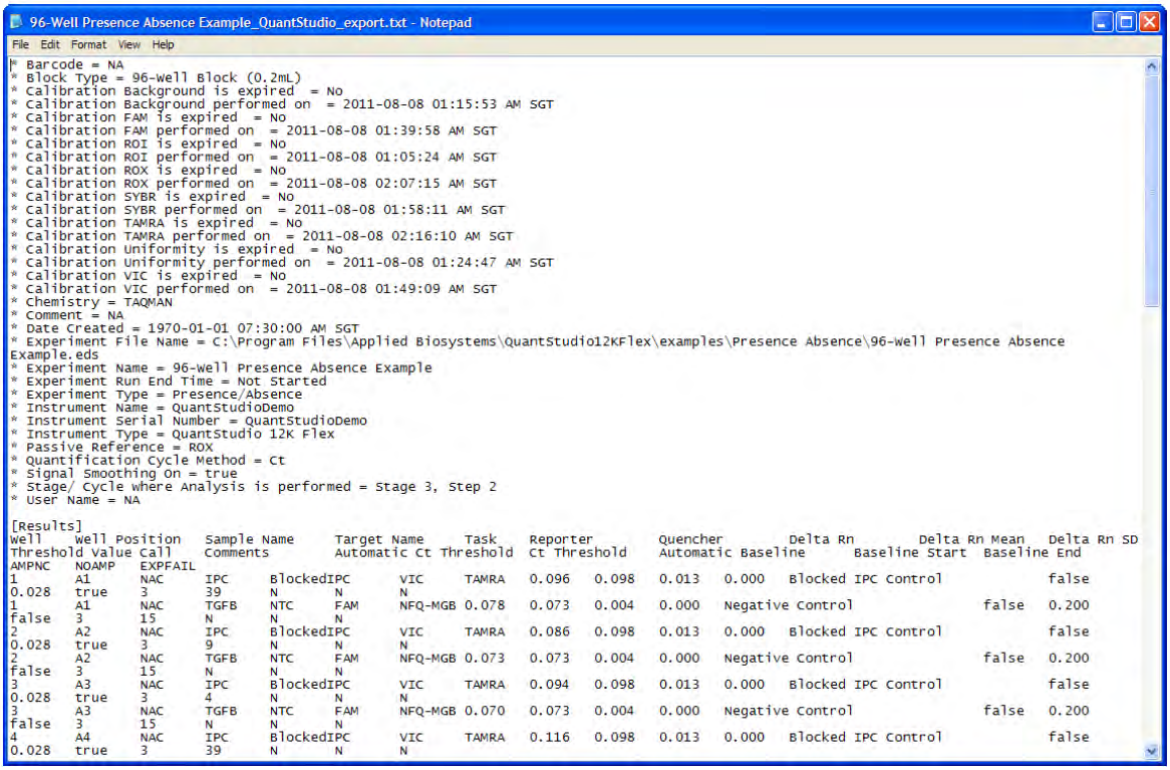
4. Complete the **Export** dialog box as shown below.

Field or Selection	Entry
Select Data to export/Select Content	Results
Export Data To options	**One File** radio button
Export File Name field	***96-Well Presence Absence Example_QuantStudio_export***
File Type dropdown list	***.txt**
Export File Location field	Use the default file location or click **Browse** to select a different location



Well	Well Position	Sample Name	Target Name	Task	Reporter	Quencher	D
1	A1	NAC	IPC	BlockedIPC	VIC	TAMRA	
1	A1	NAC	TGFB	NTC	FAM	NFQ-MGB	
2	A2	NAC	IPC	BlockedIPC	VIC	TAMRA	
2	A2	NAC	TGFB	NTC	FAM	NFQ-MGB	
3	A3	NAC	IPC	BlockedIPC	VIC	TAMRA	
3	A3	NAC	TGFB	NTC	FAM	NFQ-MGB	
4	A4	NAC	IPC	BlockedIPC	VIC	TAMRA	
4	A4	NAC	TGFB	NTC	FAM	NFQ-MGB	
5	A5	NTC	IPC	NTC	VIC	TAMRA	
5	A5	NTC	TGFB	IPC	FAM	NFQ-MGB	
6	A6	NTC	IPC	NTC	VIC	TAMRA	
6	A6	NTC	TGFB	IPC	FAM	NFQ-MGB	
7	A7	NTC	IPC	NTC	VIC	TAMRA	
7	A7	NTC	TGFB	IPC	FAM	NFQ-MGB	
8	A8	NTC	IPC	NTC	VIC	TAMRA	
8	A8	NTC	TGFB	IPC	FAM	NFQ-MGB	
13	B1	(+)	IPC	IPC	VIC	TAMRA	
13	B1	(+)	TGFB	UNKNOWN	FAM	NFQ-MGB	
14	B2	(+)	IPC	IPC	VIC	TAMRA	
14	B2	(+)	TGFB	UNKNOWN	FAM	NFQ-MGB	
15	B3	(+)	IPC	IPC	VIC	TAMRA	
15	B3	(+)	TGFB	UNKNOWN	FAM	NFQ-MGB	
16	B4	(+)	IPC	IPC	VIC	TAMRA	
16	B4	(+)	TGFB	UNKNOWN	FAM	NFQ-MGB	
17	B5	(+)	IPC	IPC	VIC	TAMRA	
17	B5	(+)	TGFB	UNKNOWN	FAM	NFQ-MGB	
18	B6	(+)	IPC	IPC	VIC	TAMRA	
18	B6	(+)	TGFB	UNKNOWN	FAM	NFQ-MGB	
19	B7	(+)	IPC	IPC	VIC	TAMRA	
19	B7	(+)	TGFB	UNKNOWN	FAM	NFQ-MGB	

Figure 112 Export screen



96-Well Presence Absence Example_QuantStudio_export.txt - Notepad

File Edit Format View Help

```
* Barcode = NA
* Block Type = 96-well Block (0.2mL)
* Calibration Background is expired  = No
* Calibration Background performed on  = 2011-08-08 01:15:53 AM SGT
* Calibration FAM is expired  = No
* Calibration FAM performed on  = 2011-08-08 01:39:58 AM SGT
* Calibration ROI is expired  = No
* Calibration ROI performed on  = 2011-08-08 01:05:24 AM SGT
* Calibration ROX is expired  = No
* Calibration ROX performed on  = 2011-08-08 02:07:15 AM SGT
* Calibration SYBR is expired  = No
* Calibration SYBR performed on  = 2011-08-08 01:58:11 AM SGT
* Calibration TAMRA is expired  = No
* Calibration TAMRA performed on  = 2011-08-08 02:16:10 AM SGT
* Calibration Uniformity is expired  = No
* Calibration Uniformity performed on  = 2011-08-08 01:24:47 AM SGT
* Calibration VIC is expired  = No
* Calibration VIC performed on  = 2011-08-08 01:49:09 AM SGT
* Chemistry = TAQMAN
* Comment = NA
* Date Created = 1970-01-01 07:30:00 AM SGT
* Experiment File Name = C:\Program Files\Applied Biosystems\QuantStudio12KFlex\examples\Presence Absence\96-Well Presence Absence
Example.eds
* Experiment Name = 96-Well Presence Absence Example
* Experiment Run End Time = Not Started
* Experiment Type = Presence/Absence
* Instrument Name = QuantStudioDemo
* Instrument Serial Number = QuantStudioDemo
* Instrument Type = QuantStudio 12K Flex
* Passive Reference = ROX
* Quantification Cycle Method = Ct
* Signal Smoothing On = true
* Stage/ Cycle where Analysis is performed = Stage 3, Step 2
* User Name = NA

[Results]
Well    Well Position   Sample Name     Target Name     Task    Reporter        Quencher        Delta Rn        Delta Rn Mean   Delta Rn SD
Threshold Value Call    Comments        Automatic Ct Threshold  Ct Threshold    Automatic Baseline      Baseline Start  Baseline End
AMPNC   NOAMP   EXPFAIL
1       A1      NAC     IPC     BlockedIPC      VIC     TAMRA   0.096   0.098   0.013   0.000   Blocked IPC Control             false
0.028   true    3       39      N       N       N
1       A1      NAC     TGFB    NTC     FAM     NFQ-MGB 0.078   0.073   0.004   0.000   Negative Control                false   0.200
false   3       15      N       N       N
2       A2      NAC     IPC     BlockedIPC      VIC     TAMRA   0.086   0.098   0.013   0.000   Blocked IPC Control             false
0.028   true    3       9       N       N       N
2       A2      NAC     TGFB    NTC     FAM     NFQ-MGB 0.073   0.073   0.004   0.000   Negative Control                false   0.200
false   3       15      N       N       N
3       A3      NAC     IPC     BlockedIPC      VIC     TAMRA   0.094   0.098   0.013   0.000   Blocked IPC Control             false
0.028   true    3       4       N       N       N
3       A3      NAC     TGFB    NTC     FAM     NFQ-MGB 0.070   0.073   0.004   0.000   Negative Control                false   0.200
false   3       15      N       N       N
4       A4      NAC     IPC     BlockedIPC      VIC     TAMRA   0.116   0.098   0.013   0.000   Blocked IPC Control             false
0.028   true    3       39      N       N       N
```

Figure 113 Exported file

Part VII

Running melt curve experiments

33 Overview of melt curve experiments

IMPORTANT! First-time users of the QuantStudio™ 12K Flex Real–Time PCR System, see the part "Getting started" on page 17 and Appendix A, "Documentation and support". The sections provide information and general instructions that are applicable to all the experiments described in this document.

Note: For more information about any of the topics discussed in this guide, access the Help from within the QuantStudio™ 12K Flex Software by pressing **F1,** clicking in the toolbar, or selecting **Help ▸ QuantStudio 12K Flex Software Help**.

Overview

A Melt Curve, also known as dissociation curve, is a plot of data collected during the Melt Curve stage of an experiment. Melt Curve experiments are performed to determine the melting temperature (Tm) of a target nucleic acid sequence or to identify nonspecific PCR amplification.

Melting temperature (Tm) is the temperature at which 50% of the target DNA is double-stranded and 50% is dissociated into single-stranded DNA.

The melting temperature and non-specific PCR amplification can be identified as peaks in the melt curve stage of an experiment.

About the melt curve reactions

With Melt Curve experiments, the reactions consist of completed PCR reactions that contain amplified products and SYBR Green™ dye to detect double-stranded DNA.

The QuantStudio™ 12K Flex Software detects the number of fluorescence peaks, determines the melting temperature (Tm) for each peak, and plots the results in a melt curve.

The fluorescence data collected during the QuantStudio™ 12K Flex Real-Time PCR Instrument run are stored in an experiment data file (EDS).

There are two types of reactions in a Melt Curve experiment:

- **Unknowns**—Wells containing PCR product with an unknown melting temperature or unknown melting temperatures.
- **Negative Controls**—Wells containing buffer or water instead of sample. The negative control wells should contain no double-stranded DNA.

About the example experiment

To illustrate how to perform Melt Curve experiments, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with a QuantStudio™ 12K Flex Real–Time PCR System.

The objective of the example Melt Curve experiment is to investigate the melting temperature of Target 1, and verify that no extraneous peaks appear. The SYBR Green™ reagent is used to detect the melting temperature stage.

Note: The example experiment performs a melt curve analysis on PCR products from a PCR performed on the QuantStudio™ 12K Flex Real–Time PCR System or on another thermal cycler.

34 Design the experiment

This chapter explains how to design the example experiment from the **Setup** menu in the **Experiment Menu** pane.

Note: To automatically export the analyzed data to a specified location, select the **Auto Export** checkbox in the **Export** screen, before running the experiment. For more information on Auto Export, see the part "Getting started" on page 17.

Define the experiment properties

In the **Experiment Menu** pane, click **Setup** ▸ **Experiment Properties**.

Enter or select the following information.

Field or selection	Entry
Experiment Name field	Enter ***384-Well Melt Curve Example***.
Barcode field	Leave the **Barcode** field empty.
User Name field	Enter ***Example User*** or enter a user name.
Comments field	Enter ***Melt Curve example***.
Block	Select **384-Well**.
Experiment Type	Select **Melt Curve**.
Reagents	Select **SYBR Green Reagents**.
Ramp speed	Select **Standard**.
Include PCR checkbox	Uncheck the **Include PCR** checkbox.

Save the experiment.

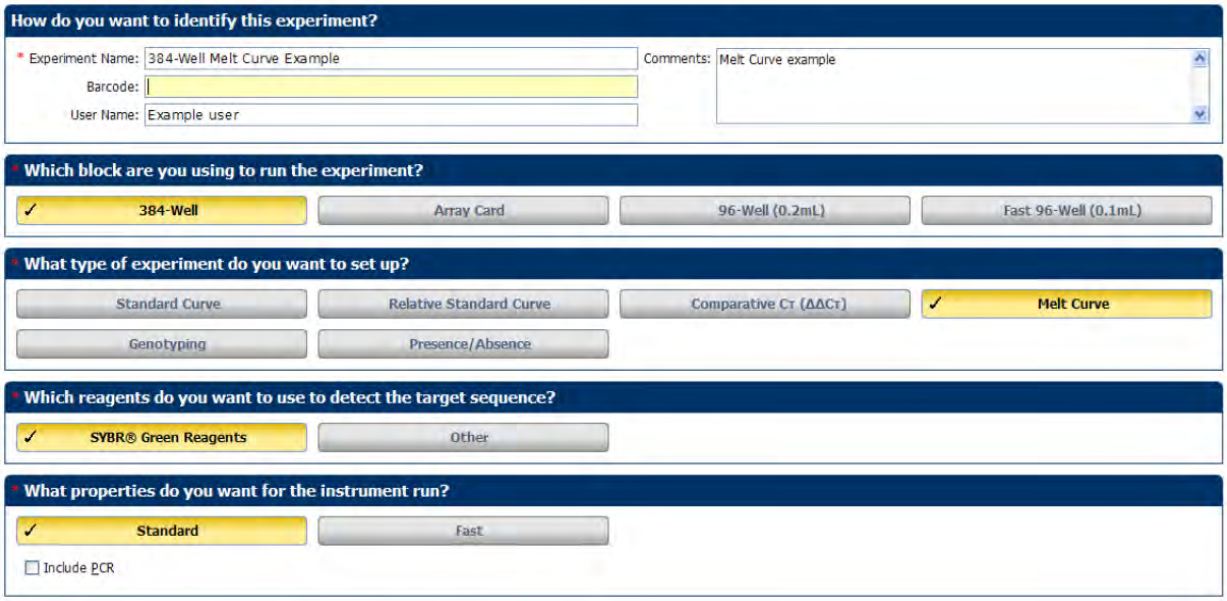


Figure 114 Experiment Properties screen

Define targets and samples

1. Click **Define** to access the **Define** screen.
2. Enter the following target information.

Target name	Reporter	Quencher	Color
Target 1	**SYBR**	**None**	

3. Enter the following sample information.

Sample name	Color
Sample 1	

4. In the **Passive Reference** dropdown list, select **ROX** .

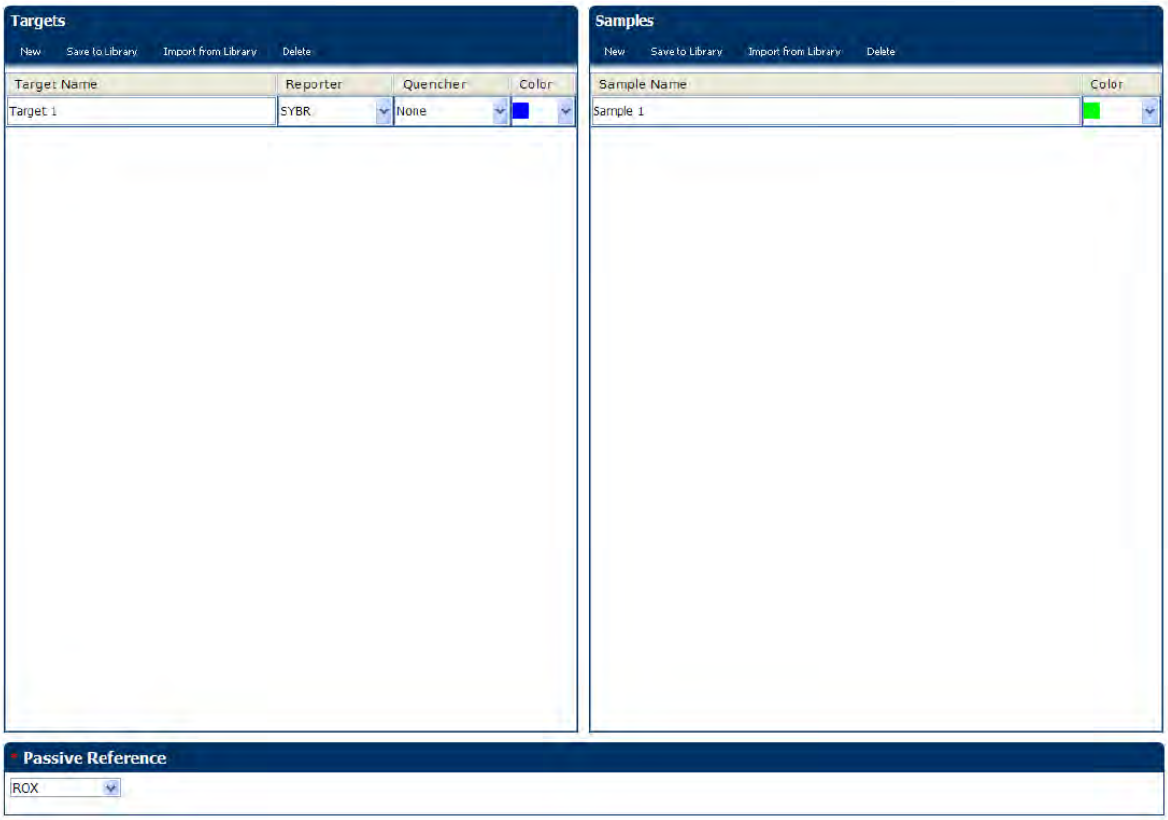


Figure 115 Define screen

Assign targets and samples

Click **Assign** to access the **Assign** screen.

Enter the following targets and samples.

Target name	Sample	Well Number	Task
SYBR	Sample 1	A1-P2 (Columns 1 and 2), A3-G3 (Column 3)	Unknown

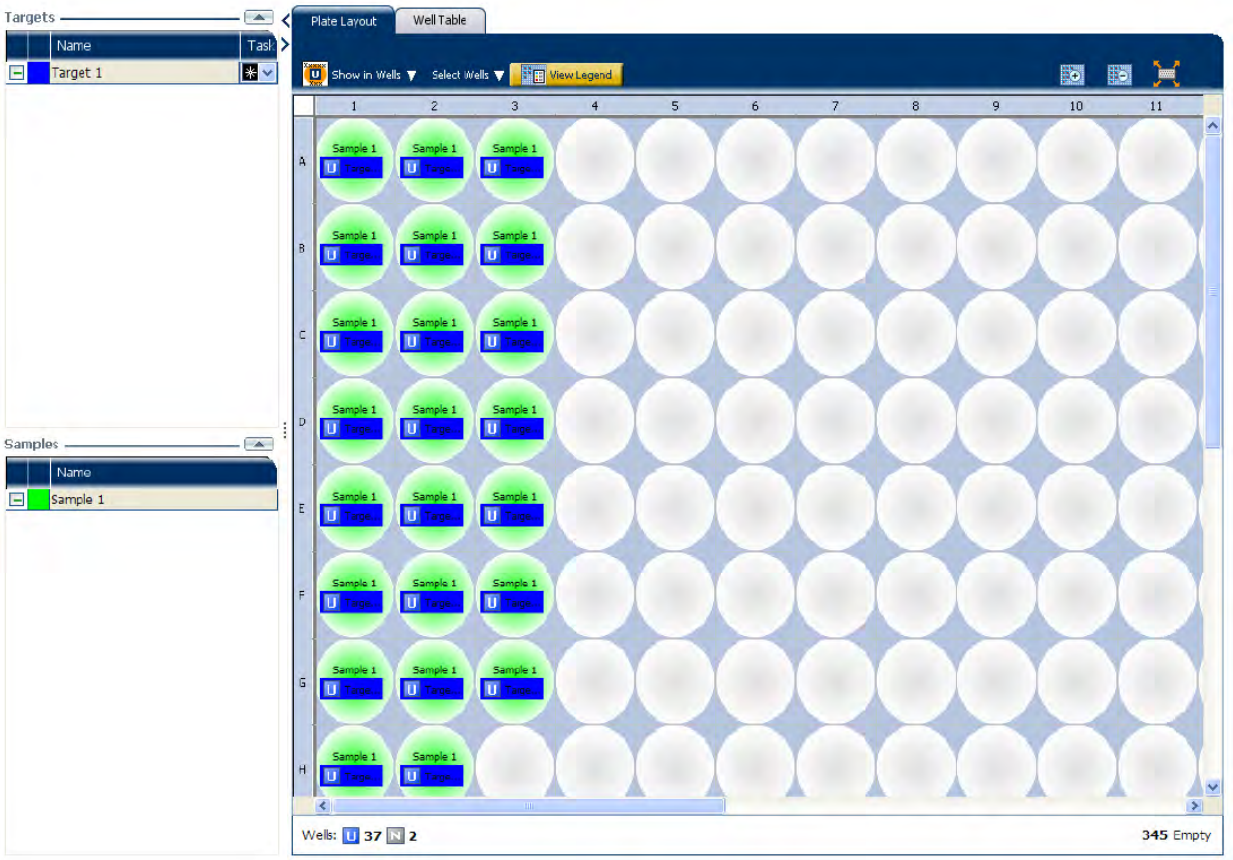


Figure 116 Assign screen

Set up the run method

Set the thermal profile

Click **Run Method** to access the **Run Method** screen. Set the thermal profile under the **Graphical View** tab. Enter the following information:

- Reaction Volume Per Well: ***20 µL***
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Melt Curve Stage	Step 1	1.6°C/s	95°C	15 seconds
	Step 2	1.6°C/s	60°C	1 minute
	Step 3 (Dissociation)	0.05°C/s	95°C	15 seconds

Edit the ramp increment

Edit the ramp increment for a melt curve (dissociation) step.

1. Select a melt curve ramp increment method.
   - **Step and Hold** radio button—Increases or decreases the ramp temperature in 0.1°C increments over the time (duration) for the melt curve ramp.
   - **Continuous** radio button (default)—Increases or decreases the ramp rate in 0.005°C per second increments.
2. If you selected the Step and Hold ramp increment method, edit the melt curve ramp time.
   - To increase or decrease the time in 1-minute or 1-second increments, click the **Step and Hold** field, select the minutes or seconds, then use the up or down arrow keys or click the up or down buttons in the field until you reach the desired time.
   - To enter the desired time, click the **Step and Hold** field, select the minutes or seconds, then enter the desired time.
3. Edit the melt curve ramp increment.
   - To increase or decrease the ramp increment, click the melt curve (dissociation) ramp increment in the thermal profile, then use the up or down arrow keys or click the up or down buttons in the field until you reach the desired value.
   - To enter the desired ramp increment, click the melt curve (dissociation) ramp increment in the thermal profile, select the value in the field, then enter the desired value.

Note: To view the maximum and minimum allowed values, place the cursor over melt curve (dissociation) ramp increment in the thermal profile and wait for the tooltip to pop up.

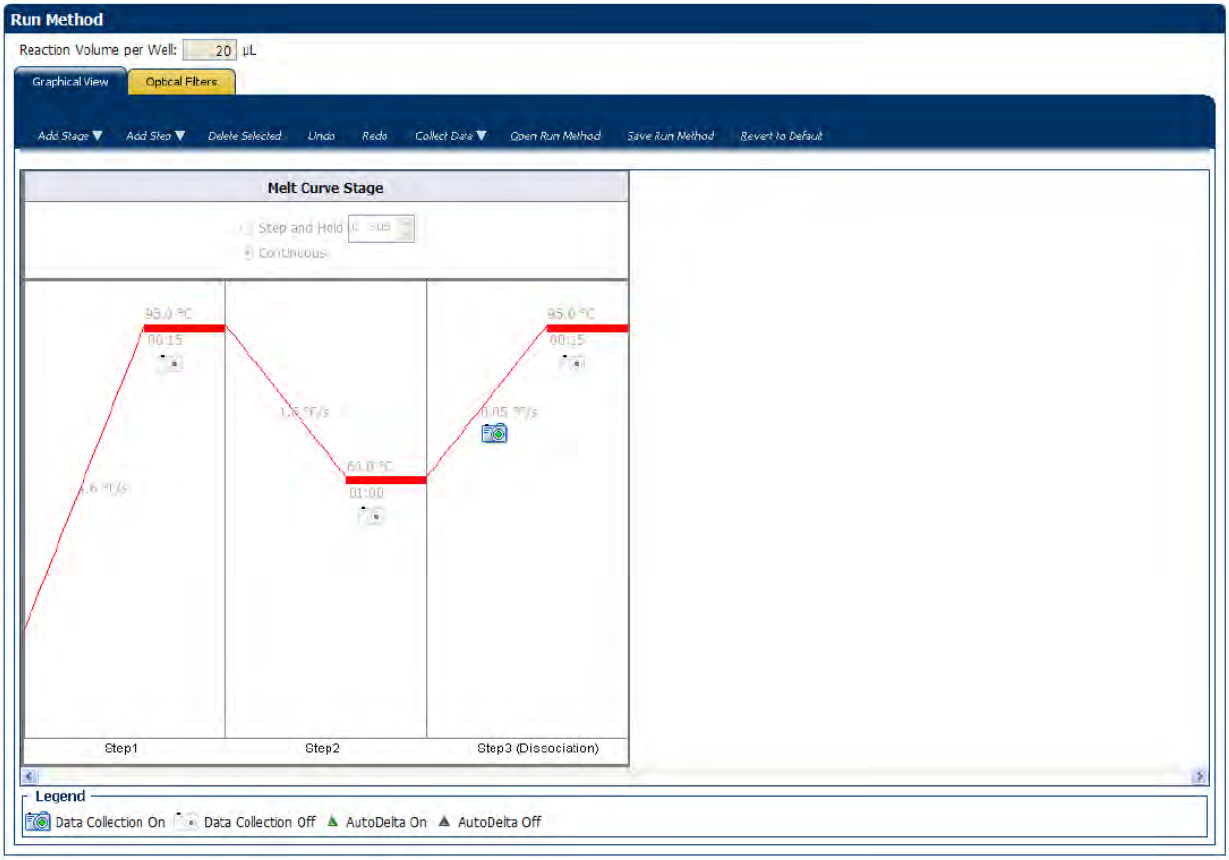


Figure 117 Run Method screen

For more information

Information	Reference
Consumables	Chapter 1, “General information and instructions”
Using alternative setup	Chapter 2, “Experiment shortcuts”

35 Prepare the reactions

This chapter explains how to prepare the reactions for running a PCR prior to running a Melt Curve.

To perform a Melt Curve experiment without running a PCR, use the reaction plate containing the PCR product.

Note: The example experiment performs a melt curve analysis on PCR products from a PCR performed on the QuantStudio™ 12K Flex Real–Time PCR System or on another thermal cycler.

Assemble required materials

- Items listed in the part "Getting started" on page 17
- Sample 1
- Example experiment reaction mix components:
  - *Power* SYBR™ Green Master Mix
  - Target—Assay Mix Forward primer (10 µM)
  - Target—Assay Mix Reverse primer (10 µM)

Prepare the sample dilutions

The stock concentration of each sample is 100 ng/µL. After you dilute the sample according to the following table, the sample will have a concentration of 10 ng/µL. Add 2µL to each reaction.

Sample name	Sample volume	Diluent volume	Total volume of diluted sample
Sample 1 (Amplified PCR Product)	10 µL	90 µL	100 µL

Prepare the reaction mix

The following table lists the universal assay conditions [volume and final concentration for using the *Power* SYBR™ Green Master Mix (2X)].

Reaction Component	Volume for 1 reaction	Volume for 40 reactions
Power SYBR™ Green Master Mix (2X)	10 µL	400 µL
Forward primer (10 µM)	0.1 µL	4 µL
Reverse primer (10 µM)	0.1 µL	4 µL
Water	7.8 µL	312 µL
Total reaction mix volume	**18 µL**	**720 µL**

Procedure

1. Label an appropriately sized tube for the reaction mix: *Power* SYBR™ Green Master Mix Reaction Mix.
2. Add the required volume of each rection mix component to the tube.
3. Mix the cocktail thoroughly by gently pipetting up and down several times, then cap the tube.
4. Centrifuge the tube briefly to remove air bubbles.
5. Place the reaction mix on ice until you prepare the reaction plate.

Calculations

Determine the quantity of primer to be added to the reaction mix by performing the following calculation:

Concentration (initial) C1 x Volume (primer stock) V1 = Concentration (final) C2 x Volume (final reaction) V2

(10µM) x (V1) = (0.05µM) (20µL)

V1 = (0.05 x 20) / 10 = 0.1

Prepare the reaction plate

1. Add reaction mix and sample to a tube.
   a. Add the volumes of reaction mix and sample listed below to an appropriately-sized tub.

Tube	Unknown reaction	Reaction mix	Reaction mix volume	Sample	Sample volume
1	Target 1	*Power* SYBR™ Green Master Mix reaction mix[1]	720 µL	Sample 1	80 µL

[1] Prepared in "Prepare the reaction mix" on page 371.

   b. Mix the reactions by gently pipetting up and down, then cap the tubes.

   c. Centrifuge the tubes briefly to remove air bubbles.

2. Pipette 20 µL of the unknown (sample) reaction to each well in the reaction plate.
3. Seal the reaction plate with optical adhesive film.
4. Centrifuge the reaction plate briefly to remove air bubbles.
5. Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.
6. Until you are ready to perform the PCR run, place the reaction plate at 4°C, in the dark.
7. Run the PCR.
8. After the PCR is completed, use the same reaction plate containing the PCR product to run the Melt Curve as described in Chapter 36, "Run the experiment".

For more information

Information	Reference
Assigning the reaction plate components	Chapter 1, "General information and instructions"
Sealing the reaction plate	Chapter 1, "General information and instructions"

36 Run the experiment

This chapter explains how to run the example experiment on the QuantStudio™ 12K Flex Real-Time PCR Instrument.

IMPORTANT! Run the experiment at the same ambient temperature at which you calibrated the instrument. Extreme variations in ambient temperature can affect the heating and cooling of the instrument and influence experimental results.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the instrument is in operation.

Start the run

1. Open the Melt Curve example file that you created.

   See Chapter 34, “Design the experiment”.

   IMPORTANT! The example experiment includes the melt curve analysis of a PCR product from PCR on the QuantStudio™ 12K Flex Real–Time PCR System or another thermal cycler. To run a Melt Curve on the example file you created in Chapter 34, “Design the experiment”, ensure that PCR has already been performed on the reaction plate you load into the instrument. Absence of the PCR product will lead to no results in the Dissociation Step of the Melt Curve Stage.

2. Load the reaction plate, containing the PCR product, into the instrument.

3. Start the run.

Monitor the run

You can monitor an experiment run in three ways:

- From the **Run** screen of the QuantStudio™ 12K Flex Software, while the experiment is in progress
- From the **Instrument Console** screen of the QuantStudio™ 12K Flex Software, to monitor an experiment started from another computer or from the instrument touchscreen (see “Monitor a run from the software Instrument Console screen” on page 374)
- From the instrument touchscreen (see “Monitor a run from the instrument touchscreen” on page 378)

Monitor a run from the software Instrument Console screen

1. In the **Instrument Console** screen, select the icon of the instrument that you are using to run the experiment.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. In the **Instrument Manager** screen, click **Monitor Running Experiment** to access the **Run** screen.

View the melt curve

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the QuantStudio™ 12K Flex Software for potential problems.

Click **Melt Curve** from the **Run Experiment** menu, select the **Plate Layout** tab, then select the wells to view.

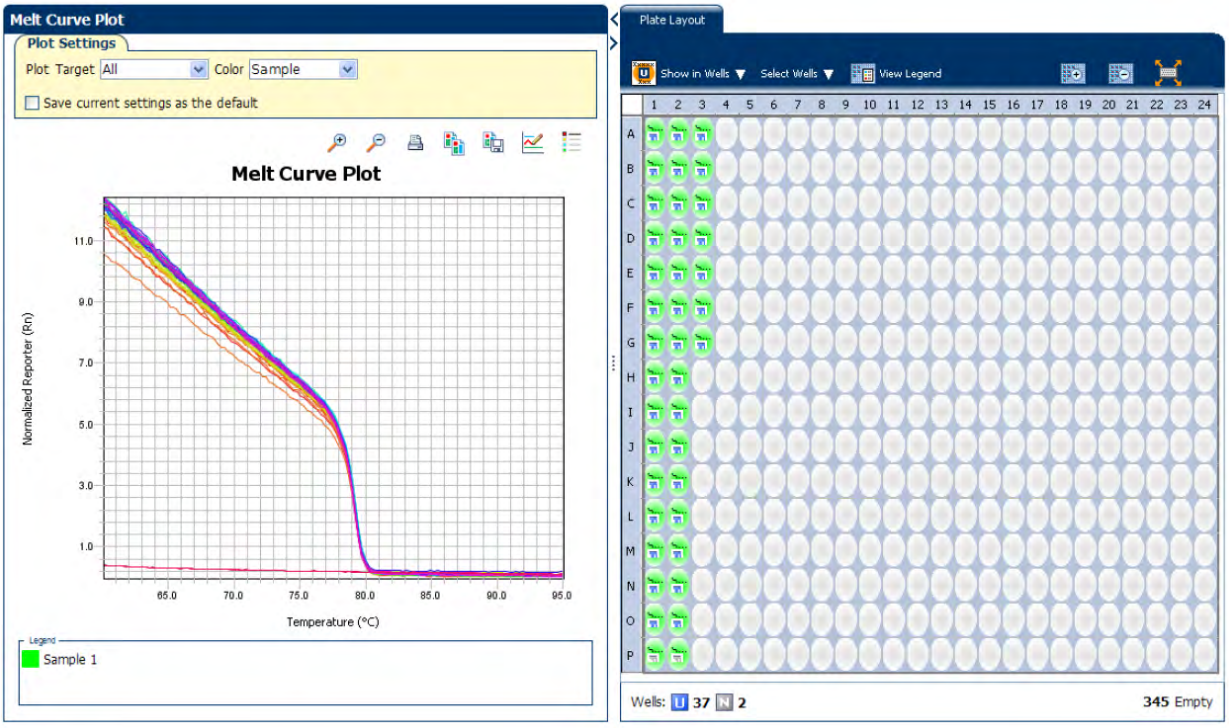


Figure 118 The Melt Curve Plot screen as it appears at the end of the example experiment

View the temperature plot

Click **Temperature Plot** from the **Run Experiment** menu.

Figure 119 The Temperature Plot screen as it appears during the example experiment

Note: The sample temperature displayed in the **Current Temperatures** group is an estimated value.

View the run method

Click **Run Method** from the **Run Experiment** menu.

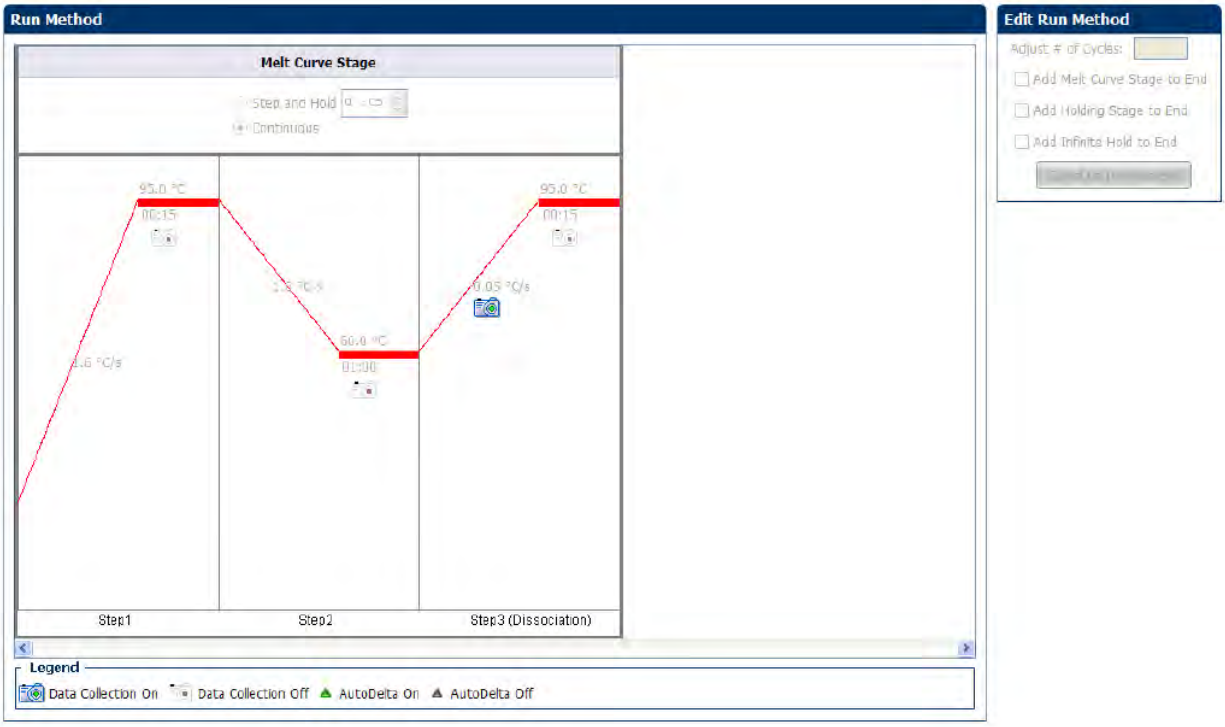


Figure 120 The Run Method screen as it appears in the example experiment

View run data

Click **View Run Data** from the **Run Experiment** menu.

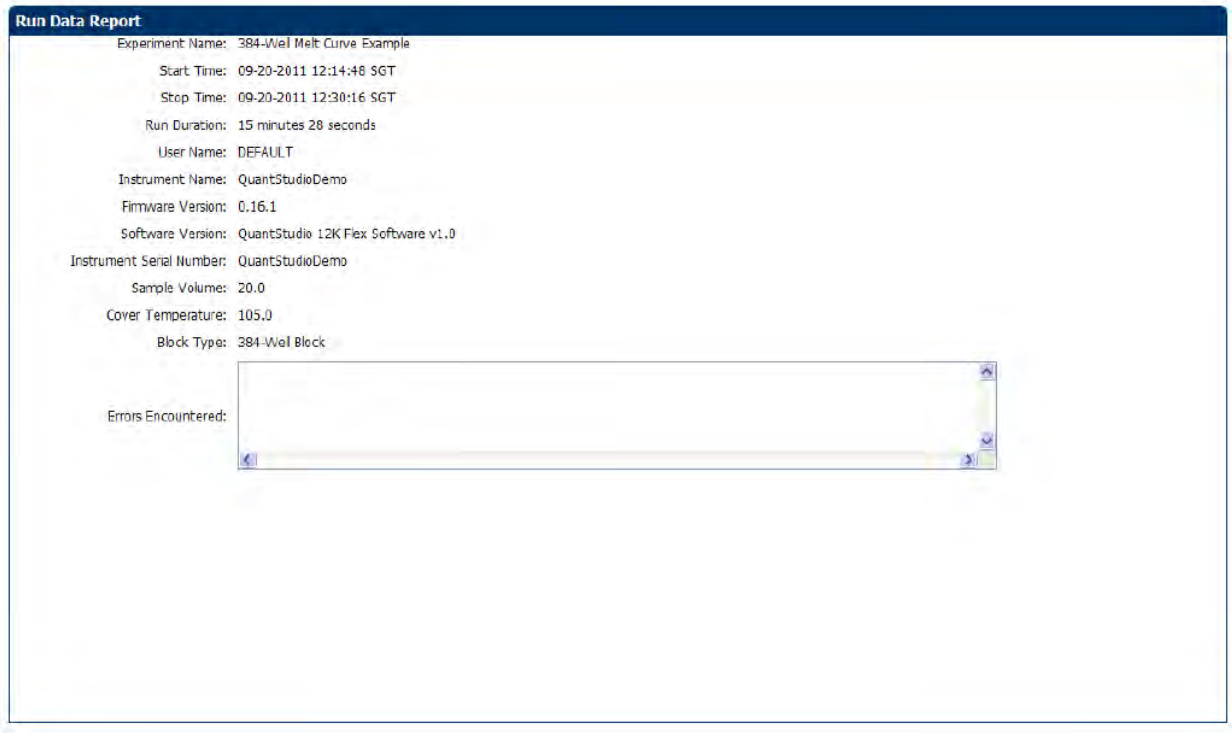


Figure 121 The View Run Data screen as it appears in the example experiment

Monitor a run from the instrument touchscreen

You can view the progress of the run from the instrument touchscreen.

The following figures are for visual representation only. Actual results vary with the experiment.

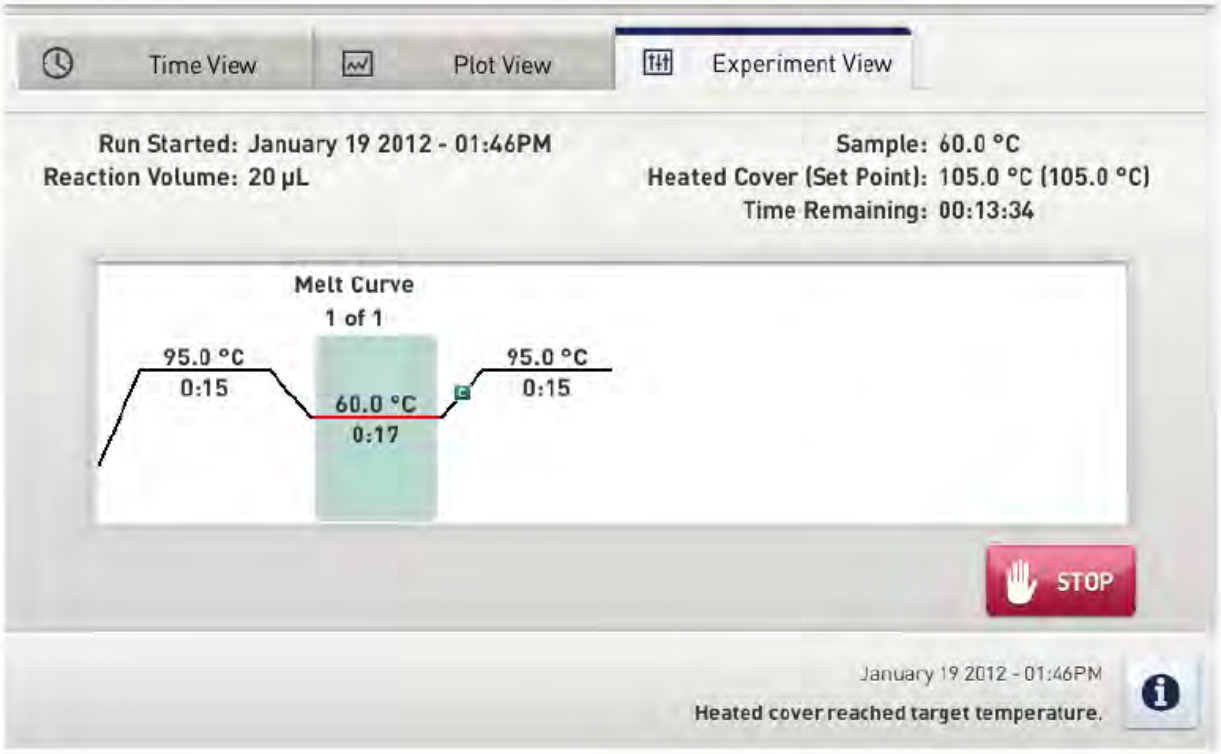


Figure 122 Experiment View tab

Figure 123 Time View tab

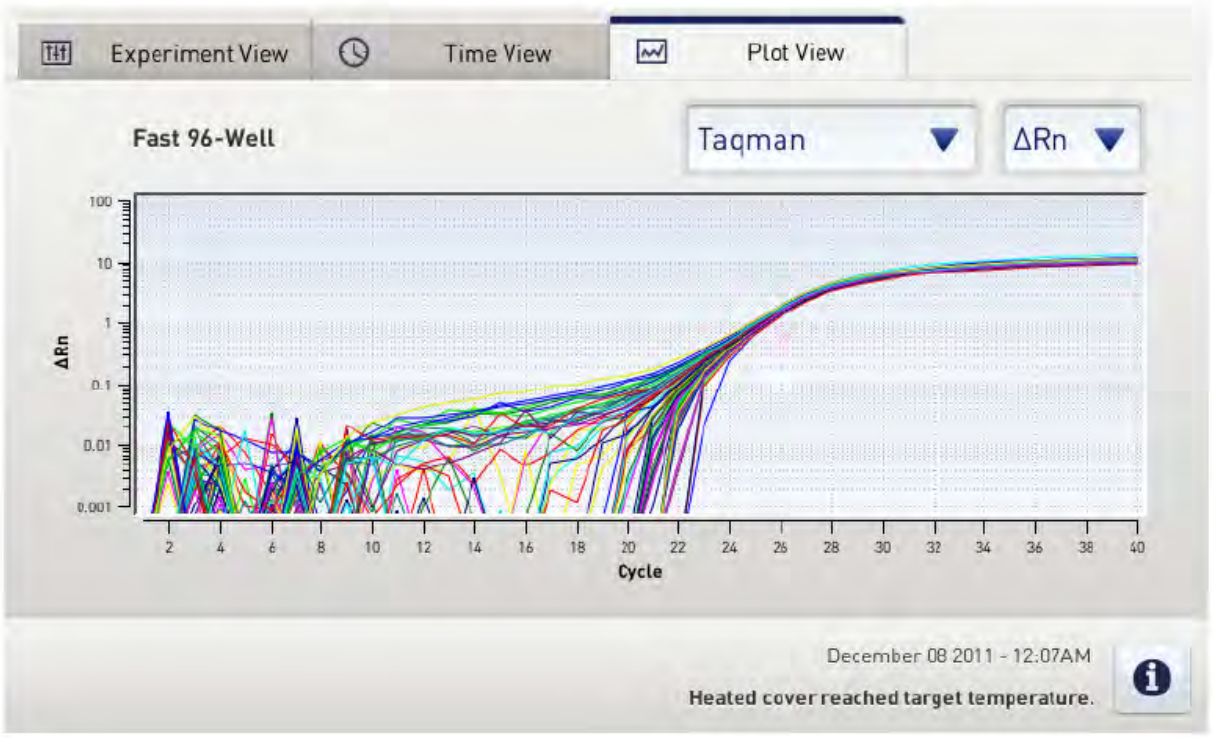


Figure 124 Plot View tab

Note: You will see the Plot View only if your experiment includes the PCR process.

37 Review results and adjust experiment parameters

The following topics are covered in this chapter:

- Review the analyzed data using several of the analysis screens and publish the data (see “Review results” on page 380)
- Modify experiment parameters to troubleshoot problems with experiment results before rerunning an experiment (see “Adjust parameters for re-analysis of your own experiments” on page 392)

Review results

Analyze the example experiment

1. Open the example experiment file.
   See Chapter 36, “Run the experiment”.
2. Click **Analyze**.

Note: You can also access the experiment to analyze from the **Home** screen.

The software analyzes the data using the default analysis settings.

View the melt curve plot

View the Melt Curve Plot as the Derivative Reporter (-Rn) versus the Temperature Plot generated by the target.

The **Melt Curve Plot** screen displays the melt curve of the targets in the selected wells. Use the Melt Curve plots to confirm the results of the experiment:

- **Normalized Reporter (Rn) vs. Temperature**—This plot displays the fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference. You can use this plot to see the change in Rn with change in the temperature. You cannot use this plot to determine the Tm of the target.
- **Derivative Reporter (-Rn) vs. Temperature**—This plot displays the derivative reporter signal in the y-axis. The peaks in the plot indicate significant decrease in SYBR Green™ signal, and therefore the Tm of the target.

Purpose

The purpose of viewing the Melt Curve Plot for the example experiment is to review the melting temperature of the target.

View and assess the melt curve

1. In the **Experiment Menu** pane, click **Analysis** ▸ **Melt Curve Plot**.

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

2. Select the following items in the **Plot Settings** menu.

Item	Selection
Plot dropdown list	**Derivative Reporter**
Target dropdown list	**All**
Color dropdown list	**Target**
Click **Show a legend for the plot** (default). (This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.)	Check (default)

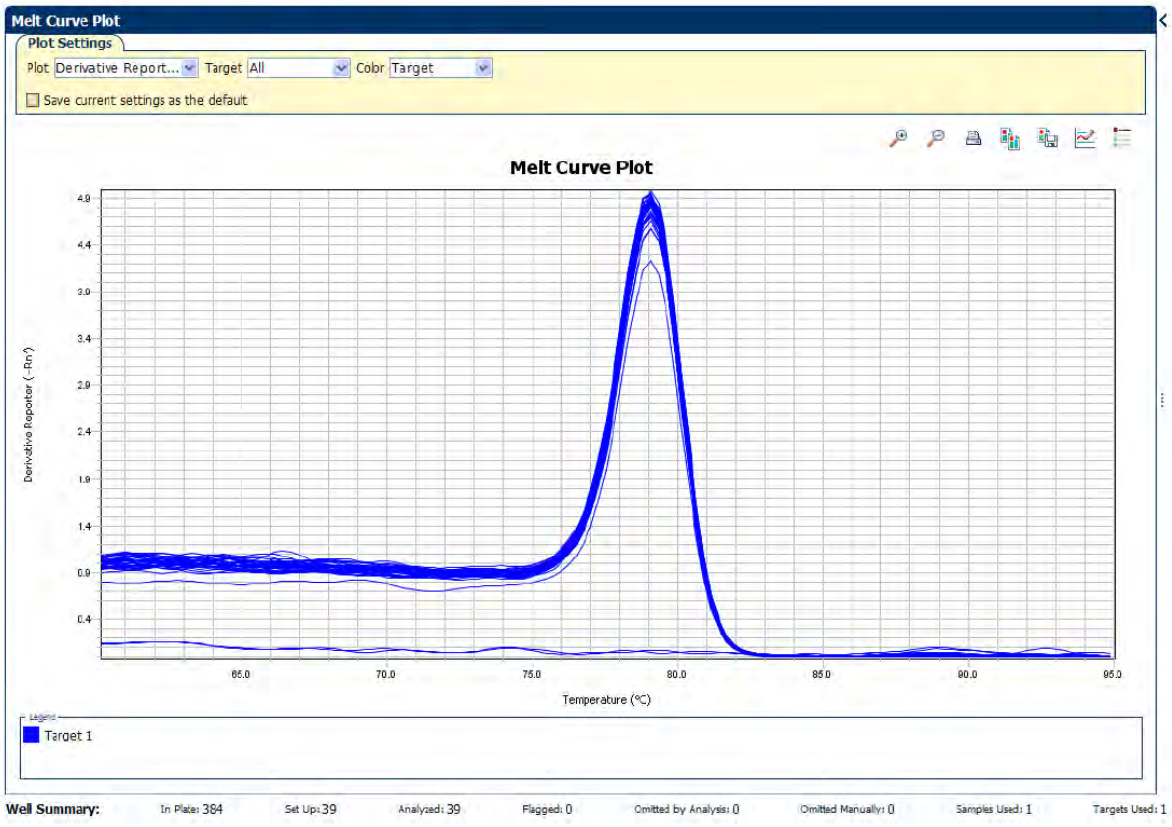


Figure 125 Melt curve plot

Tips for viewing melt curves in your own experiments

When you analyze your own Melt Curve experiment, look for wells with multiple peaks, indicating non-specific amplifications or primer dimer formation.

If your experiment does not amplify properly or indicates non-specific amplification, troubleshoot by manually adjusting the Melt Curve settings (see “Adjust analysis settings” on page 392).

Identify well problems using the well table

Review the details of the experiment results in the well table and identify any flagged wells. The well table displays the assay-specific setup and analysis properties for the experiment in a tabular format.

Example experiment values and flags

For the example experiment, confirm that no wells of the reaction plate triggered QC flags (⚠).

View the well table

1. Select the **Well Table** tab.
2. Click the **Flag** column header to sort the data so that the wells that triggered flags appear at the top of the table.
3. Confirm the integrity of the controls.
   a. In the **Group By** menu, select **Task** to organize the table rows by their function on the reaction plate.

b. Confirm that each of the controls do not display flags (▲).

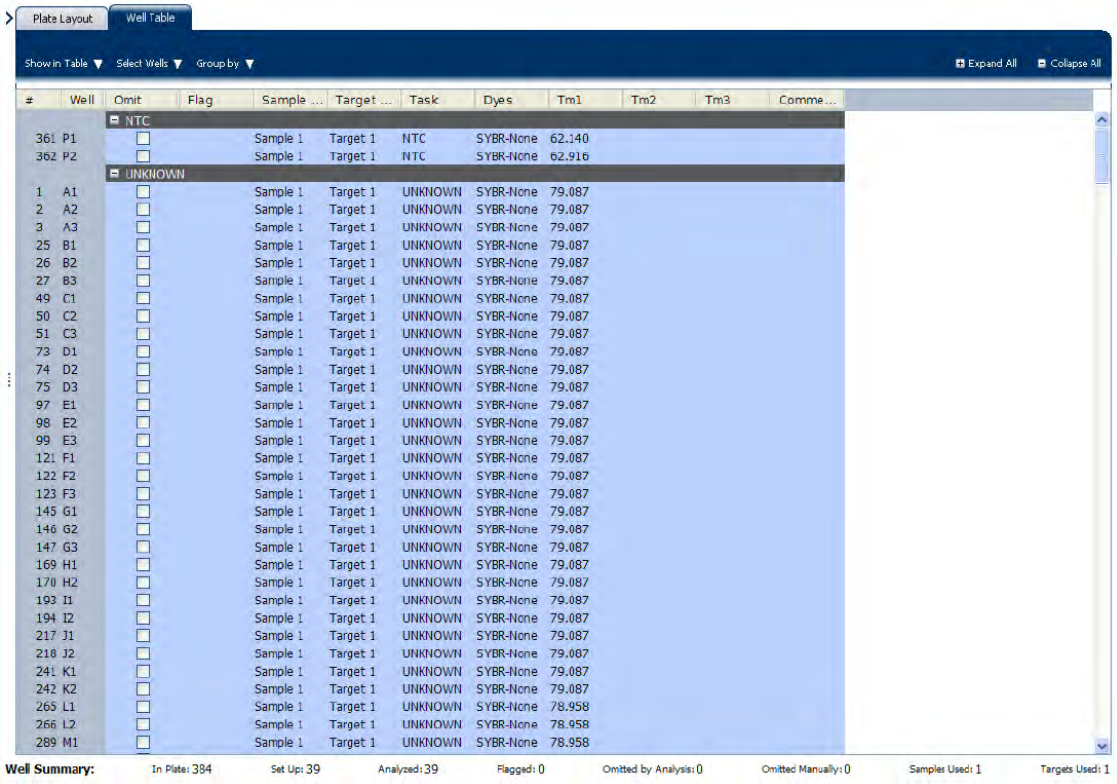


Figure 126 Well table of the example Melt Curve experiment

The table below gives the description of each column in the well table.

Column	Description
Well column	The position of the well on the reaction plate.
Omit column	A check mark indicates that the well has been removed from the analysis.
Flag column	A ▲ symbol indicates that the well triggered the number of flags listed inside the symbol.
Sample Name column	The name of the sample.
Target Name column	The name of the target evaluated by the well.
Task column	The task assigned to the well (Unknown, Negative Control, or Positive Control).
Dyes column	The name of the reporter and quencher dyes of the associated sample for the target evaluated by the well.
Tm1 column	The melting temperature of the target.
Tm2 column	The second melting temperature (for targets with multiple melting temperatures).
Tm3 column	The third melting temperature (for targets with multiple melting temperatures).
Comments column	Comments

Tips for viewing well tables your own experiments

When you analyze your own experiment:

- Review the data for the Unknown samples. For each row that displays the ⚠ symbol in the **Flag** column, note the data and the flag or the flags triggered by the associated well.
- Select areas of the table or wells of a specified type by performing the following tasks:
  - Left-clicking the mouse and dragging across the area you want to select an area of the table.
  - In the **Select Wells** dropdown list in the **Well Table** tab, select **Sample**, **Target**, or **Task**. Select the sample name, target name, or task name to select wells of a specific type using the well-selection tool.
- Group the rows of the plate layout by selecting an option in the **Group By** dropdown list. You can then collapse or expand the lists either by clicking the **+** button or the **–** button next to individual lists, or by clicking **+ Expand All** or **– Collapse All**.
- Omit a well from the analysis by selecting the **Omit** checkbox for that well. To include the well in the analysis, deselect the **Omit** checkbox.

Note: You must reanalyze the experiment each time you omit or include a well.

Confirm accurate dye signal using the multicomponent plot

The **Multicomponent Plot** screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Purpose

In the Melt Curve example experiment, you review the **Multicomponent Plot** screen for:

- ROX™ dye (passive reference)
- SYBR™ dye (reporter)
- Spikes, dips, and/or sudden changes

View the multicomponent plot

1. In the **Experiment Menu** pane, select **Analysis ▸ Multicomponent Plot.**

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

2. Display the unknown wells in the plate layout to display the corresponding data in the **Multicomponent Plot** screen.
   a. Click the **Plate Layout** tab.
   b. Select one well in the plate layout. The well is shown in the **Multicomponent Plot** screen.

      Note: If you select multiple wells, the **Multicomponent Plot** screen displays the data for all selected wells simultaneously.

3. In the **Plot Color** dropdown list, select **Dye**.

4. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

5. Check the ROX™ dye signal.

   In the example experiment, the ROX™ dye signal remains constant throughout the PCR process. A constant ROX™ dye signal indicates typical data.

6. Check the SYBR™ dye signal.

   In the example experiment, because the PCR run has already been completed, the SYBR™ dye signal shows gradual decrease throughout the run and a sudden dip in the fluorescence at one point. The sudden drop in the SYBR™ dye signal indicates the melting temperature of the target.

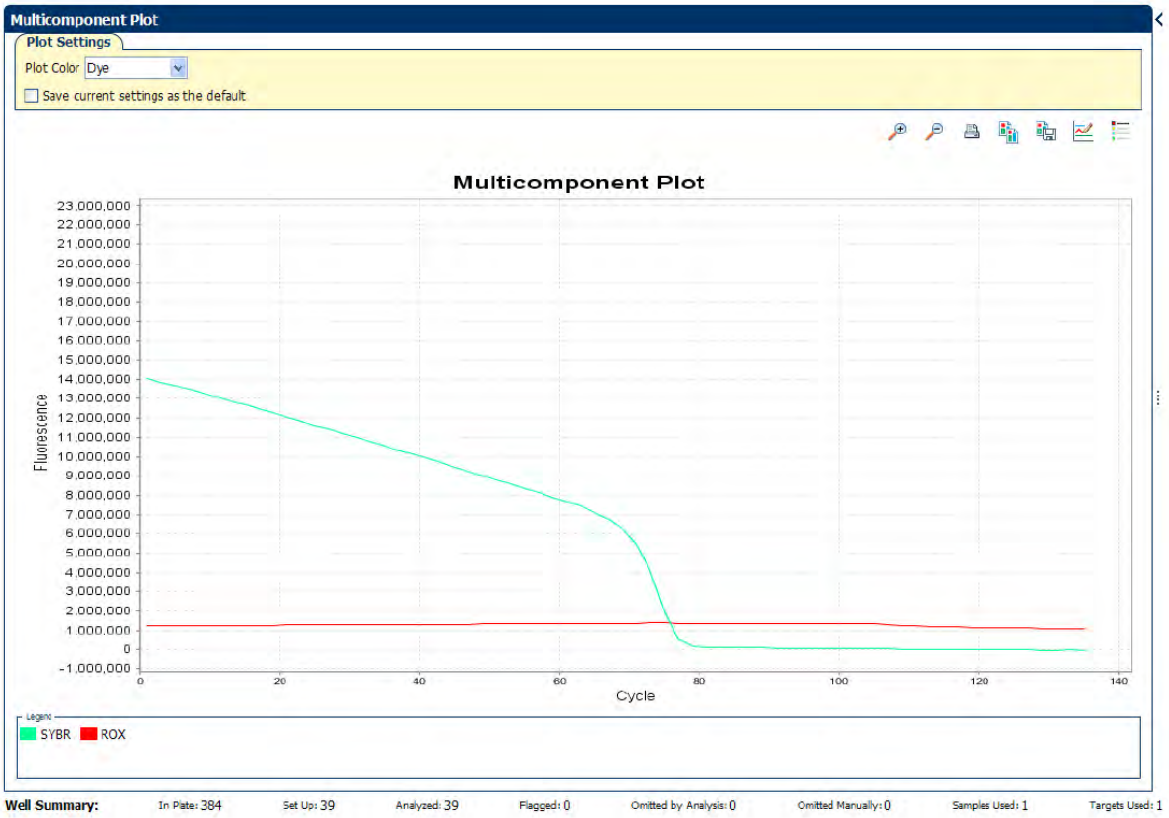


Figure 127 Multicomponent Plot screen for the example experiment

Tips for confirming dye accuracy in your own experiment

When you analyze your own Melt Curve experiment, look for the following items:

- **Passive reference**—The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye**—The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds. If the Melt Curve is being performed post-PCR, then there should be a gradual decrease in fluorescence and a sudden dip indicating the melting temperature of the target.
- **Irregularities in the signal**—There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.

Determine signal accuracy using the raw data plot

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

About the example experiment

In the Melt Curve example experiment, you review the **Raw Data Plot** screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the raw data plot

1. In the **Experiment Menu** pane, select **Analysis** ▸ **Raw Data Plot**.

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

2. Display all 384 wells in the **Raw Data Plot** screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.

3. Click **Show a legend for the plot** (default).

   Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

   The legend displays the color code for each row of the reaction plate (see the legend in the Raw Data Plot shown below).

4. Click and drag the **Show Cycle** pointer from cycle 1 to cycle 135.

   In the example experiment, the signal from filter 1, which corresponds to the SYBR™ dye filter, is stable throughout.

   Note: The readings shown below are from the example experiment. Actual results will vary with individual experiment setup.

   Note: The cycle number in the Melt Curve represents the number of data collection points for that experiment.

Figure 128 Raw Data plot for the example experiment

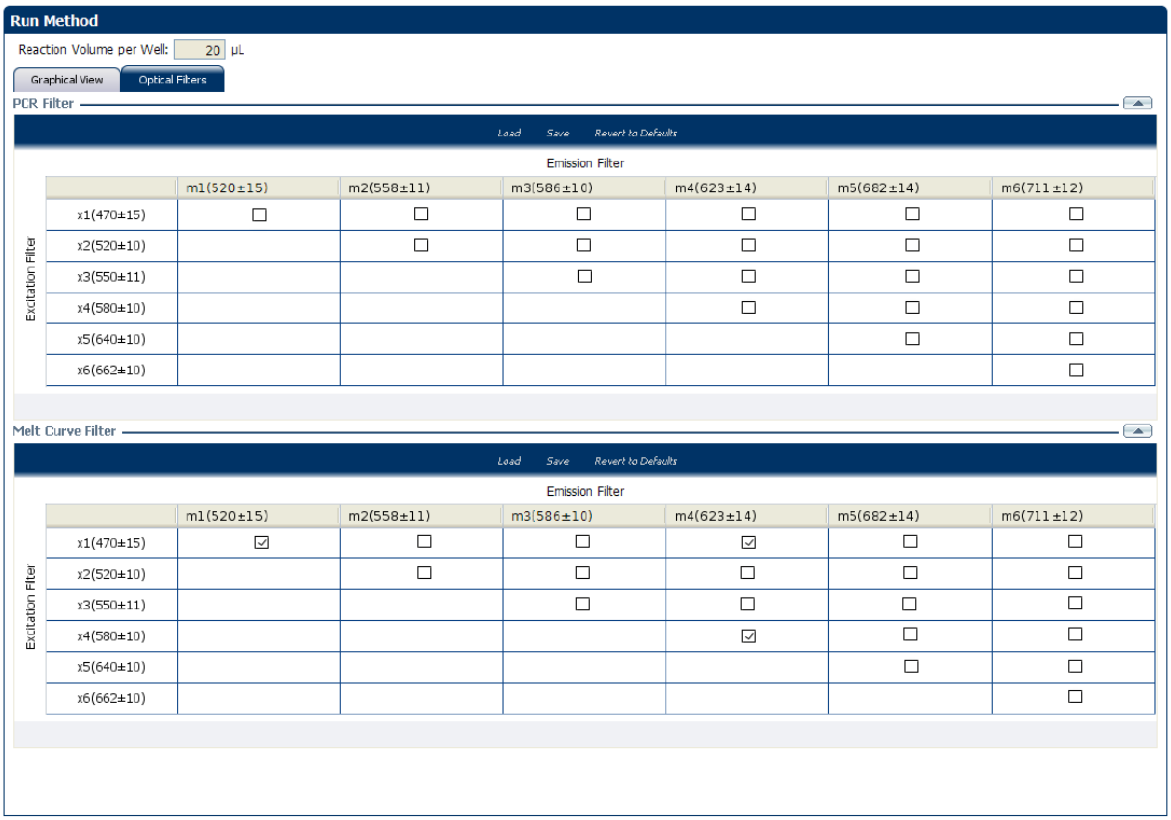


Figure 129 Filters used for the example experiment

Tips for determining signal accuracy in your own experiments

When you analyze your own Melt Curve experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

Review the flags in the QC summary

The **QC Summary** screen displays a list of the QuantStudio™ 12K Flex Software flags, including the flag frequency and location for the open experiment.

View the QC summary

1. In the **Experiment Menu** pane, select **Analysis ▸ QC Summary**.

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

2. Review the Flags Summary.

   Note: A 0 displayed in the **Frequency** column indicates that the flag does not appear in the experiment. If the frequency is greater than 0, the flag appears somewhere in the experiment. The well position is listed in the **Wells** column.
   In the example experiment, there are no flagged wells.

3. In the **Flag Details** table, click each flag with a frequency greater than 0 to display detailed information about the flag.

   In the example experiment, the **Frequency** column displays 0 for the three flags NOSIGNAL, OFFSCALE, and MTP.

4. *(Optional)* For those flags with frequency greater than 0, click the troubleshooting link to view information on correcting the flag.

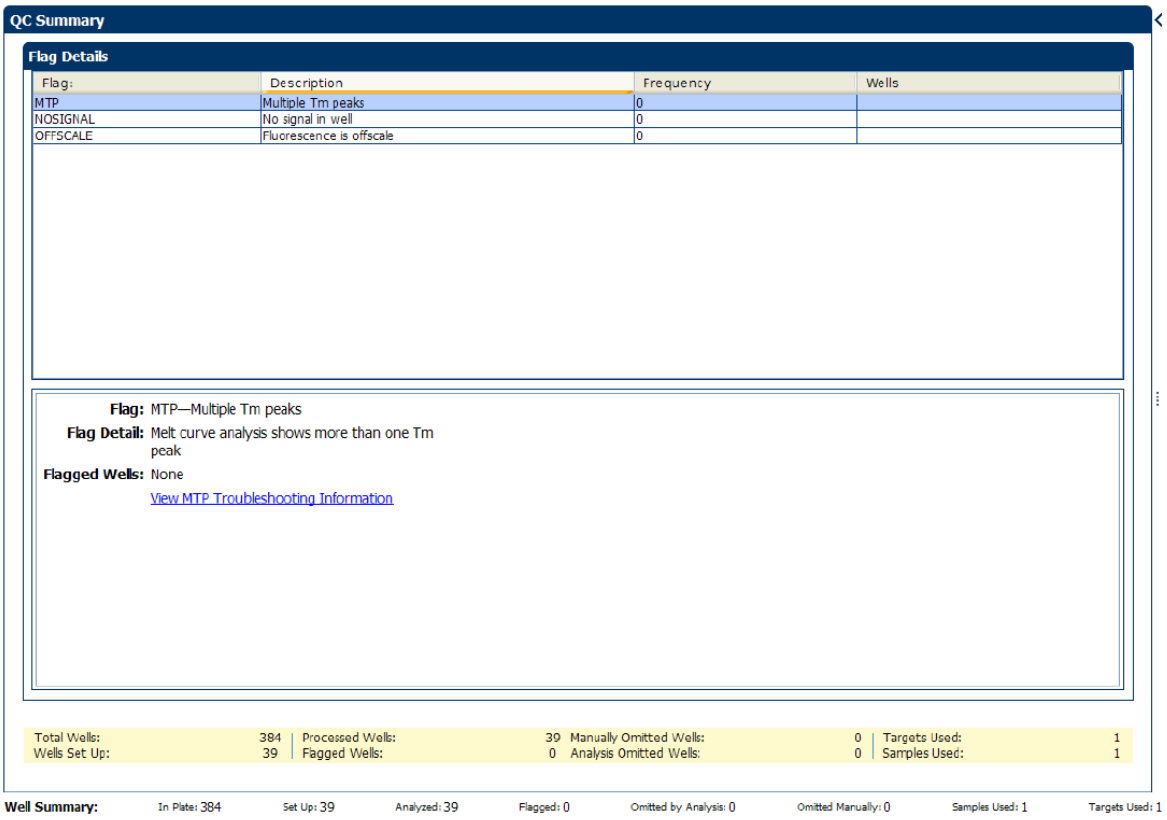


Figure 130 QC Summary screen for the example experiment

Possible flags

For Melt Curve experiments that do not include amplification, the flags listed below may be triggered by the experiment data.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flag	
NOSIGNAL	No signal in well
Secondary analysis flag	
MTP	Multiple Tm peaks

For Melt Curve experiments that include amplification, the flags listed below may be triggered by the experiment data.

Flag	Description
Pre-processing flag	
OFFSCALE	Fluorescence is offscale
Primary analysis flags	
BADROX	Bad passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C_T algorithm failed
AMPSCORE	Amplification in the linear region is below a certain threshold, corresponding to the score set in the analysis settings
Secondary analysis flags	
MTP	Multiple Tm peaks
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group

Note: When you use the Relative Threshold algorithm, the EXPFAIL, BLFAIL, THOLDFAIL, and CTFAIL flags are not reported by the algorithm, but they appear in the QC Summary (by default, a 0 is displayed in the **Frequency** column for each flag).

For more information

Information	Reference
Publishing data	Chapter 1, “General information and instructions”

Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

The **Analysis Settings** dialog box displays the analysis settings for the Melt Curve and flags.

If the default analysis settings in the QuantStudio™ 12K Flex Software are not suitable for your own experiment, you can change the settings in the **Analysis Settings** dialog box, then reanalyze your experiment.

View the analysis settings

1. In the **Experiment Menu** pane, select **Analysis**.
2. Click **Analysis** ▸ **Analysis Settings** to open the **Analysis Settings** dialog box.
   In the example experiment, the default analysis settings are used for each tab:
   - Melt Curve Settings
   - C_T Settings
   - Flag Settings
   - Advanced Settings

Note: The C_T **Settings** tab and the **Advanced Settings** tab appear in the **Analysis Settings** dialog box only if the Melt Curve experiment you are performing includes the PCR process.

Note: Select the **Include PCR** checkbox in the **Experiment Properties** screen to include amplification in your Melt Curve experiment.

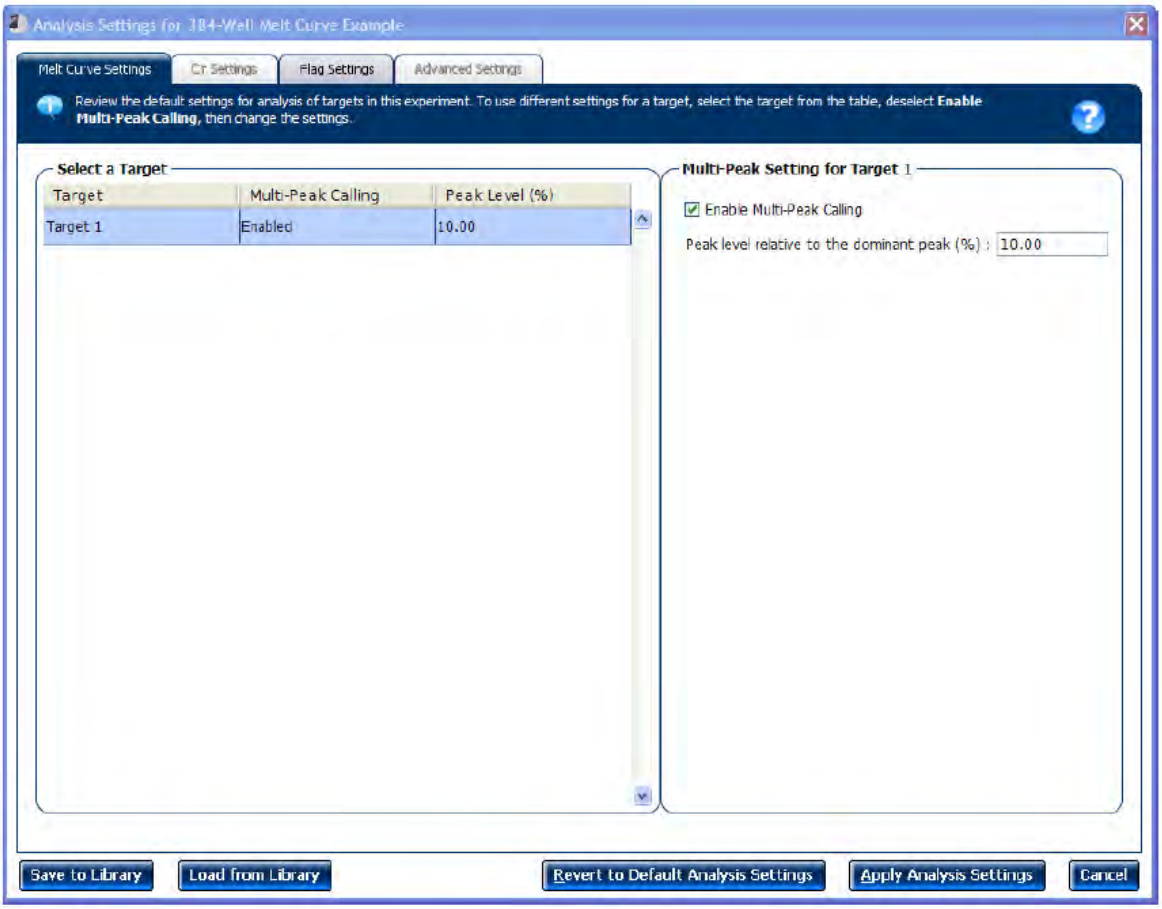


Figure 131 Analysis Settings dialog box for a Melt Curve experiment

3. View and, if necessary, change the analysis settings (see “Adjust analysis settings” on page 394).

Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see “About the analysis settings library” on page 62.

4. Click **Apply Analysis Settings** to apply the current analysis settings.

Note: You can go back to the default analysis settings, by clicking **Revert to Default Analysis Settings**.

Adjust analysis settings

Melt curve settings

- Enable or disable multi-peak calling.
  - Select the **Enable Multi-Peak Calling** checkbox if you expect to amplify more than one PCR product and you want to determine the Tm for more than one peak.
  - Deselect the **Enable Multi-Peak Calling** checkbox if you expect to amplify one PCR product and you do not want to determine the Tm for more than one peak.
- Enter a value (in percentage) for the peak level relative to the dominant peak.

  Specify a fractional level value as the peak detection threshold. The detected peaks are measured relative to the height of the tallest peak, which has a perfect fractional level 100%. The default value is initially set at 10%.

  For example, if you set a fractional level detection threshold value at 40, then only peaks above 40% of the tallest peak are reported and the peaks at lower height are regarded as noise.

C_T settings

- **Data Step Selection**

  Use this feature to select one stage/step combination for C_T analysis when there is more than one data collection point in the run method.
- **Algorithm Settings**

  You can select the algorithm that determines the C_T values. There are two algorithms: Baseline Threshold (the default) and Relative Threshold.

  The Baseline Threshold algorithm is an expression estimation algorithm that subtracts a baseline component and sets a fluorescent threshold in the exponential region for quantification.

  The Relative Threshold algorithm is a well-based analysis based on the PCR reaction efficiency and fitted to the Amplification curve. This setting is ideal for a single sample across genes with no dependence on targets, thereby reducing variability. It is not necessary to set either a baseline or a threshold when you use the Relative Threshold algorithm, so any settings for baseline or threshold will not affect the analysis.
- **Default C_T Settings**

  Use the default C_T settings feature to calculate C_T for the targets that do not have custom settings. To edit the default settings, click **Edit Default Settings**.
- **C_T Settings for Target**

  When you manually set the threshold and baseline, we recommend the following settings:

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

Note: Selecting Automatic Threshold implies selection of automatic setting of the baseline. However, if Automatic Threshold is deselected, then you can choose between setting the baseline either automatically or manually.

Flag settings

Use the **Flag Settings** tab to perform the following tasks:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the QuantStudio™ 12K Flex Software.

1. In the **Use** column, select the check boxes for flags to apply during analysis.
2. *(Optional)* If an attribute, condition, and value are listed for a flag, specify the setting for applying the flag.

Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

3. In the **Reject Well** column, select the check boxes if you want the software to reject wells with the flag.

Note: After you have rejected the flagged wells, analysis results depend on factors such as the experiment type and flag type. For example, rejecting wells flagged by HIGHSD in experiments using the Standard Deviation calculations may change the result of C_T SD. For some flags, analysis results calculated before the well is rejected are maintained.

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4. Click **Apply Analysis Settings** in the **Analysis Settings** dialog box.

   If the run status is complete, the data are reanalyzed.

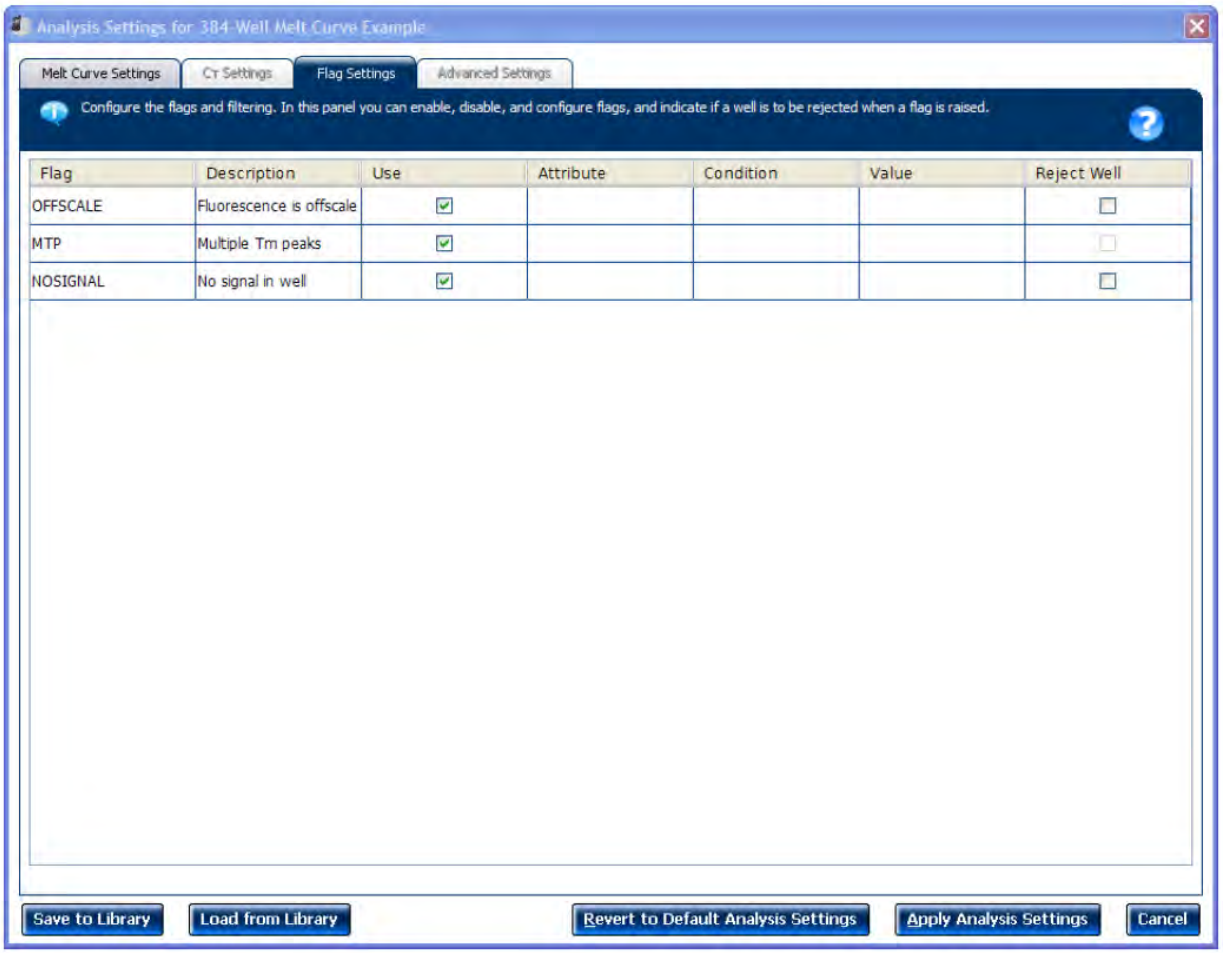


Figure 132 Flag Settings tab

Advanced settings

Use the **Advanced Settings** tab to change baseline settings well-by-well.

Note: The baseline and threshold values do not affect the analysis using the Relative Threshold setting.

The following steps describe the use of custom baseline settings for a well-target combination.

1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** checkbox.
3. Define the custom baseline settings.
   - For automatic baseline calculations, select the **Automatic Baseline** checkbox.
   - To define the baseline, deselect the **Automatic Baseline** checkbox, then enter the baseline start cycle and baseline end cycle.

38 Export analysis results

1. Open the Melt Curve example experiment file that has been analyzed.

   For information about analysis, see Chapter 37, “Review results and adjust experiment parameters”.

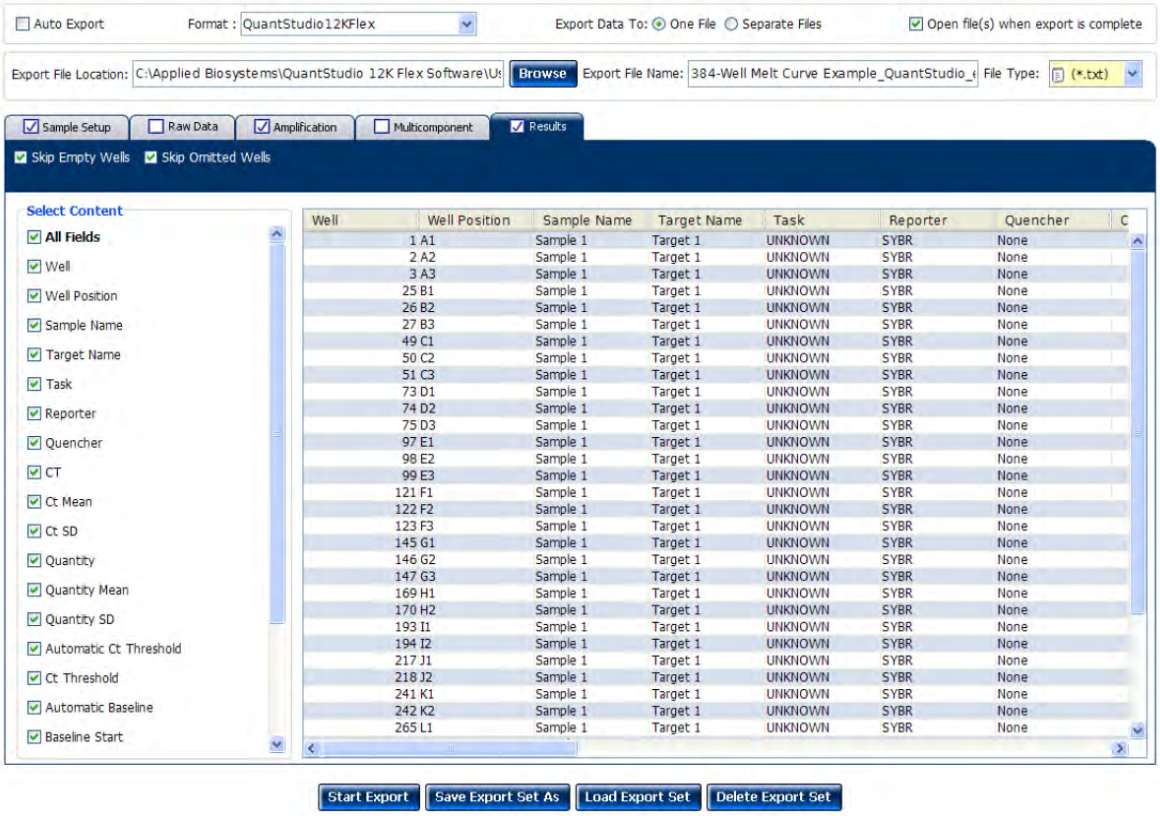
2. In the **Experiment Menu** pane, click **Export**.

   Note: To export data automatically after analysis, select the **Auto Export** checkbox during experiment setup or before running the experiment. The **Auto Export** checkbox is unchecked for the example experiment.

3. In the **Format** dropdown list, select **QuantStudio 12K Flex format**.

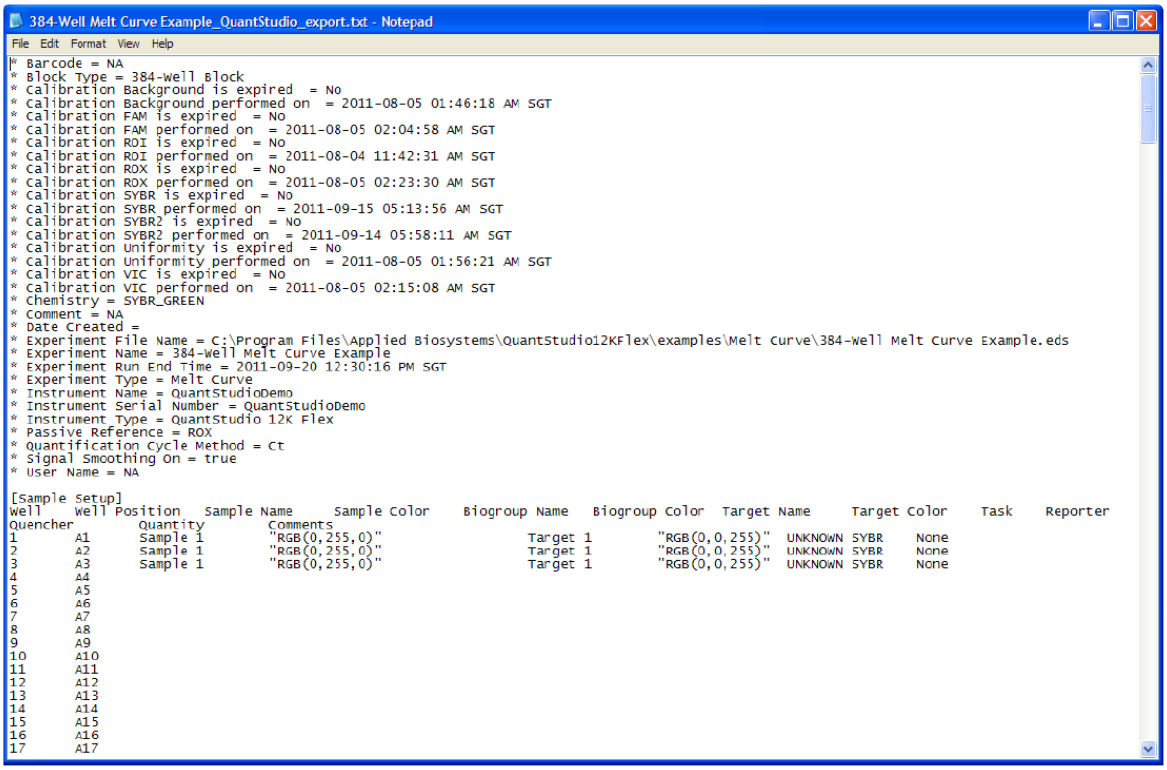
4. Complete the **Export** dialog box as shown below.

Field or selection	Entry
Select Data to export/Select Content	Results
Export Data To options	**One File** radio button
Export File Name field	***384-Well Melt Curve Example_QuantStudio_export***
File Type dropdown list	***.txt**
Export File Location field	Use the default file location or click **Browse** to select a different location



Well	Well Position	Sample Name	Target Name	Task	Reporter	Quencher
1	A1	Sample 1	Target 1	UNKNOWN	SYBR	None
2	A2	Sample 1	Target 1	UNKNOWN	SYBR	None
3	A3	Sample 1	Target 1	UNKNOWN	SYBR	None
25	B1	Sample 1	Target 1	UNKNOWN	SYBR	None
26	B2	Sample 1	Target 1	UNKNOWN	SYBR	None
27	B3	Sample 1	Target 1	UNKNOWN	SYBR	None
49	C1	Sample 1	Target 1	UNKNOWN	SYBR	None
50	C2	Sample 1	Target 1	UNKNOWN	SYBR	None
51	C3	Sample 1	Target 1	UNKNOWN	SYBR	None
73	D1	Sample 1	Target 1	UNKNOWN	SYBR	None
74	D2	Sample 1	Target 1	UNKNOWN	SYBR	None
75	D3	Sample 1	Target 1	UNKNOWN	SYBR	None
97	E1	Sample 1	Target 1	UNKNOWN	SYBR	None
98	E2	Sample 1	Target 1	UNKNOWN	SYBR	None
99	E3	Sample 1	Target 1	UNKNOWN	SYBR	None
121	F1	Sample 1	Target 1	UNKNOWN	SYBR	None
122	F2	Sample 1	Target 1	UNKNOWN	SYBR	None
123	F3	Sample 1	Target 1	UNKNOWN	SYBR	None
145	G1	Sample 1	Target 1	UNKNOWN	SYBR	None
146	G2	Sample 1	Target 1	UNKNOWN	SYBR	None
147	G3	Sample 1	Target 1	UNKNOWN	SYBR	None
169	H1	Sample 1	Target 1	UNKNOWN	SYBR	None
170	H2	Sample 1	Target 1	UNKNOWN	SYBR	None
193	I1	Sample 1	Target 1	UNKNOWN	SYBR	None
194	I2	Sample 1	Target 1	UNKNOWN	SYBR	None
217	J1	Sample 1	Target 1	UNKNOWN	SYBR	None
218	J2	Sample 1	Target 1	UNKNOWN	SYBR	None
241	K1	Sample 1	Target 1	UNKNOWN	SYBR	None
242	K2	Sample 1	Target 1	UNKNOWN	SYBR	None
265	L1	Sample 1	Target 1	UNKNOWN	SYBR	None

Figure 133 Export screen



384-Well Melt Curve Example_QuantStudio_export.txt - Notepad

File Edit Format View Help

```
* Barcode = NA
* Block Type = 384-Well Block
* Calibration Background is expired  = No
* Calibration Background performed on  = 2011-08-05 01:46:18 AM SGT
* Calibration FAM is expired  = No
* Calibration FAM performed on  = 2011-08-05 02:04:58 AM SGT
* Calibration ROI is expired  = No
* Calibration ROI performed on  = 2011-08-04 11:42:31 AM SGT
* Calibration ROX is expired  = No
* Calibration ROX performed on  = 2011-08-05 02:23:30 AM SGT
* Calibration SYBR is expired  = No
* Calibration SYBR performed on  = 2011-09-15 05:13:56 AM SGT
* Calibration SYBR2 is expired  = No
* Calibration SYBR2 performed on  = 2011-09-14 05:58:11 AM SGT
* Calibration Uniformity is expired  = No
* Calibration Uniformity performed on  = 2011-08-05 01:56:21 AM SGT
* Calibration VIC is expired  = No
* Calibration VIC performed on  = 2011-08-05 02:15:08 AM SGT
* Chemistry = SYBR_GREEN
* Comment = NA
* Date Created =
* Experiment File Name = C:\Program Files\Applied Biosystems\QuantStudio12KFlex\examples\Melt Curve\384-Well Melt Curve Example.eds
* Experiment Name = 384-Well Melt Curve Example
* Experiment Run End Time = 2011-09-20 12:30:16 PM SGT
* Experiment Type = Melt Curve
* Instrument Name = QuantStudioDemo
* Instrument Serial Number = QuantStudioDemo
* Instrument Type = QuantStudio 12K Flex
* Passive Reference = ROX
* Quantification Cycle Method = Ct
* Signal Smoothing On = true
* User Name = NA

[Sample Setup]
Well    Well Position   Sample Name     Sample Color    Biogroup Name   Biogroup Color  Target Name     Target Color    Task    Reporter
Quencher        Quantity        Comments
1       A1      Sample 1        "RGB(0,255,0)"                  Target 1        "RGB(0,0,255)"  UNKNOWN SYBR    None
2       A2      Sample 1        "RGB(0,255,0)"                  Target 1        "RGB(0,0,255)"  UNKNOWN SYBR    None
3       A3      Sample 1        "RGB(0,255,0)"                  Target 1        "RGB(0,0,255)"  UNKNOWN SYBR    None
4       A4
5       A5
6       A6
7       A7
8       A8
9       A9
10      A10
11      A11
12      A12
13      A13
14      A14
15      A15
16      A16
17      A17
```

Figure 134 Exported file

A Documentation and support

Related documentation

Document	Pub. No.
QuantStudio™ 12K Flex Real–Time PCR System Site Preparation Guide	4470654
QuantStudio™ 12K Flex Real–Time PCR System: OpenArray™ Plate Quick Reference	4478673
QuantStudio™ 12K Flex Real–Time PCR System: OpenArray™ Experiments User Guide	4470935
High Resolution Melt Software for QuantStudio™ 12K Flex Real–Time PCR System Getting Started Guide	4470692
QuantStudio™ 12K Flex Software Automation API Reference Guide	MAN0028446
QuantStudio™ 12K Flex Software v1.6 or later	
QuantStudio™ 12K Flex Real–Time PCR System v1.6 or later Maintenance and Administration Guide	MAN0018832
QuantStudio™ 12K Flex Real–Time PCR System v1.6 or later Multi-Well Plates and Array Card Quick Reference	MAN0018833
QuantStudio™ 12K Flex Software v1.4 or earlier	
QuantStudio™ 12K Flex Real–Time PCR System: Multi-Well Plates and Array Card Quick Reference	4470688
QuantStudio™ 12K Flex Real–Time PCR System v1.4 Maintenance and Administration Guide	4470689

Obtaining information from the help system

The QuantStudio™ 12K Flex Software has a help system that describes how to use each feature of the user interface. Access the help system in one of the following ways.

- Press F1.
- Click in the toolbar.
- Click **Help** ▸ **QuantStudio 12K Flex Software Help**.

You can use the Help system to find topics of interest by:

- Reviewing the table of contents
- Searching for a specific topic

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

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

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

Limited product warranty

Life Technologies Corporation and its affiliates warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have questions, contact Life Technologies at **www.thermofisher.com/support**.