USER GUIDE

applied biosystems® by life technologies™

Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System

Multi-Well Plates and Array Card Experiments

for use with: Other real-time PCR systems

ExpressionSuite Software
TaqMan® Genotyper Software

MULTI-WELL PLATES AND ARRAY CARD EXPERIMENTS USER GUIDE

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life technologies™

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Roadmap

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BOOKLET 2 **Running Standard Curve Experiments**

BOOKLET 3 **Running Relative Standard Curve and Comparative C_T Experiments**

- **PART I: Running Relative Standard Curve Experiments**
- **PART II: Running Comparative C_T Experiments**

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BOOKLET 5 **Running Presence/Absence Experiments**

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GETTING STARTED GUIDE

applied biosystems®
by life technologies™

Booklet 1 - Getting Started with QuantStudio™ 12K Flex System Multi-Well Plates and Array Card Experiments

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About This Guide

CAUTION! ABBREVIATED SAFETY ALERTS. Hazard symbols and hazard types specified in procedures may be abbreviated in this document. For the complete safety information, see the "Safety" appendix in this document.

IMPORTANT! Before using this product, read and understand the information the "Safety" appendix in this document.

Revision history

Revision	Date	Description
A	March 2012	New document

Purpose

The *QuantStudio™ 12K Flex Real-Time PCR System Multi-Well Plates and Array Card Experiments User Guide Binder* 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 Array Card consumables on the QuantStudio™ 12K Flex System.

Prerequisites

This user guide is intended for personnel who have been specifically trained by Life Technologies. 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 System, please read this booklet, *Getting Started with QuantStudio™ 12K Flex System 96-Well, 384-Well, and Array Card Experiments* thoroughly. The booklet provides information and general instructions that are applicable to all the experiments described in this binder.

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 on the installation CD. The following booklets are provided:

- *Getting Started with QuantStudio™ 12K Flex System 96-Well, 384-Well, and Array Card Experiments* – introductory information and experiment workflow common to all experiments.
- *Running Standard Curve Experiments* – designing, running, and analyzing a Standard Curve experiment.
- *Running Relative Standard Curve and Comparative C_T Experiments* – designing, running, and analyzing Relative Standard Curve and Comparative C_T experiments.
- *Running Genotyping Experiments* – designing, running, and analyzing a Genotyping experiment.
- *Running Presence/Absence Experiments* – designing, running, and analyzing a Presence/Absence experiment.
- *Running Melt Curve Experiments* – designing, running, and analyzing a Melt Curve experiment.
- *QuantStudio™12K Flex System Multi-Well Plates and Array Card Experiments - Appendixes* – common information such as ordering information, additional documentation, and glossary.

  Note: In all booklets, 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 on the installation CD. 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.

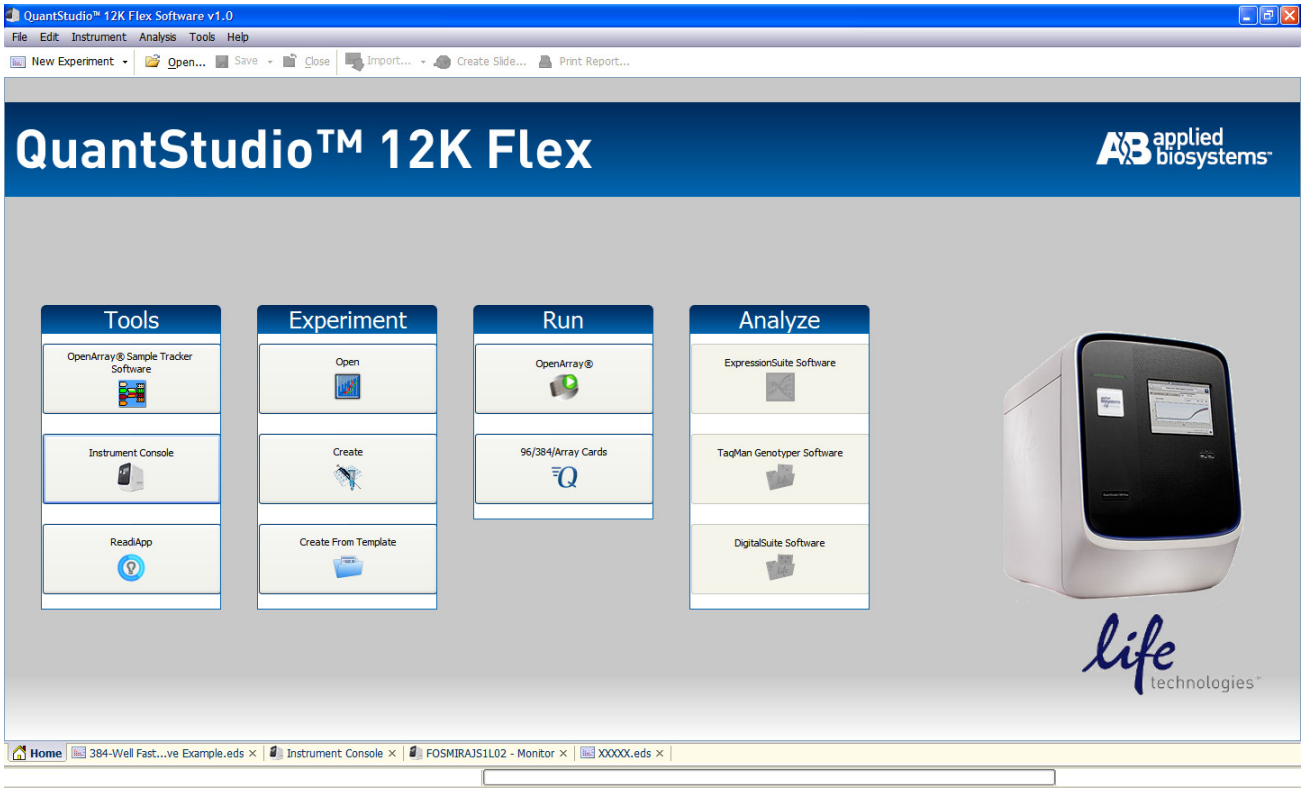
How to access an example experiment

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 hand side of the screen.

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



Note: The icons in the Analyze menu appear active only if you've downloaded the ExpressionSuite Software, TaqMan® Genotyper Software and DigitalSuite Software on your computer.

Open an example experiment

From the Home screen, click **Open**, to navigate to the **experiments** folder (default):

C:\Program Files\Applied Biosystems\QuantStudio 12K Flex Software\examples, and 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

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 IMPORTANTs, 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 for descriptions of the symbols.

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

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 and from the Experiment menu, click the Create icon. 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

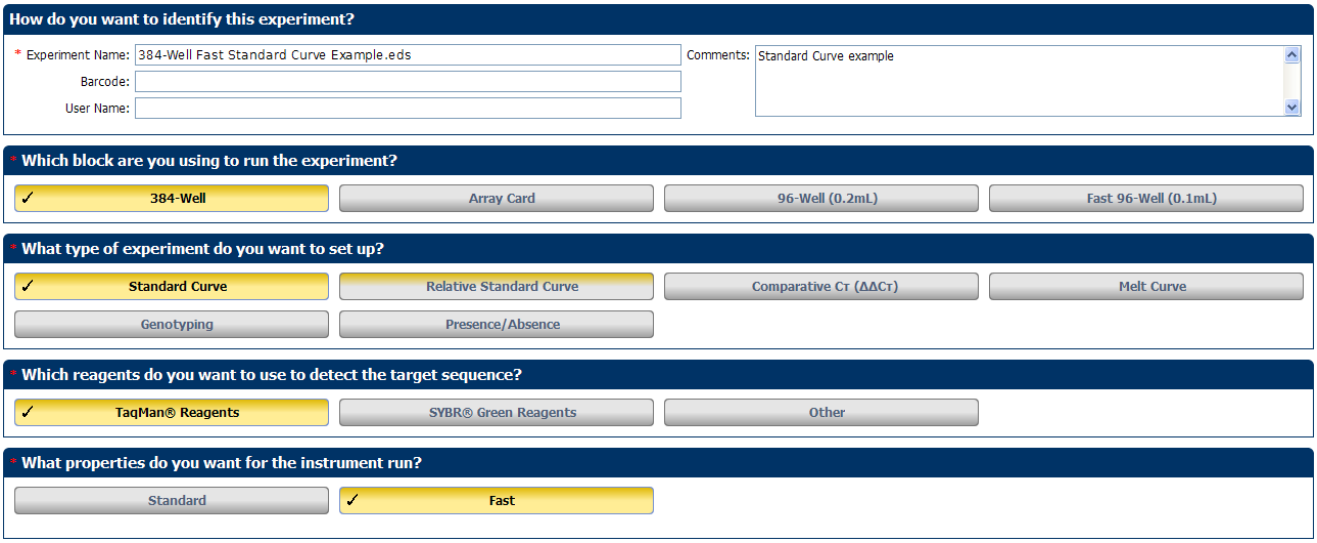
   Go to **Tools** ▸ **Preferences**. Click the Experiment tab. Select the Auto Analysis and Auto Save check boxes for the QuantStudio™ 12K Flex Software to automatically analyze and save experiment results. You can also edit the following default baseline settings:

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 19 for information on target libraries.

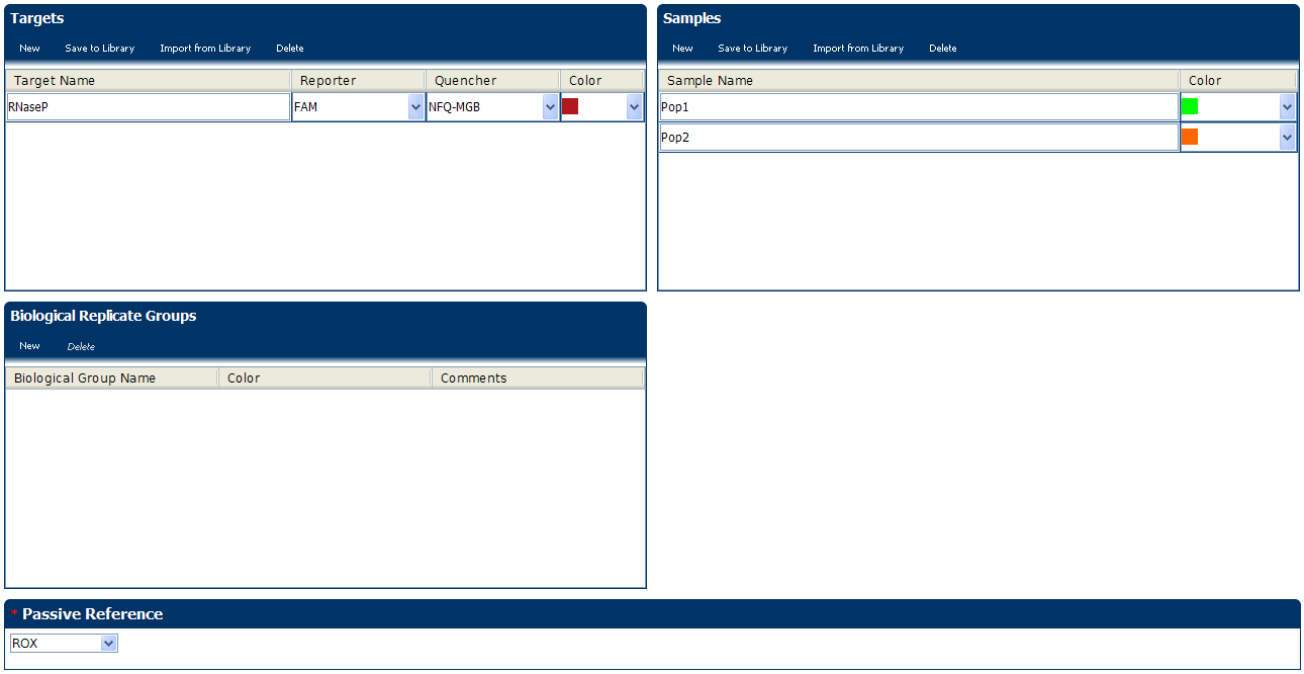
f. Click **Import from Library** to add targets from the target library.

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 19 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).

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
	~~I~~	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.

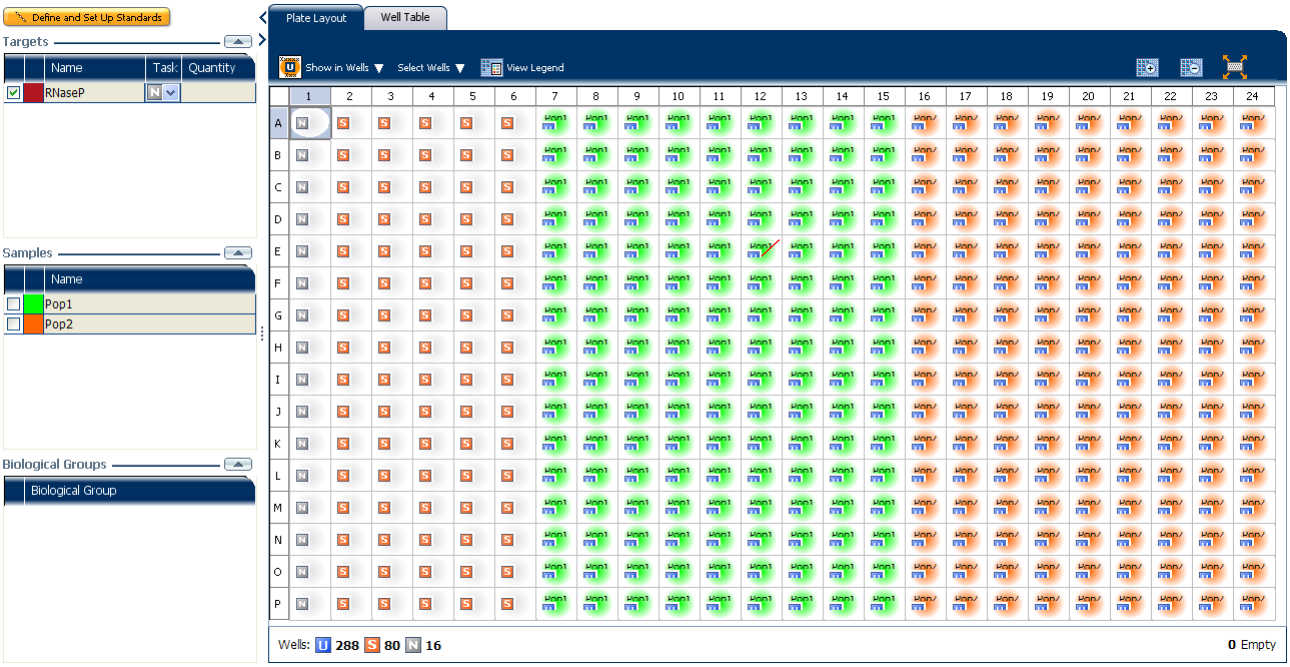
Note: You can assign only one sample to a well.

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.

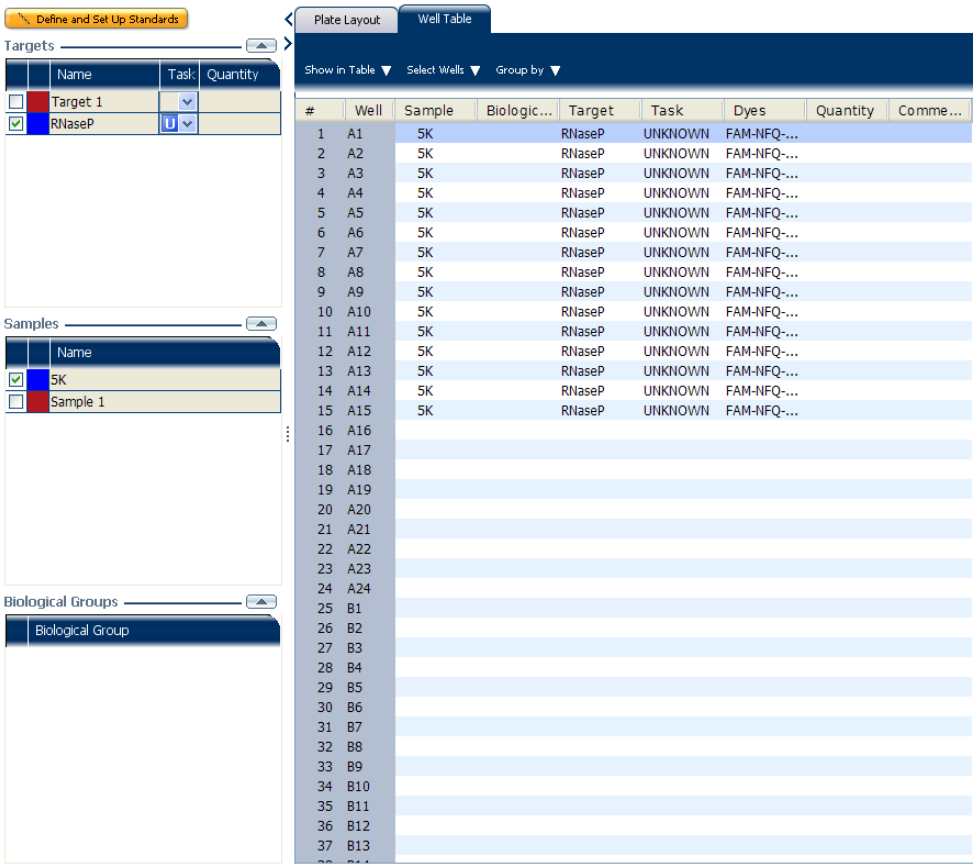
Note: You must select the header, and the Well Number and Well Position columns while copying information from the ***.xls** file.

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

A1 | fx | Well

	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		

Copy and paste the selected information



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 19 for information on run method libraries.

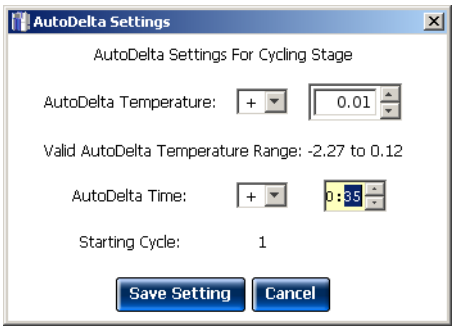
2. Enter a number from 1 to 20 for the reaction volume per well. The QuantStudio™ 12K Flex Instrument supports the following maximum reaction volumes for the consumables listed below:
   - MicroAmp® Optical 384-Well Reaction Plate - 30 µL
   - Applied Biosystems Array Card - 1 µL
   - MicroAmp® Optical 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.

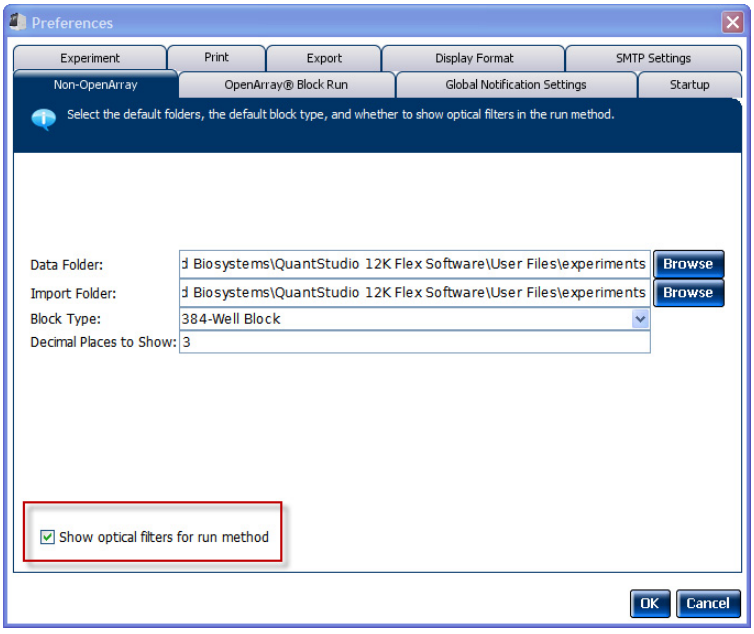
- Edit the PCR Stage.
- Change the Number of Cycles for the PCR stage.
- Select the **Enable AutoDelta** check box, to increase or decrease the temperature and/or hold time for each subsequent cycle or to change the Starting Cycle for AutoDelta. Enabling AutoDelta displays the ▲ icon. Click the AutoDelta 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.

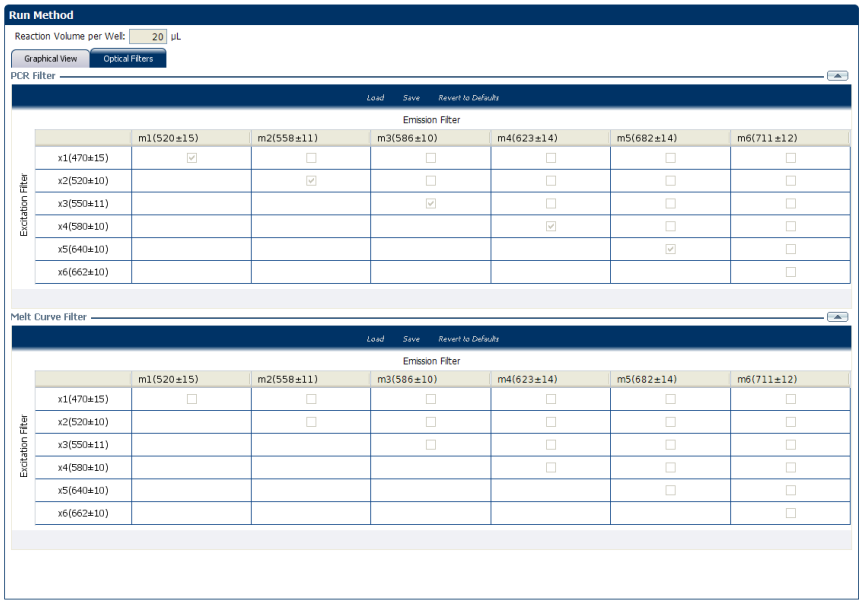
4. Complete the tasks on the Optical Filters tab:

   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 check box 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. Refer to the *Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Maintenance and Administration Guide* 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**.

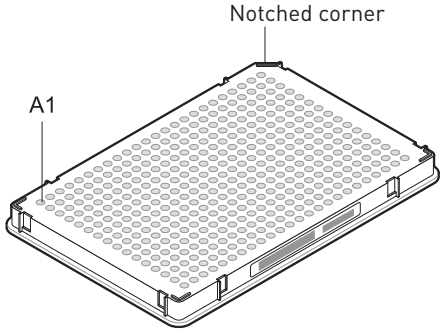
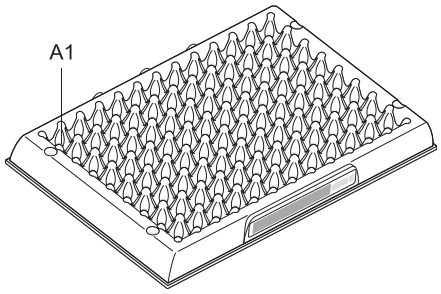
To select a run method from the Run Method Library

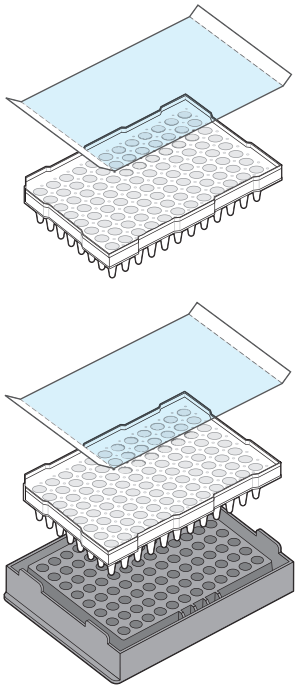
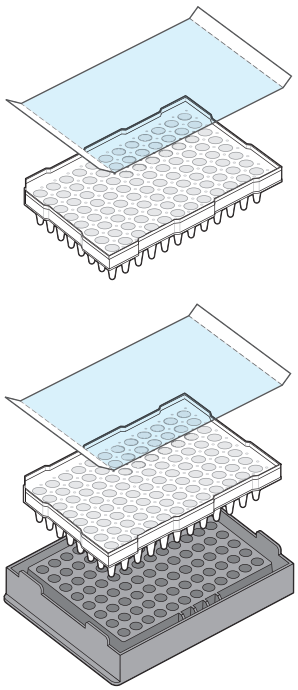
Click **Open Run Method** on the Run Method screen, and select one from the saved run methods.

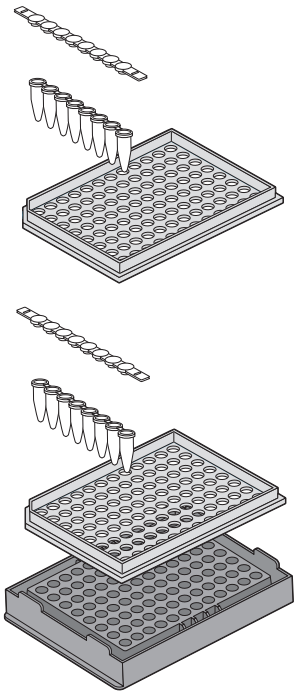
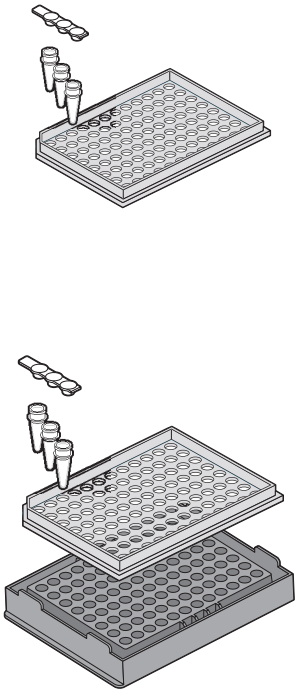
Prepare reactions

Supported consumables

The QuantStudio™ 12K Flex Instrument is optimized for Applied Biosystems consumables. These can be ordered from the Life Technologies website. Use the consumables appropriate for the sample block of your instrument.

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

Sample block	Consumable		Maximum reaction volume (µL) supported	Recommended reaction volume (µL)
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	10-100
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	10-30

Sample block	Consumable		Maximum reaction volume (µL) supported	Recommended reaction volume (µL)
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 for Fast 96-well plate • 200 for 96-well plate	• 10-30 for Fast 96-well plate • 10-100 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 for Fast 96-well plate • 200 for 96-well plate	• 10-30 for Fast 96-well plate • 10-100 for 96-well plate

WARNING! 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

Life Technologies supports the reagents listed below for experiments performed on the QuantStudio™ 12K Flex System.

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

Note: Fast Universal 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

Applied Biosystems TaqMan® Reagents

Description

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

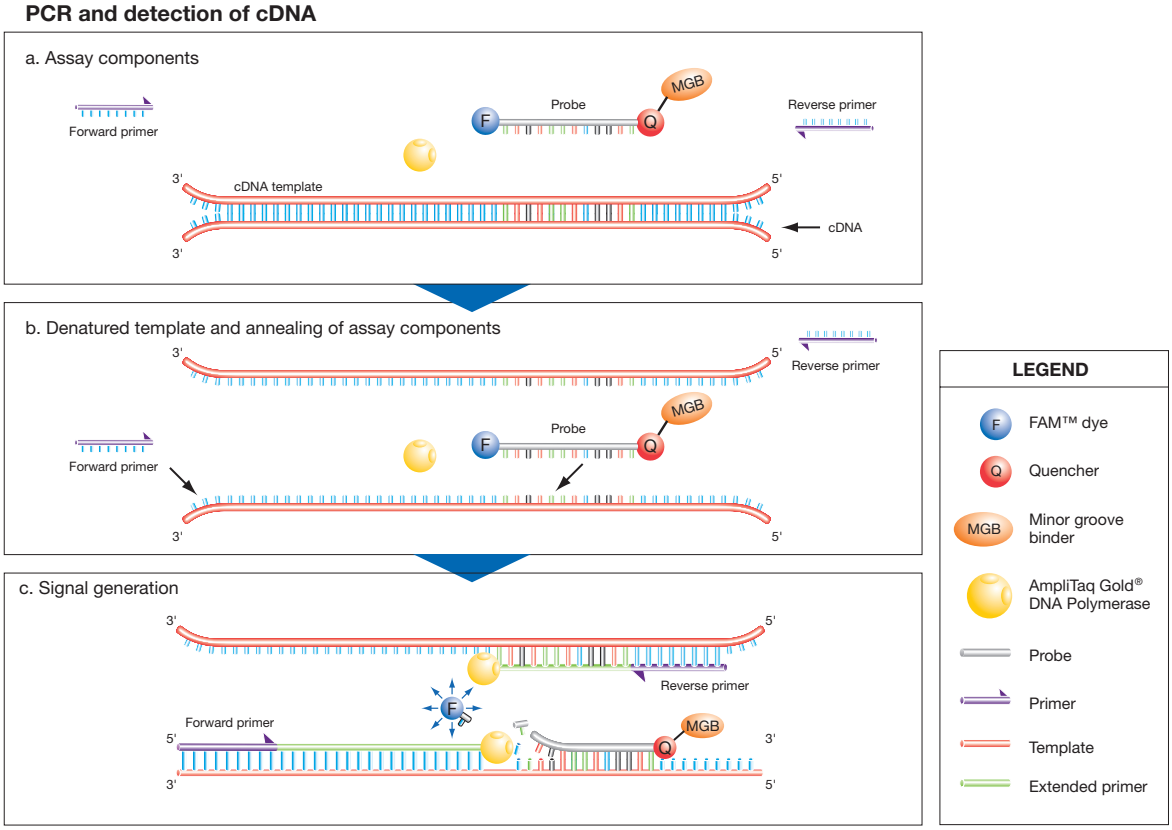
Advantages

- 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.

TaqMan® Reagents detection process



Applied Biosystems SYBR® Green reagents

Description

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

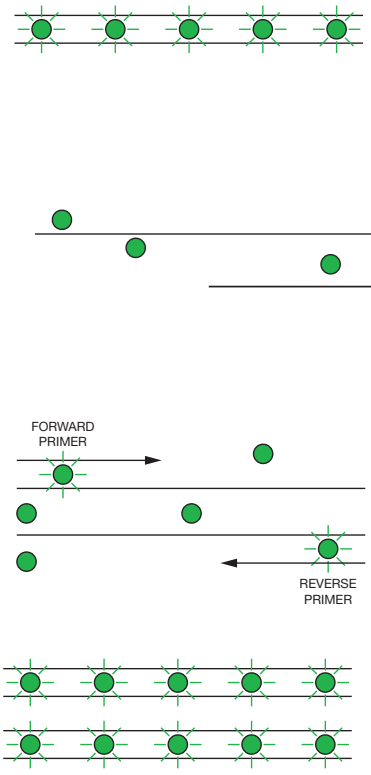
Advantages

- 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.

SYBR® Green detection process



Step 1: Reaction setup
The SYBR® Green I dye fluoresces when bound to double-stranded DNA.

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.

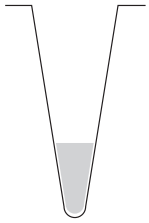
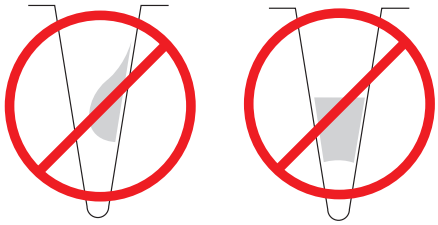
Materials required while preparing the dilutions

- 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.

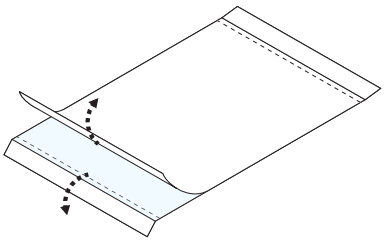
1. Load the reaction plate using the plate layout described in "Assign targets, samples, and biological replicate groups" on page 14.

   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.

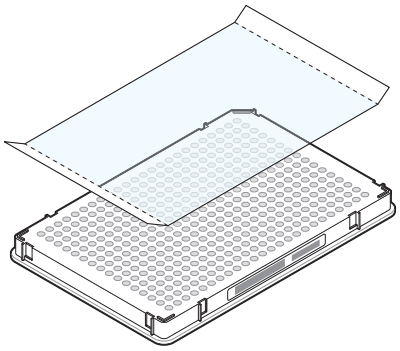
2. Remove a single optical adhesive film from the box. Bend both end-tabs upward. Hold the film backing side up.

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

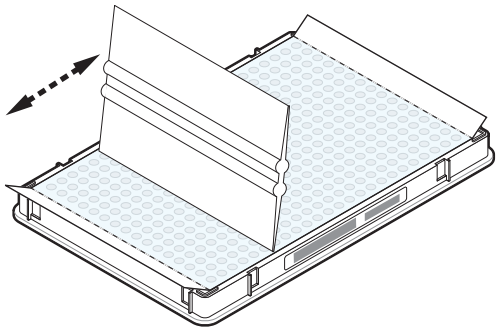


IMPORTANT! Improper peeling of the optical adhesive film may result in haziness, but it will not affect results. Haziness disappears when the film comes into contact with the heated cover in the instrument.

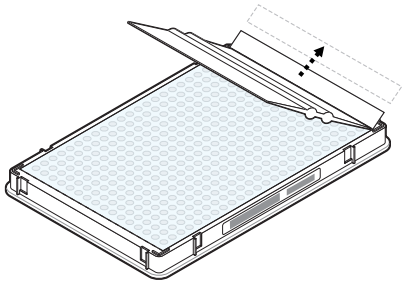
4. Holding the film by the end-tabs, lower the film onto the reaction plate (adhesive side facing the reaction plate). Make sure that the film completely covers all wells of the reaction plate.



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.

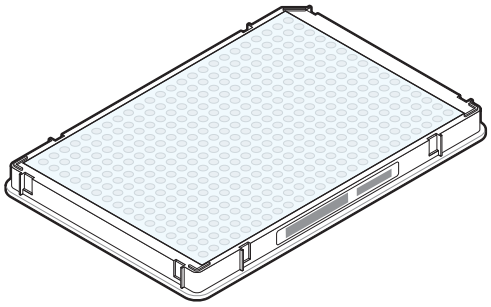


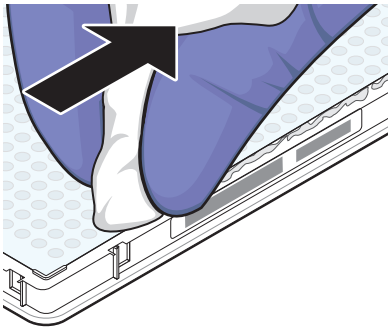
Note: Ensure clean removal of both end-tabs from the dotted line. Improper peeling of the end-tab can cause sticking of plate on the heated cover assembly.

7. To ensure a tight, evaporation-free seal, repeat 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.step 5. 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 the gripper of the Twister® Robot or to the sample block of the QuantStudio™ 12K Flex Instrument.

Fill and seal the array card

Fill and spin the array card

IMPORTANT! Wear powder-free gloves while preparing the Arrays.

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.

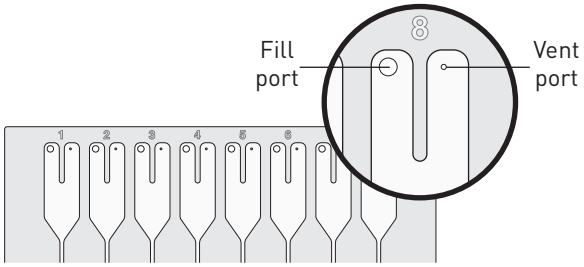
   For each transfer:

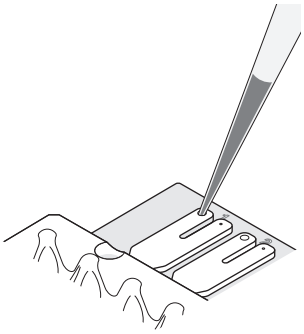
   a. Place the array card on a lab bench, with the foil side down.

   b. Load 100 µL of fluid into a pipette.

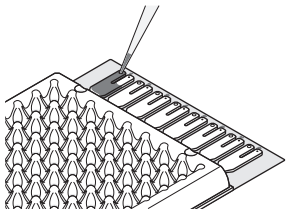
   c. Hold the pipette in an angled position (~45 degrees) and place the tip into the fill port. There is a fill port on the left arm of each fill reservoir – it is the larger of the two holes.

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



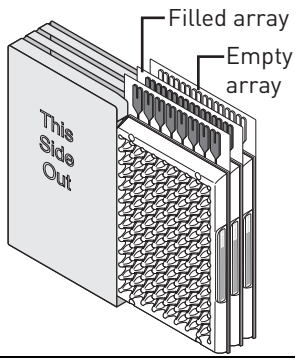


   d. Dispense the fluid so that it sweeps in and around the fill reservoir toward the vent port. Pipette fluid into the fill reservoir, but **do not** go past the first stop of pipettor plunger when pipetting the reagents into the array card, or you may blow the solution out of the port.



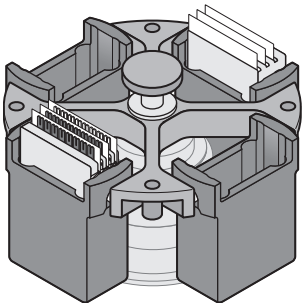
IMPORTANT! Do not allow the tip to contact and possibly damage the coated foil beneath the fill port.

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.

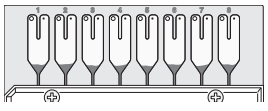
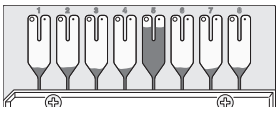
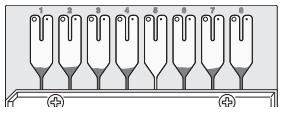


IMPORTANT! You must run the centrifuge with all four buckets in place and each of the two carriers filled with the array card. Place empty array cards (4-pack PN 4334812 and 1-pack PN 4351471) into unfilled slots.

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.

Correct	Incorrect	
		

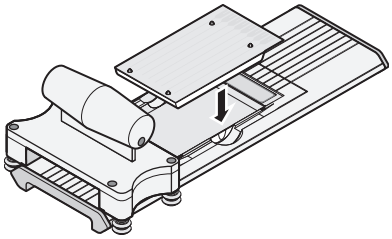
9. If necessary, centrifuge the array card(s) for an additional minute to fill any unfilled wells. Do not exceed three 1-minute runs or centrifuge the array card for longer than 1 minute at a time.

   Note: Visit the Life Technologies website, log on to store, and view an online video of loading, centrifuging, and sealing an array card.

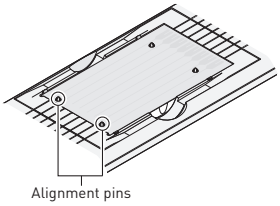
Seal the array card(s)

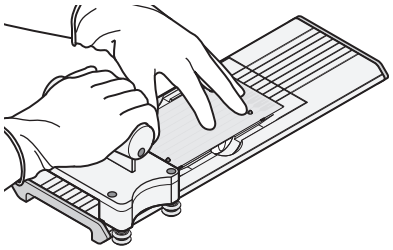
1. With the carriage (roller assembly) of the TaqMan® Array Micro Fluidic Card Sealer in the Start position, place a filled array card into the fixture with the foil side up so that the fill reservoirs are the farthest away from the carriage.

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

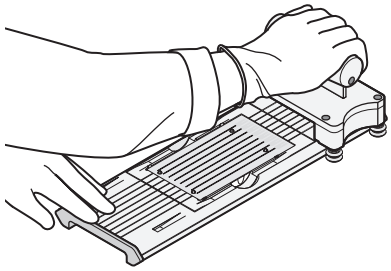


3. Use the two alignment pins in the fixture to position the array card correctly.

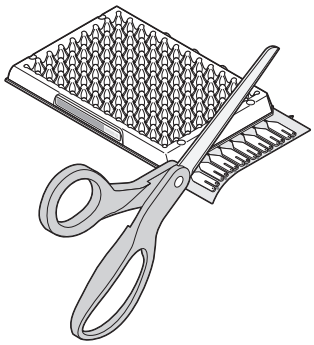




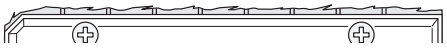
4. Seal the array card by running the carriage slowly over it, in one direction only. Do not apply downward force on the carriage as you move it forward over the card.



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	Incorrect
	

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

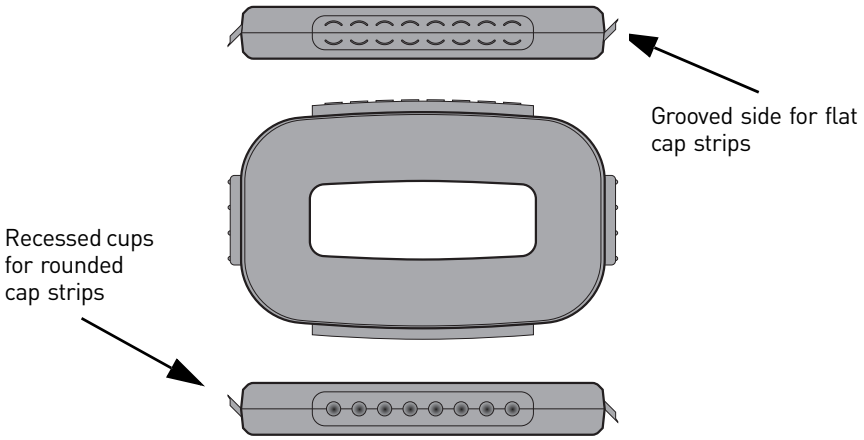
Note: Make sure that you secure the caps on the tubes and tube-strips tightly to avoid sample evaporation.

If you use the 96-well MicroAmp® Optical 8-Tube Strips or MicroAmp® Optical Tubes without Cap, use the MicroAmp® Cap Installing Tool (PN 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

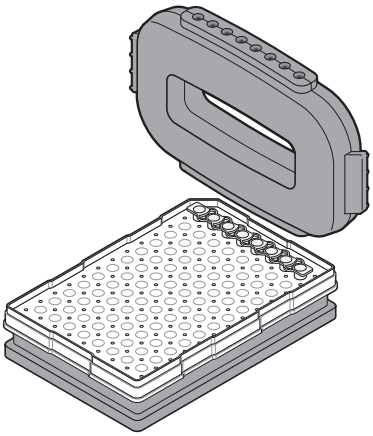


MicroAmp® Cap Installing Tool

Apply the MicroAmp® Optical 8-Cap Strip (flat)

1. Grasp the Cap Installing Tool so that the grooved side is exposed.

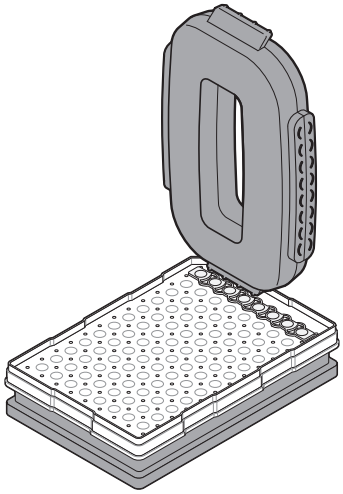
2. Hold the strip of caps over the tube strip or the row of tubes.
3. Use the grooved side (shown) of the Cap Installing Tool to push and seat each cap firmly in place. Use a rocking motion to properly seat each cap.



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. To remove the cap or cap strip:

1. Insert the small protrusions on the side of the Cap Installing Tool under the webbing between the caps on a cap strip.
2. Slowly pry the strip from the plate or Tray/ Retainer assembly.



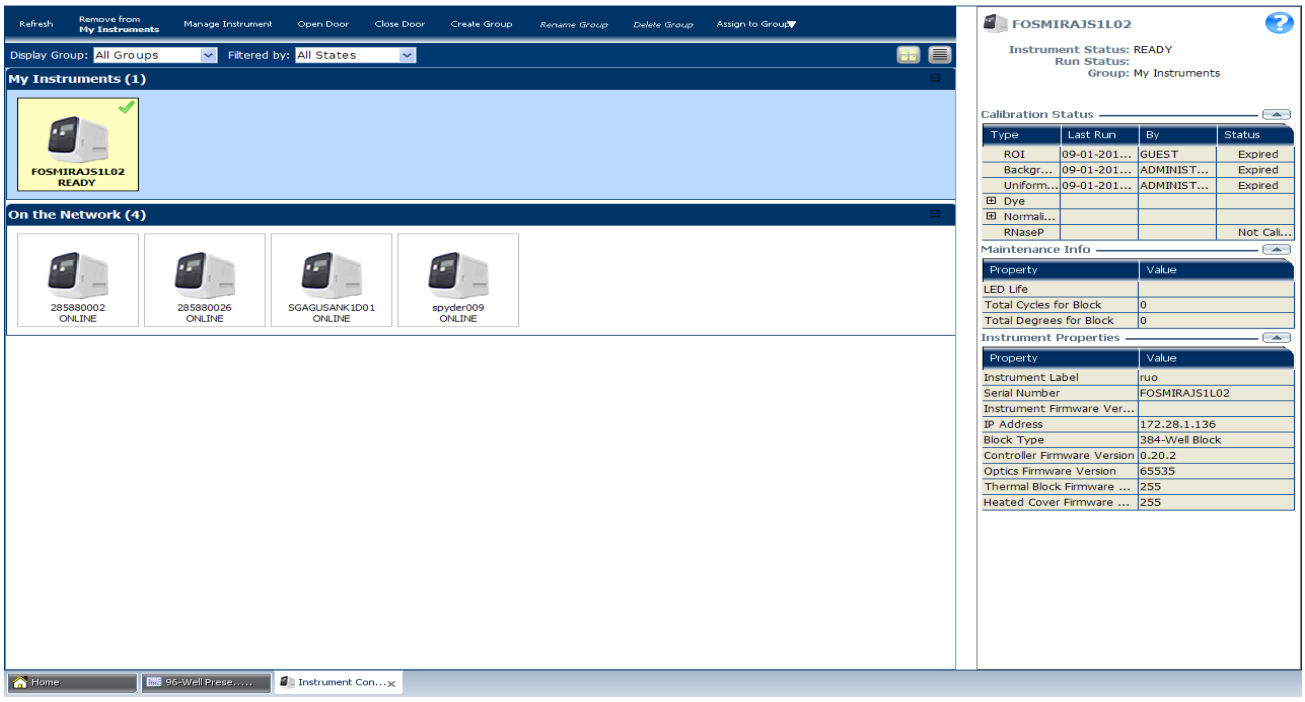
Start the experiment

To start an experiment:

1. Access the Instrument Console.
2. Load the reaction plate or array card into the instrument, as shown on page 37.
3. Run the experiment as shown on page 39.

Instrument Console

The Instrument Console displays all the QuantStudio™ 12K Flex Instruments discovered on a network.



Left panel

The features on the left panel of the Instrument Console allow:

- Instrument access: Open and close the QuantStudio™ 12K Flex Instrument door from the QuantStudio™ 12K Flex Software user interface.
- Group management:
  - Create, rename, and delete groups and assign instruments to the groups.
  - Add and remove instruments to and from My Instruments.

    Note: To add instruments, select the icon of the QuantStudio™ 12K Flex Instrument that you want to add to the My Instruments list. Then click **Add to My Instruments**. Similarly, click **Remove from My Instruments** to remove an instrument from the My Instruments list. You can also drag and drop the instrument icon into My Instruments or into the group created by you.
  - Display instrument groups from the Display Group drop-down menu. according to their activity. Select the status from the Filtered By drop-down menu. For more information on the status of an instrument, see "Monitor the experiment" on page 40.
- 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 40.
  - Maintain instruments (check the calibration status of instruments and perform different calibrations). For more information on Instrument maintenance, refer to *Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Maintenance and Administration Guide*.
  - 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 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** and select the setup folder you want to shift the setup file into.

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

Right panel

The right panel of the Instrument Console displays:

- 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

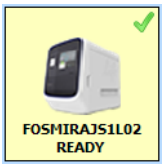
You can monitor the instrument status and view calibration and other information in the Instrument Console.

QuantStudio™ 12K Flex 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 on the Instrument Console. An instrument displays the status when you place the instrument icon under My Instruments or under the Group(s) that you created.

To monitor the instrument status:

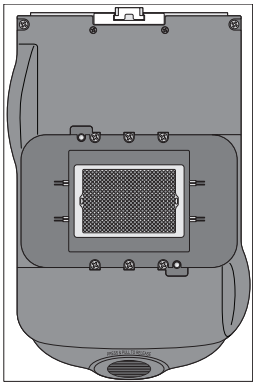
1. On the Home tab (), select **Instrument Console**. If you do not see an instrument, click **Refresh** in the instrument console toolbar.
2. If needed, move the instrument from the On the Network group to a group which can be monitored:
   a. Click the instrument of interest, then click **Assign to 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 under My Instruments or a group you created.
	Run in process (The time remaining for the run is shown to the left of the icon.)
	Unavailable
	Incompatible firmware version
	No longer connected to the network

Icon	Instrument status
	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.



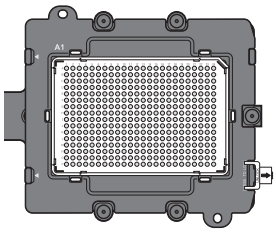
Sample block

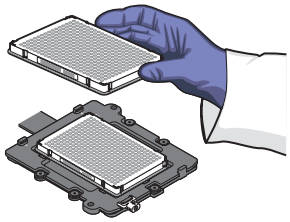
IMPORTANT! Wear powder-free gloves when you handle the **reaction plate or array card**.

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 QuantStudio™ 12K Flex Instrument touchscreen or click **Open Door** in the Instrument Console screen of the QuantStudio™ 12K Flex 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).

WARNING! 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 QuantStudio™ 12K Flex Instrument touchscreen or click **Close Door** in the Instrument Console screen of the QuantStudio™ 12K Flex 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 QuantStudio™ 12K Flex Instrument begins and completes a run, or if an error occurs during a run.

Note: For details on using the Notification Settings feature, refer to the *Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Maintenance and Administration Guide*.

Run the experiment

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

- From the QuantStudio™ 12K Flex Software
- From the QuantStudio™ 12K Flex Instrument touchscreen

Note: The example experiments in each of the getting started guide booklets start a run from the QuantStudio™ 12K Flex Software.

IMPORTANT! Make sure that instrument calibration is up-to-date. If a calibration has expired, you will get a warning when you start a run. For information on calibrating the QuantStudio™ 12K Flex Instrument, refer to *Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Maintenance and Administration Guide*.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the QuantStudio™ 12K Flex Instrument is in operation.

From the QuantStudio™ 12K Flex Software

1. In the QuantStudio™ 12K Flex Software, click 

Run in the navigation pane.
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.

From the QuantStudio™ 12K Flex Instrument touchscreen

1. Touch the QuantStudio™ 12K Flex 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 

View/Edit to view or edit the experiment before starting the run.

   Note: You can start a run immediately by clicking 

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

6. *(Optional)* Modify the experiment parameters as needed. You can use the:
   - 

Add and 

Delete buttons to add and delete a stage or step to the thermal profile.
   - 

Add Melt Curve button to add a melt curve to the thermal profile.
   - 

Save button to save the experiment you modify.

7. In the Save Experiment screen, touch each field to edit the:
   - 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 47.

Monitor the experiment

Note: If there is loss of connection during an experiment, remove and then add the instrument to the My Instruments list. You may then resume monitoring the experiment.

You can monitor an experiment run in three ways:

- From the QuantStudio™ 12K Flex Instrument touchscreen, in the same way that you run the experiment (see "From the QuantStudio™ 12K Flex Instrument touchscreen" on page 39).
- From the Run screen of the QuantStudio™ 12K Flex Software, while the experiment is in progress, as shown below.
- From the Instrument Console of the QuantStudio™ 12K Flex Software (to monitor an experiment started from another computer or from the QuantStudio™ 12K Flex Instrument touchscreen) as described in "From the QuantStudio™ 12K Flex Software Instrument Console" on page 41.

From the QuantStudio™ 12K Flex 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.

From the QuantStudio™ 12K Flex Software Instrument Console

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. On the Instrument Manager screen, click **Monitor Running Instrument**.

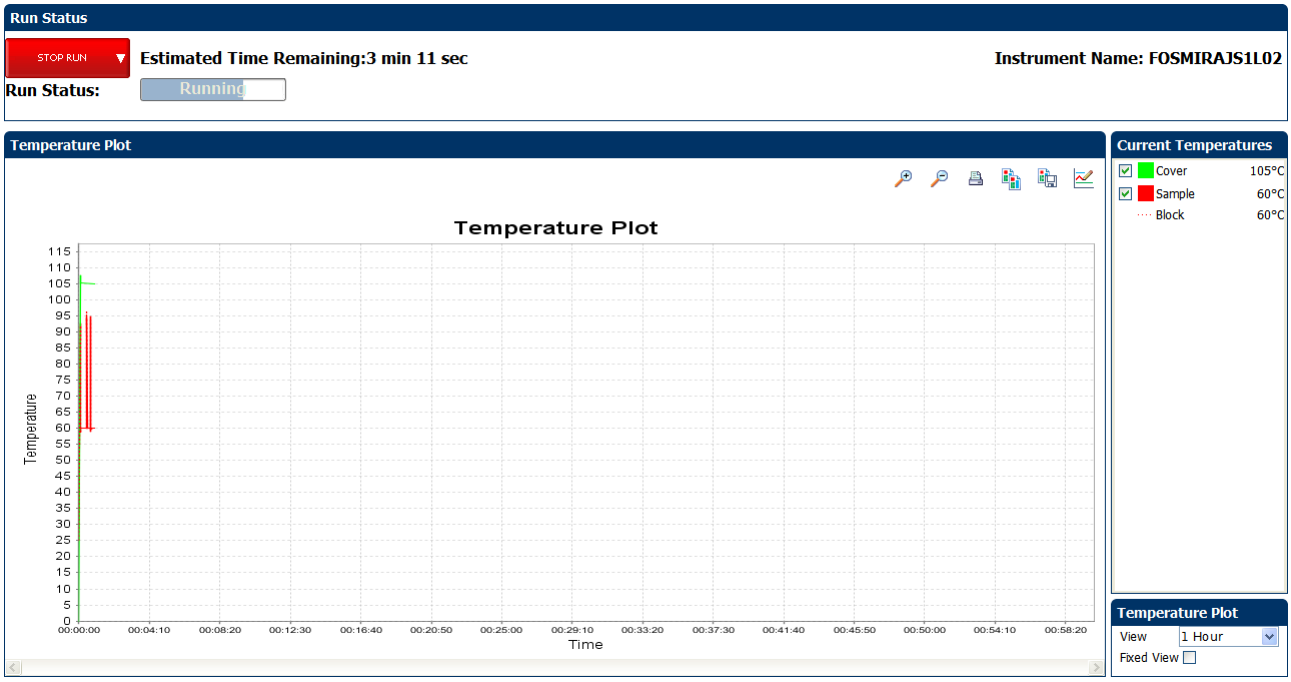
You can view the progress of the run in real time from the Run screen. During the run, periodically view the Amplification Plot, Temperature Plot and Run Method (see page 42) available from the QuantStudio™ 12K Flex Software for potential problems.

To...	Action
Stop the run	• In the QuantStudio™ 12K Flex Software, click **STOP RUN**. • In the Stop Run dialog, click one of the following: – **Stop Immediately** to stop the run immediately. – **Cancel** to continue the run.
View amplification data in real time	Select **Amplification Plot**. See "To monitor the Amplification Plot" on page 42.
View temperature data for the run in real time	Select **Temperature Plot**. See "To monitor the Temperature Plot" on page 42.
View progress of the run in the Run Method screen	Select **Run Method**. See "To monitor the Run Method" on page 43.
Enable/disable the Notification Settings	Select or deselect **Enable Notifications**. See "Enable or change the Notification Settings" on page 38.

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 QuantStudio™ 12K Flex 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.

To...	Action
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 menu, 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 menu, 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 and Block plots for abnormal behavior.

- The Sample and Block plots should mirror each other approximately. A significant deviation of the plots may 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 QuantStudio™ 12K Flex 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.

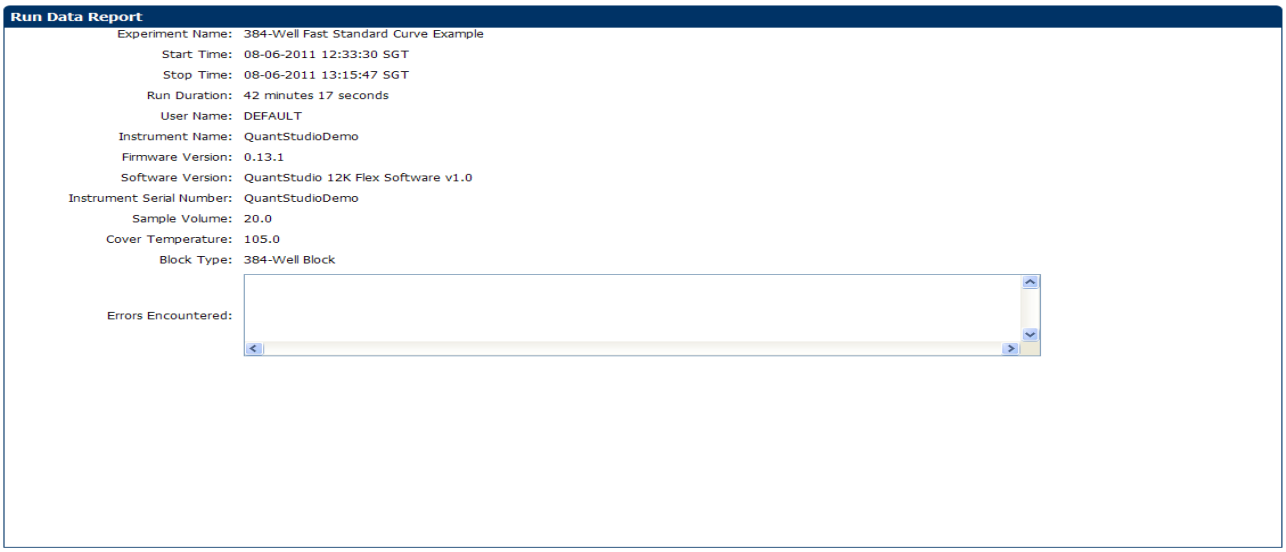
To...	Action
Change the number of cycles	In the **Adjust # of Cycles** field, enter the number of cycles to apply to the Cycling Stage.
Add a melt curve stage to the end of the run	Select **Add Melt Curve Stage to End**.
Add a Hold stage to the end of the run	Select **Add Holding Stage to End**.

To...	Action
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 QuantStudio™ 12K Flex 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.

From the QuantStudio™ 12K Flex Instrument touchscreen

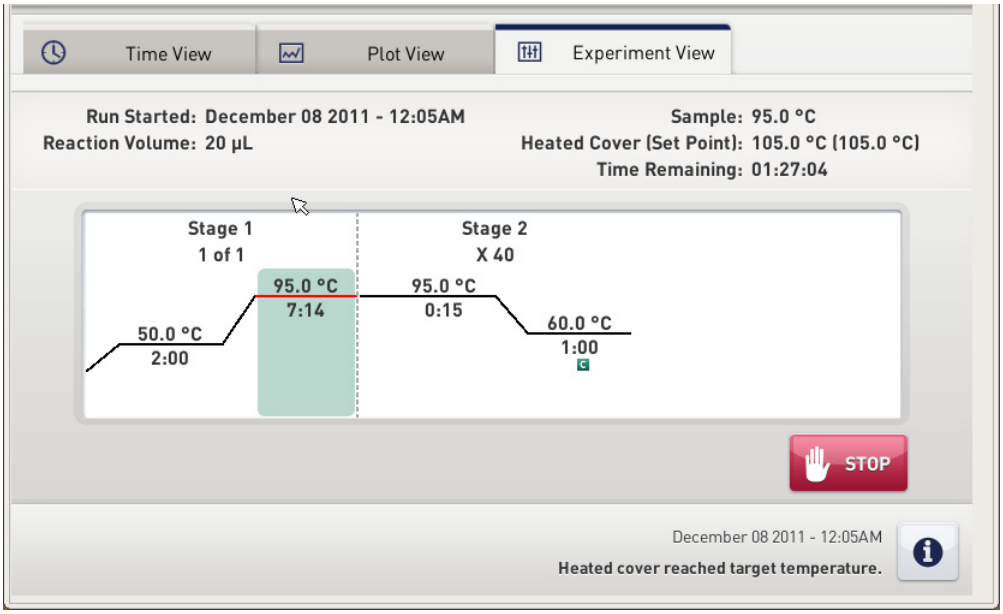
The touchscreen displays the method for the experiment, the date and time at which the run started, the time remaining in the run, and other information.

To...	Action
Display a graphical view of the run	Touch **Experiment View**.
Show the Amplification Plot for the run	Touch the **Plot View**, then touch **Experiment View** to return to the Run Method screen.

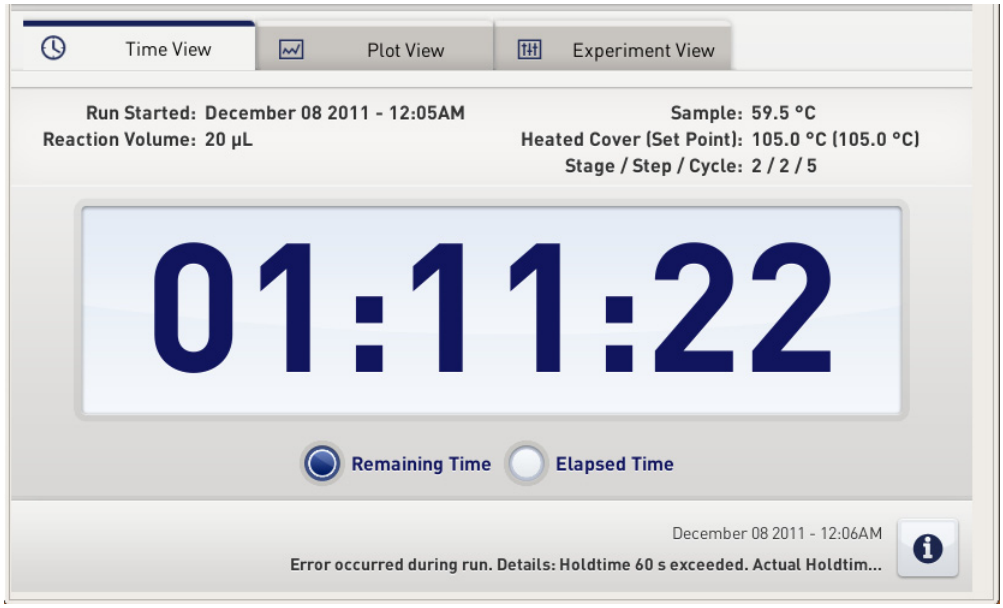
To...	Action
Display the time elapsed and the time remaining in the run	Touch the **Time View tab**, then touch **Experiment View** tab to return to the Run Method screen.
Stop the run	Touch **STOP** to stop the protocol run immediately.
View the Events Log	Touch the status bar to display the events log.

The run method on the QuantStudio™ 12K Flex Instrument touchscreen is shown in the following graphics:

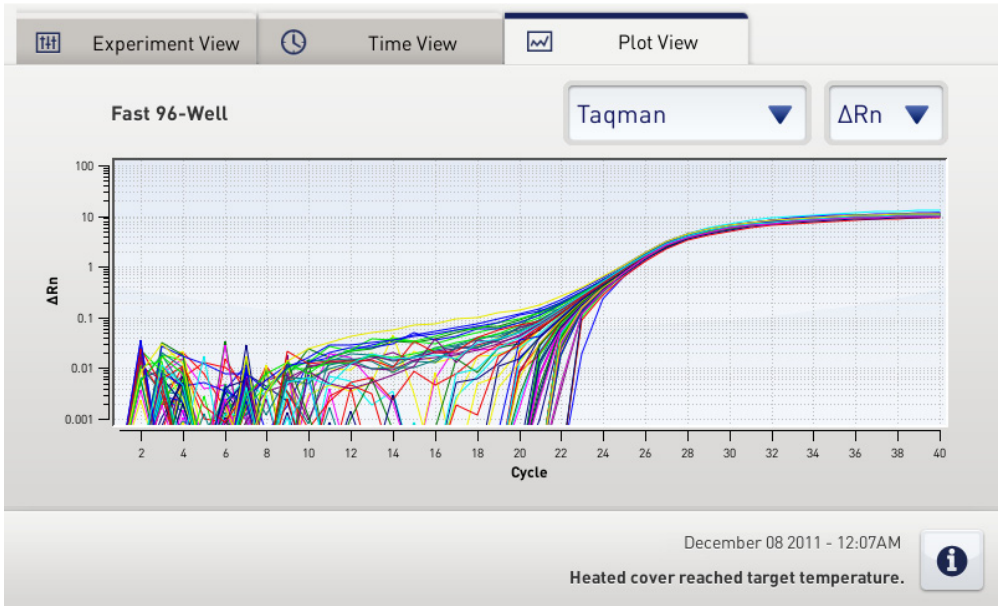
Experiment View



Time View



Plot View



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

Touch...	To...
Rn ▼	Change the data displayed on the y axis. Select either **Rn** (normalized reporter) or **ΔRn** (baseline-corrected normalized reporter).

Touch...	To...
FAM	Change the reporter dye displayed in the plot. Only dyes used in your experiment are shown.
	View the run events that occurred during the run. Touch again to close the event list..

Unload the instrument

When your QuantStudio™ 12K Flex 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. Touch on the QuantStudio™ 12K Flex Instrument touchscreen or click **Open Door** in the Instrument Console screen of the QuantStudio™ 12K Flex Software.
2. Remove the reaction plate or array card from the plate adapter.
3. Touch or click **Close Door** to retract the plate adapter back into the instrument.

   If the QuantStudio™ 12K Flex Instrument does not eject the plate, remove the plate as follows:

   a. Power off the QuantStudio™ 12K Flex Instrument.
   b. Wait for 15 minutes, then power on the QuantStudio™ 12K Flex Instrument and eject the plate.
   c. If the plate does not eject, power off and unplug the QuantStudio™ 12K Flex Instrument, then open the access door.
   d. Wearing powder-free gloves, reach into the QuantStudio™ 12K Flex Instrument and remove the plate from the heated cover, then close the access door.
   e. Perform a background calibration to confirm that the sample block has not been contaminated.

Transfer experiment results

You can transfer the experiment results in either of the following two ways:

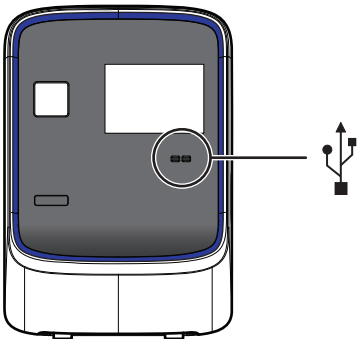
Download the experiment from the QuantStudio™ 12K Flex Instrument over the network

1. In the QuantStudio™ 12K Flex Software, select **Instrument** ▸ **Instrument Console**.
2. Select the instrument icon of the **QuantStudio™ 12K Flex Instrument** you just used to run the experiment from the My Instruments list.
3. Click **Manage Instrument** to open the Instrument Manager.
4. In the Instrument Manager, click **Manage Files**.

5. In the Experiments panel, select the experiment to download. Click **Download**.
6. In the Save dialog box, select the folder to hold the experiment results and click **Save**. The experiments folder is located at:
   <*drive*>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments\
   where, <*drive*> is the computer hard drive on which the QuantStudio™ 12K Flex Software is installed. The default installation drive for the software is the C: drive.

Transfer the experiment from the QuantStudio™ 12K Flex Instrument to the computer via a USB drive:

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



2. Touch the QuantStudio™ 12K Flex Instrument touchscreen to awaken it.
3. If the touchscreen is not at the Main Menu screen, touch  .
4. In the Main Menu, touch  **Collect Results** to save the data to the USB drive.
5. Select one or multiple experiments (by touching them). Then touch  **Save to USB** to copy selected 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.
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:
   <*drive*>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments\

where <*drive*> is the computer hard drive on which the QuantStudio™ 12K Flex Software is installed. The default installation drive for the software is the C: drive.

Review experiment results

About analysis results

Immediately after a run, the QuantStudio™ 12K Flex 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** and select the **Auto Analysis** check box.

Note: For Genotyping experiments, the QuantStudio™ 12K Flex Software displays the Allelic Discrimination Plot screen.

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

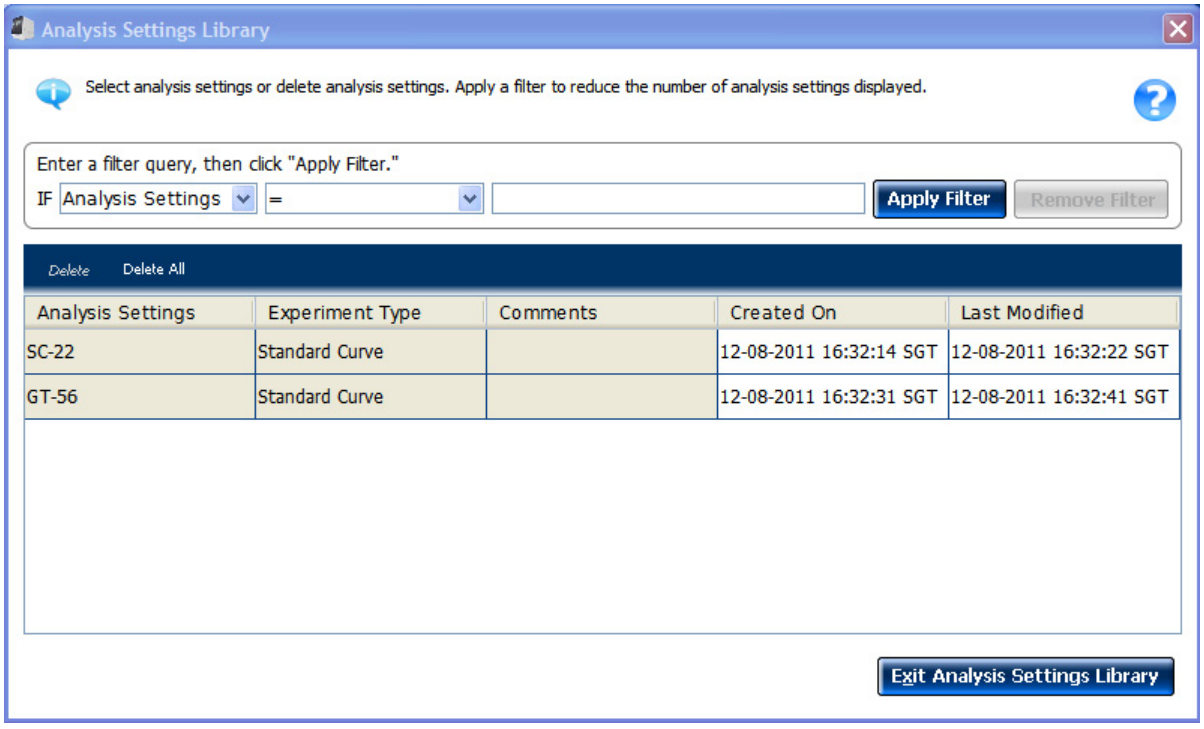
About the Analysis Settings Library

Analysis Settings are different for each experiment type. If the default analysis settings in the 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.

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.

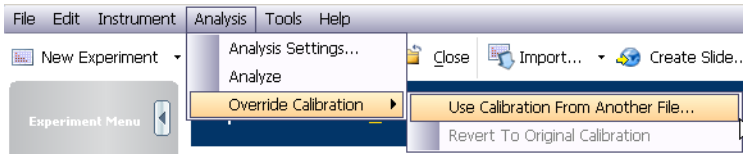
To override calibration

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

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

To use the calibration data of another experiment

1. Open the experiment file (.eds), in which you want to import the calibration data from another QuantStudio™ 12K Flex Instrument, in the QuantStudio™ 12K Flex Software.
2. Go to **Analysis** ▸ **Override Calibration** ▸ **Use Calibration From Another File....**

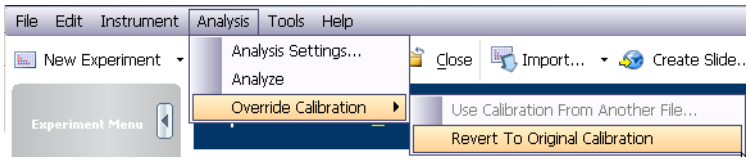


3. Browse to experiment file (.eds) from which you want to use the calibration data.

   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**.

To 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. Go to **Analysis** ▸ **Override Calibration** ▸ **Revert To Original Calibration.**



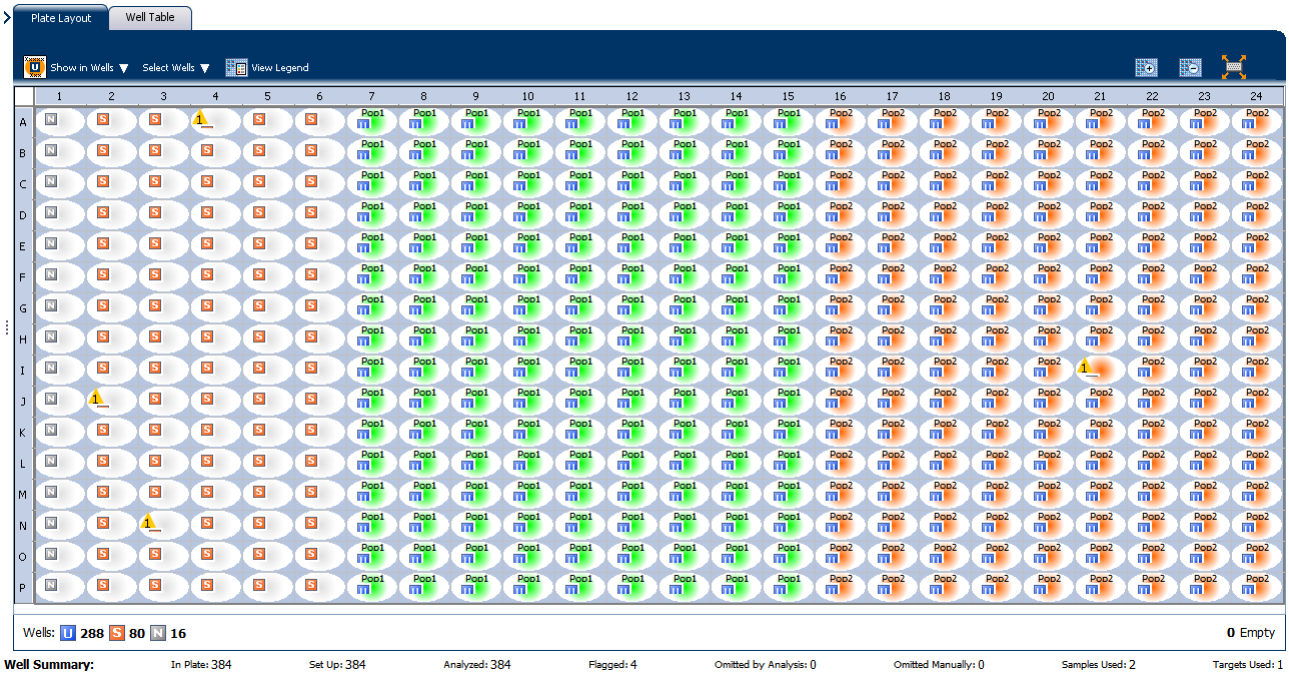
The experiment file will display analysis results as per the calibration data of the QuantStudio™ 12K Flex Instrument that the experiment was run on.

To 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 drop-down menus: 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 **Ctrl-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:

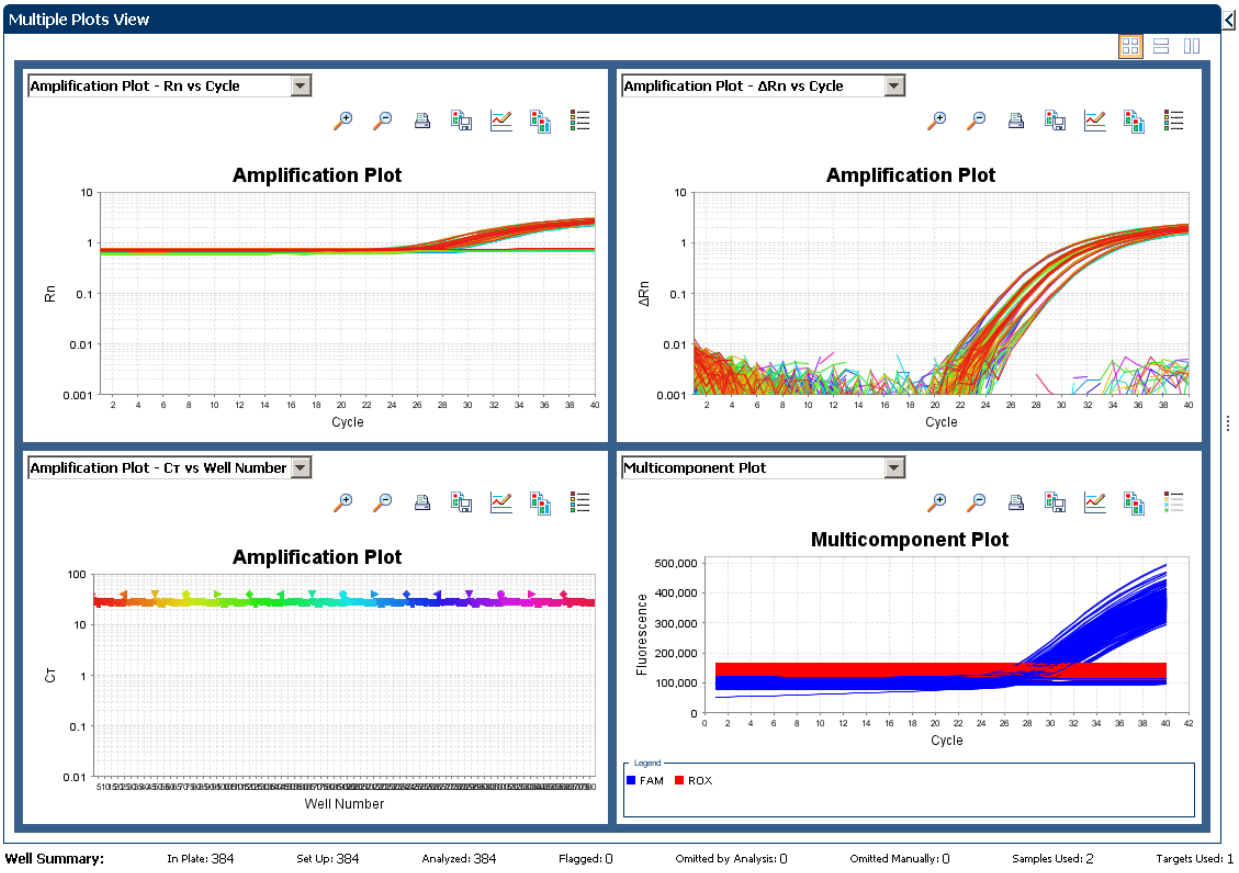


To display multiple plots

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**.
- Similarly, to display two plots in rows, click **Show plots in two rows**. and to display two plots vertically, click **Show plots in two columns**.
- To display a specific plot, select the plot from the drop-down menu above each plot display.

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



To 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.

To edit plot properties

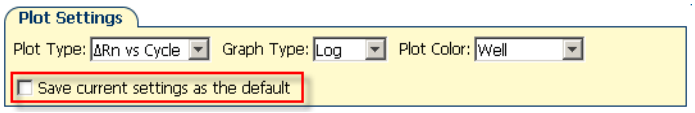
Use the Plot Properties dialog box on the Analysis screen to edit plot settings such as the font and color of the plot text, and the labels on the X axis and Y Axis.

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, X Axis, and 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**.

To 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** check box on the respective plot screens under the Analysis Experiment Menu.



To publish the analyzed data

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

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

Export an experiment

About exporting an experiment

The Export feature of 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

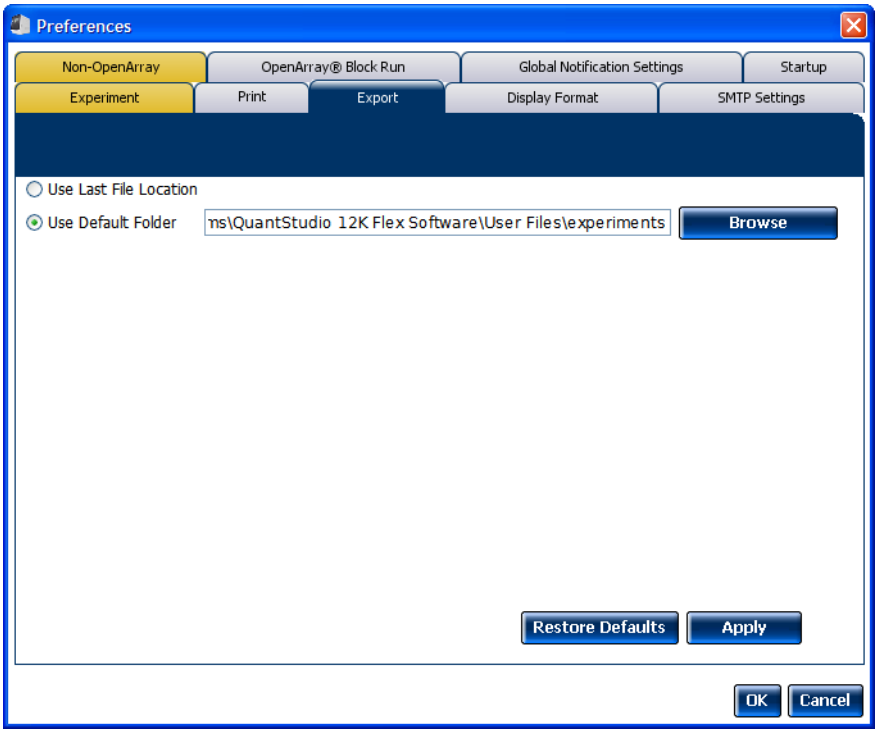
Note: If you choose the Auto Export option during experiment setup or before running an experiment, the data is automatically exported to the location you specified. If you did not set the Auto Export option, the analyzed data is not exported automatically.

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 data).
   - **7900 format** - Single experiments are exported in the SDS 2.4 detector centric export format of the 7900 Sequence Detecting System.
     The 7900 format supports only the .txt type of data.
   - **RDML format** - Real Time Data Markup Language (supports only .xml type of data).
3. Select 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, *.xlsx or *.txt) to export from the **File Type** drop-down menu.

     Note: You cannot use an exported ***.xls** or an ***.xlsx** file when importing plate setup information.
4. *(Optional)* Select the **Open file(s) when export is complet**e check box 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**. Click **Browse** if you do not want to save the export file in the default export folder.

Note: To set up the Export File Location, go to **Tools** ▸ **Preferences**, and select the **Export** tab. You can select the **Use Last File Location** or **Use Default Folder** check box.



6. Select the data to export:

Select...	To 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.

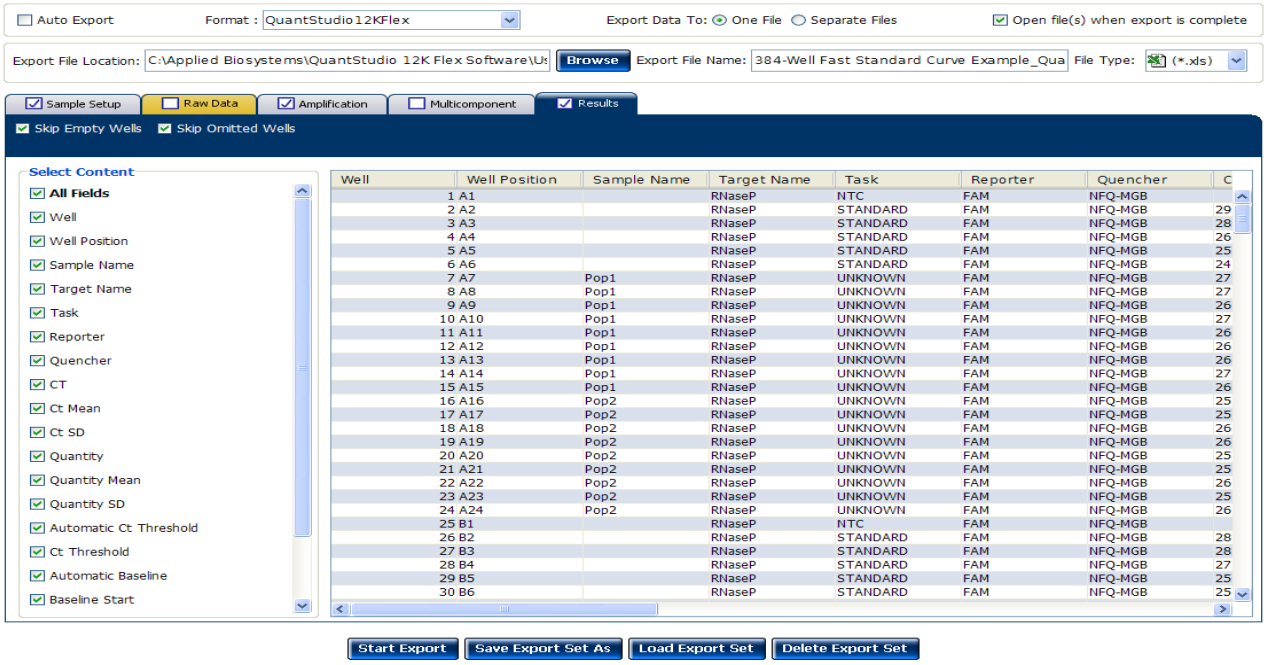
7. (*Optional*) For Standard Curve experiments, select the external application, **CopyCaller® Software** if the Software is installed on your computer.

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 the external Applied Biosystems application, **CopyCaller® Software** for further analysis.

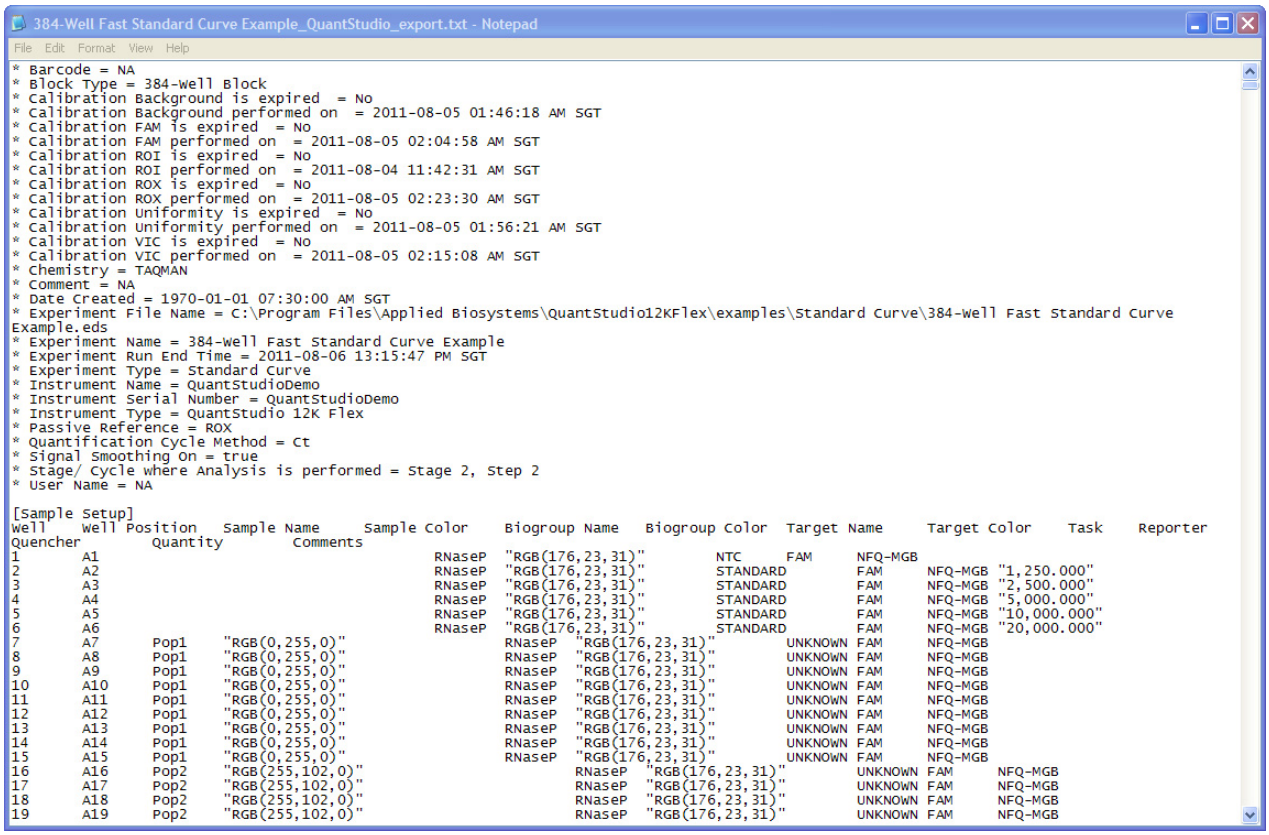
9. Click **Start Export**.

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



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

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 59
- QuickStart an experiment 60
- Import plate setup for an experiment 62
- Import sample information 63
- Use a template to create a batch of experiments 66
- Create an experiment using ReadiApp 68

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 QuantStudio™ 12K Flex Instrument touchscreen.

Note: To access the QuantStudio™ 12K Flex Software example templates, navigate to the templates folder located at <drive>:\Program Files\Applied Biosystems\QuantStudio12KFlex\templates.

To create a template

1. Log in to the QuantStudio™ 12K Flex Software and, from the 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 10.

2. Select **File** ▸ **Save As Template**.
3. Enter a file name, select a location for the template, then click **Save** and **Close**.

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

To 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.

To create an experiment using a template on the QuantStudio™ 12K Flex Instrument touchscreen

You can run experiments using templates from the QuantStudio™ 12K Flex 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.

To run a pre-existing template

1. Touch **View Template** on the Home screen of the QuantStudio™ 12K Flex Instrument touchscreen.
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 to go back to View Template screen. Touch **Start Run**.

To 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. Touch **Save & Exit** to save and exit the experiment or touch **Save & Start Run** to save and start an experiment run.

QuickStart an experiment

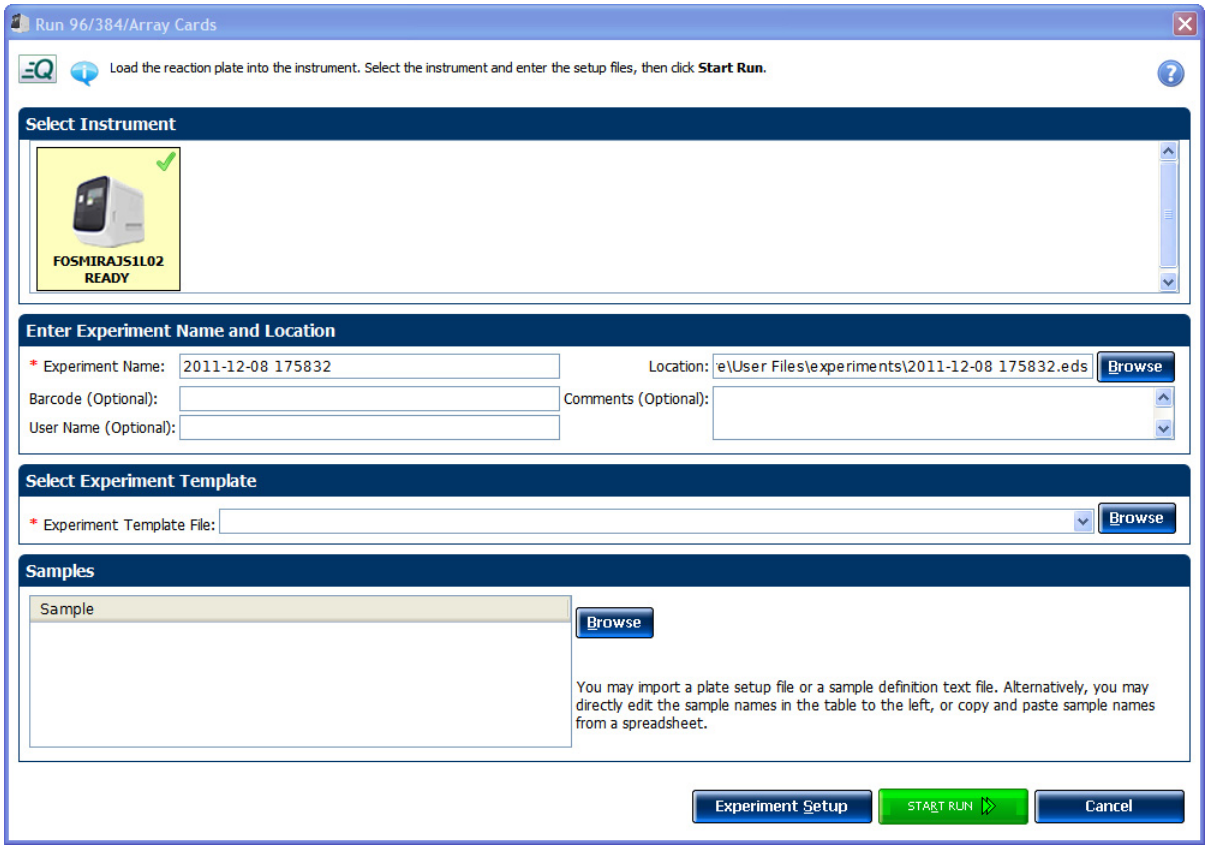
You can use a template to run an experiment with the QuantStudio™ 12K Flex Software Quickstart feature:

QuickStart from the QuantStudio™ 12K Flex Software

1. Prepare the reactions.
2. Log in to the QuantStudio™ 12K Flex Software and, from the Home screen, click **96/384/Array Cards** to access the Run 96/384/Array Cards dialog box.
3. In the QuickStart dialog box, enter or select the:
   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.

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

   The 96/384/Array Cards dialog box looks like this:



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

QuickStart from the QuantStudio™ 12K Flex Instrument touchscreen

You can QuickStart an experiment from the QuantStudio™ 12K Flex 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 QuantStudio™ 12K Flex 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 pre-defined experiment or folder buttons.

To create a shortcut button for a preferred experiment or a folder that contains experiments:

1. Touch **Settings** to open the Settings Menu.
2. Touch **Set Up Shortcuts** to list the Shortcut Targets.
3. On the Shortcut Targets list 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:

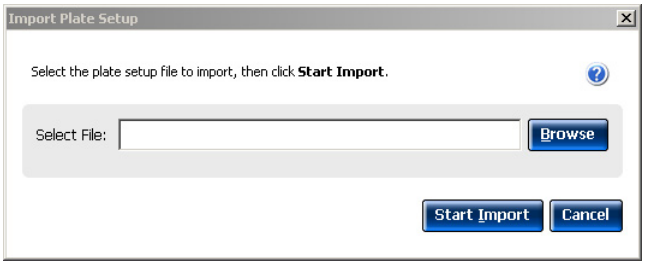
- *.txt - Text format
- *.xml - XML format
- *.csv - Comma separated values format
- *.sdt - Sequence detecting system (sds) template files format
- *.sds - 7900 v2.3 format

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 54.

To Import the plate setup data:

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 drop-down menu in the toolbar and 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 such as 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

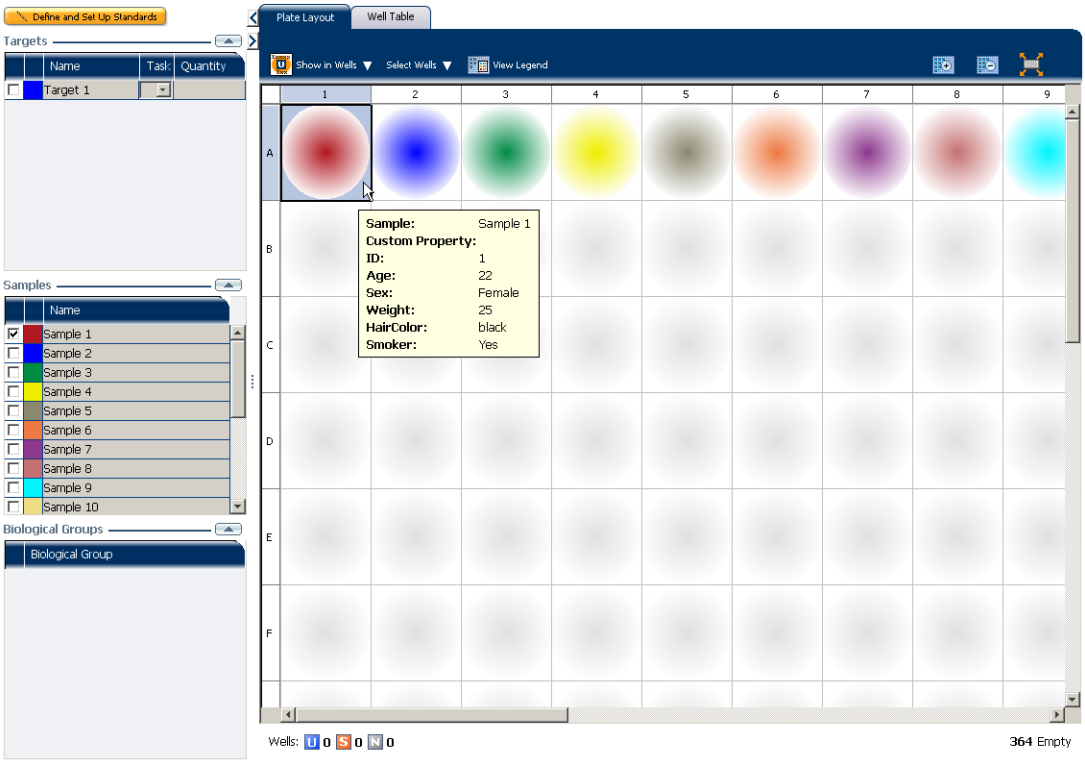
Import sample information from a sample definition file

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.

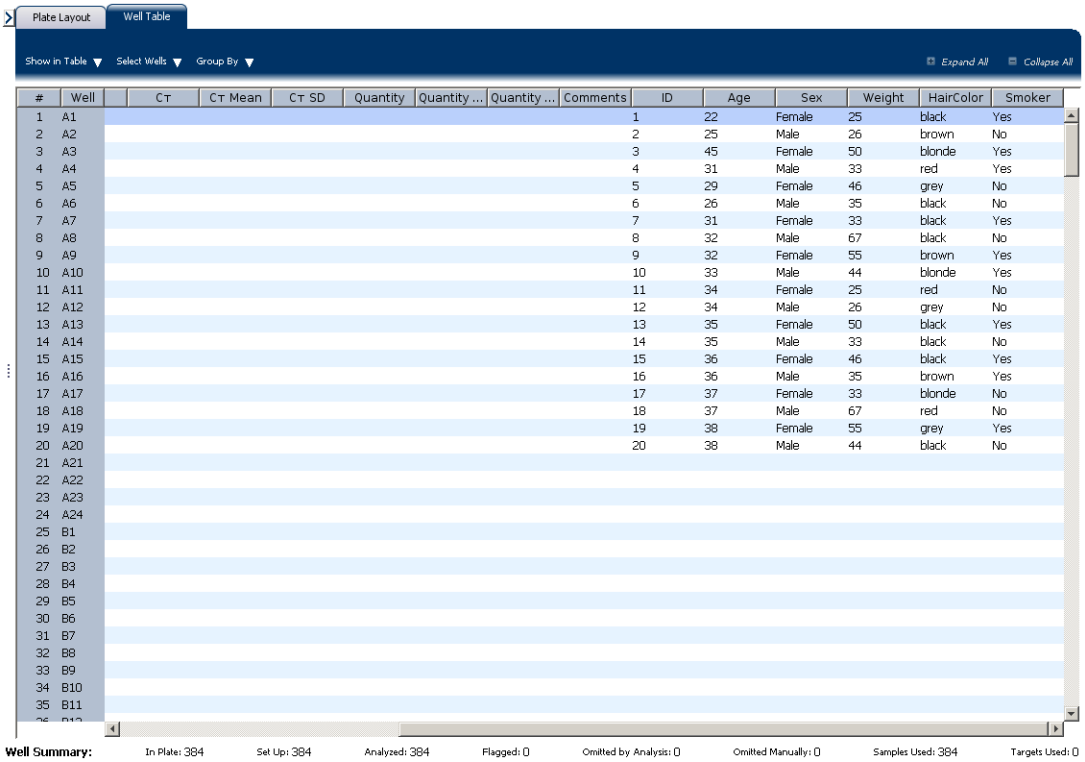
The samples appear in the Samples table for the experiment. All samples and well assignments in the experiment are replaced with those in the file. If defined, the custom sample properties also appear in the Well Table of the Analysis Section, and also in the Plate Layout tooltips in both the Setup and Analysis screens. 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:

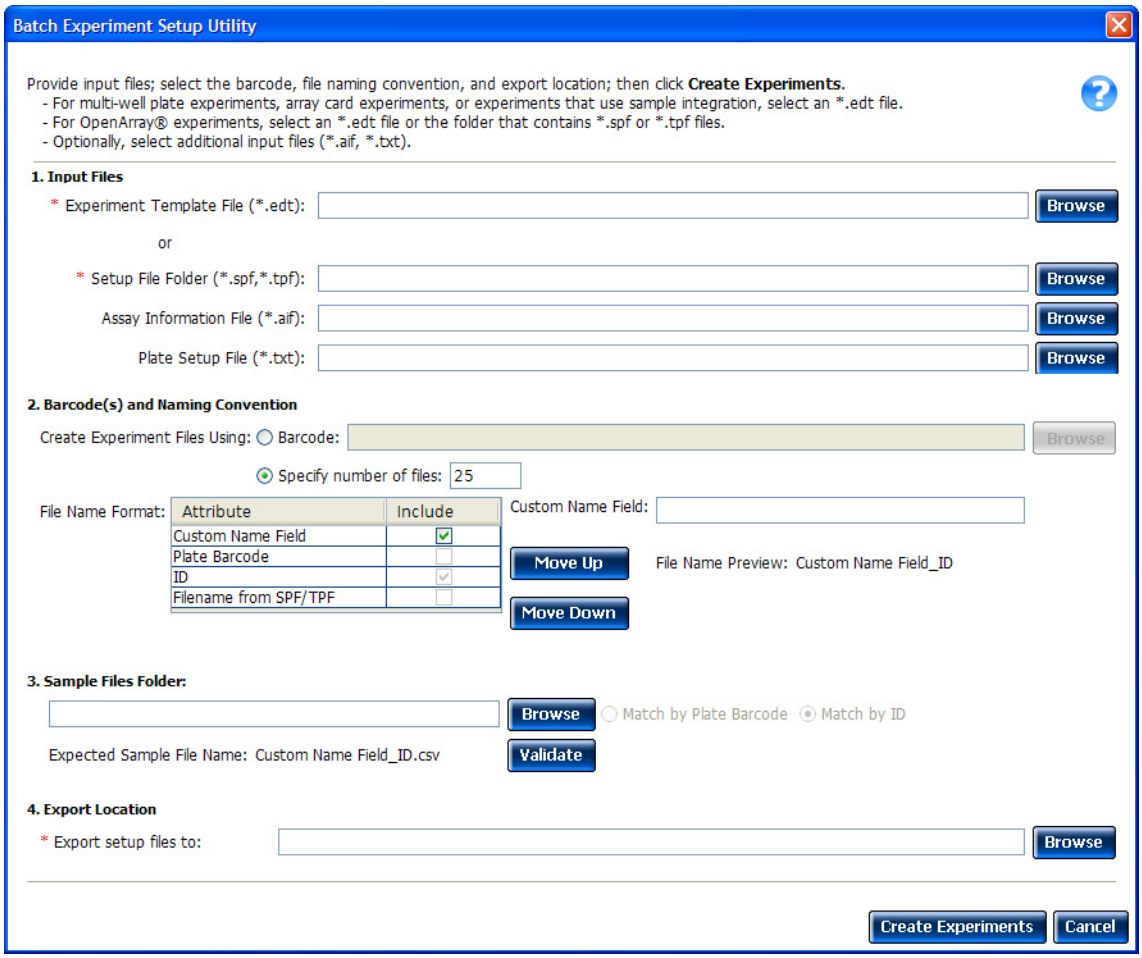


#	Well	CT	CT Mean	CT SD	Quantity	Quantity ...	Quantity ...	Comments	ID	Age	Sex	Weight	HairColor	Smoker
1	A1								1	22	Female	25	black	Yes
2	A2								2	25	Male	26	brown	No
3	A3								3	45	Female	50	blonde	Yes
4	A4								4	31	Male	33	red	Yes
5	A5								5	29	Female	46	grey	No
6	A6								6	26	Male	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 Experiment Setup.

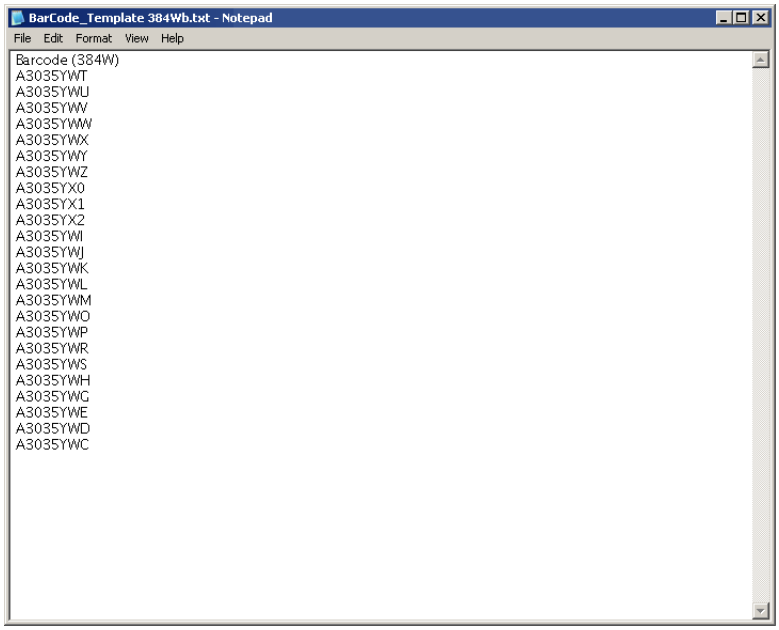
1. In the menu bar, select **Tools** ▸ **Batch Experiment Setup**.The Batch Experiment Setup Utility dialog box looks like this:



2. Select the file(s) 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 (template) or an *.spf/ *.tpf file to import, then click **Select**.
   e. *(Optional)* Repeat **steps 2a** and **2b** 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** - Enter a number from 1 to 100.
   - **Barcode** - Click **Browse** and 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. An example Barcode File looks like this:



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 and 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 then 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 appears when the batch of experiments has been created.

Create an experiment using ReadiApp

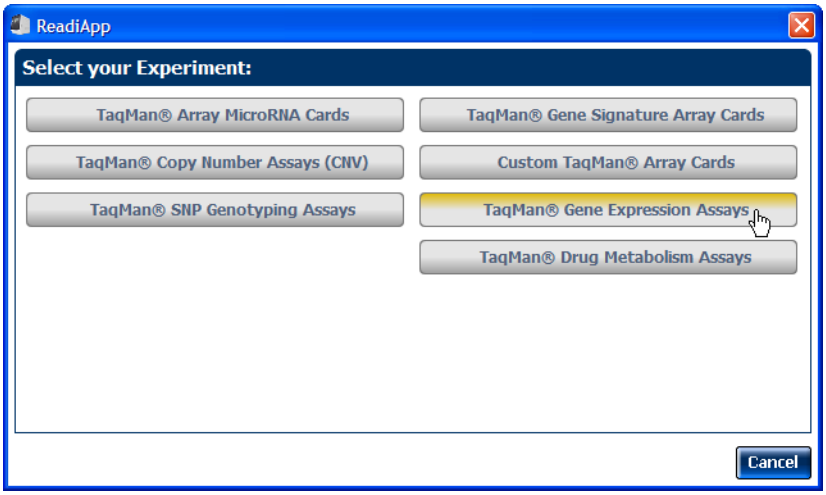
You can use the ReadiApp feature to set up an experiment in the QuantStudio™ 12K Flex Software. The ReadiApp feature provides a shortcut to create experiments for the assays purchased from Life Technologies.

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

- TaqMan® Gene Signature Array Cards
- Custom TaqMan® Array Cards
- TaqMan® Gene Expression Assays
- TaqMan® Drug Metabolism Assays
- TaqMan® array MicroRNA Cards
- TaqMan® Copy Number Assays (CNV)
- TaqMan® SNP Genotyping Assays

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.

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GETTING STARTED GUIDE

applied biosystems by life technologies™

Booklet 2 - Running Standard Curve Experiments

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life technologies™

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1 About Standard Curve Experiments

This chapter covers:

- Before you begin 5
- About the example experiment 6

IMPORTANT! First-time users of the QuantStudio™ 12K Flex System please read Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* and Booklet 7, *QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes* of this binder thoroughly. The booklets provide information and general instructions that are applicable to all the experiments described in this binder

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.

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 and multiplex PCR (page 6)

  and
- 1-step and 2-step RT-PCR (page 6)

Singleplex and Multiplex PCR

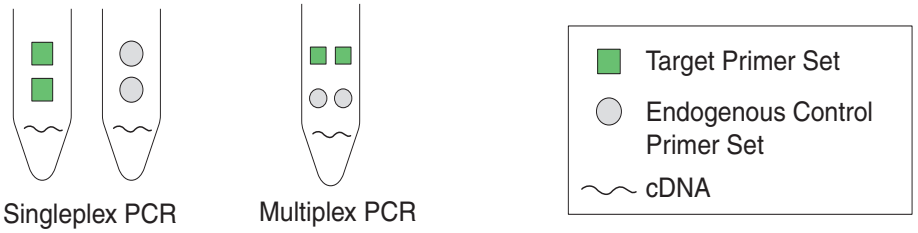
You can perform a PCR reaction using either:

- **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. Typically, a probe labeled with FAM™ dye detects the target and a probe 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 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.

  Note: 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 Life Technologies 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 (PN 4331348).

2 Design the Experiment

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

This chapter covers:

- Define the experiment properties. 9
- Define targets, samples, and biological replicates 10
- Assign targets, samples, and biological groups 11
- Set up the run method 13
- For more information 15

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 on Auto Export, refer to Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.

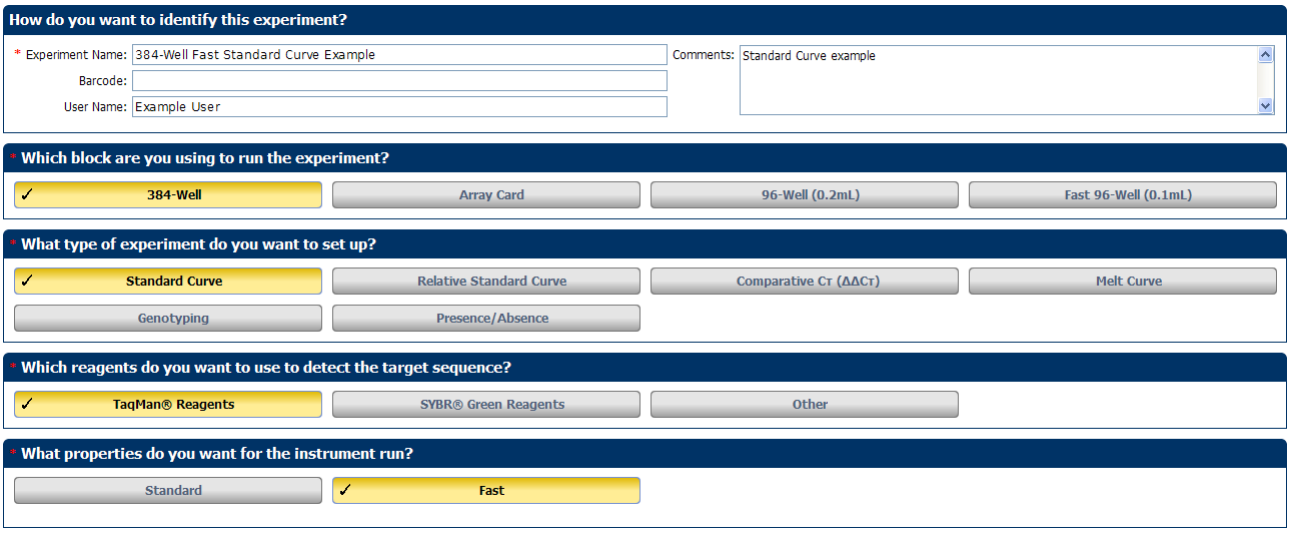
Define the experiment properties

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

Field or selection	Entry
Experiment Name	384-Well Fast Standard Curve Example
Barcode	Leave field empty
User Name	Example User
Comments	Standard Curve example
Block	384-Well
Experiment Type	Standard Curve
Reagents	TaqMan® Reagents
Ramp speed	Fast

Save the experiment.

Your Experiment Properties screen should look like this:



Define targets, samples, and biological replicates

Click **Define** to access the Define screen. Enter:

1. Targets

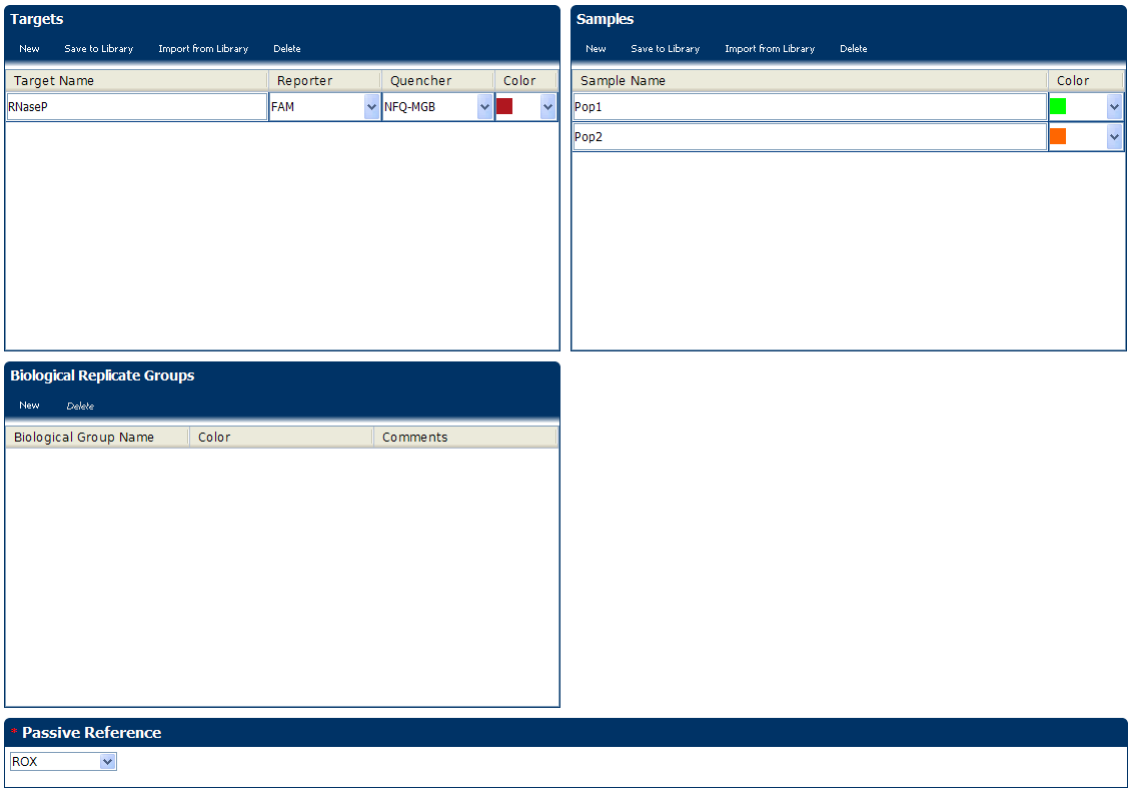
Target name	Reporter	Quencher	Color
RNaseP	FAM	NFQ-MGB	

2. Samples

Sample name	Color
Pop1	
Pop2	

3. Dye to be used as a Passive Reference
ROX

Your Define screen should look like this:



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

Assign targets, samples, and biological groups

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

1. Define and set up standards.
   a. Click **Define and Set Up Standards** on the Assign screen.
   b. Select a target.

Field	Select
Select a target for this standard curve	RNaseP

   c. Define the standard curve.

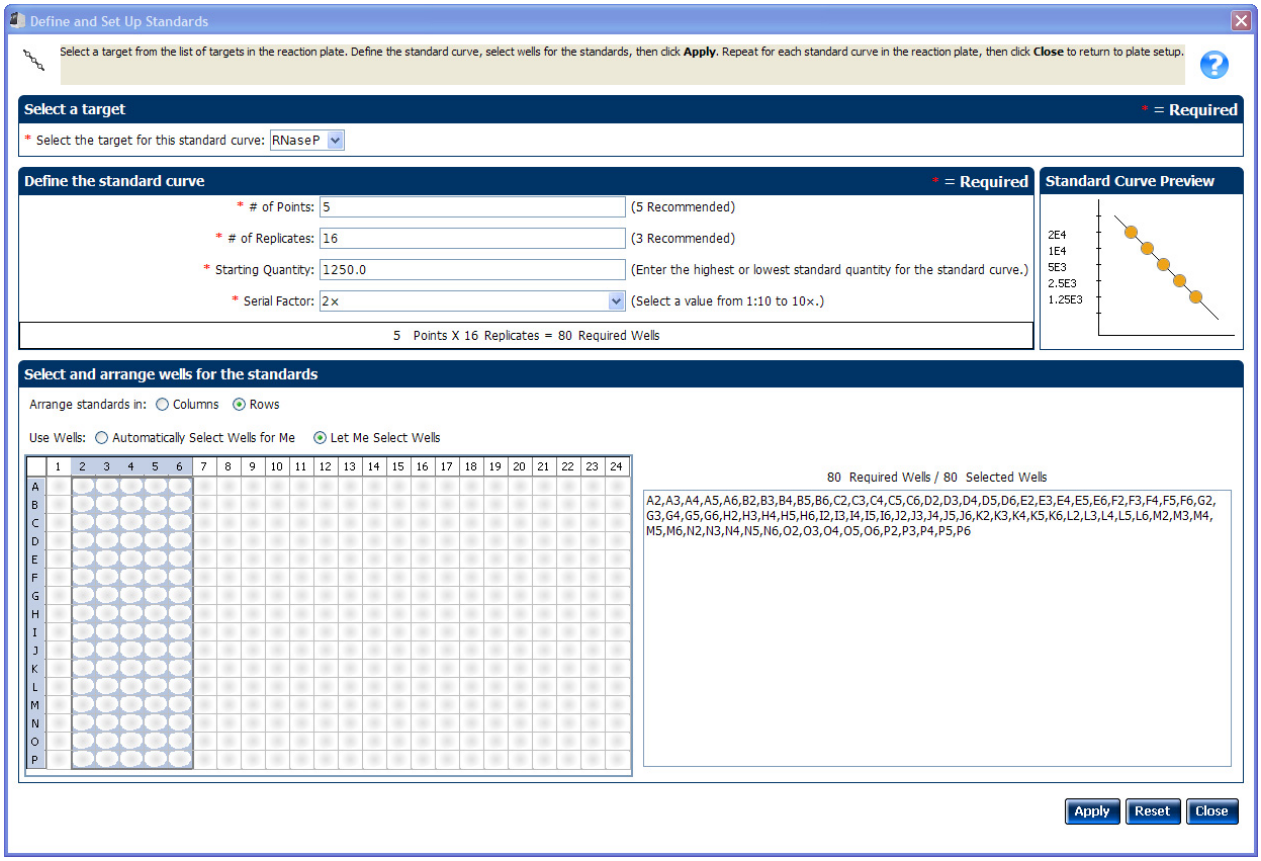
Field	Enter
# of Points	5
# of Replicates	16
Starting Quantity	1250.0
Serial Factor	2x

d. Select and arrange wells for the standards.

Field	Select
Use Wells	Let Me Select Wells

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

Your Define and Set Up Standards dialog box should look like this:

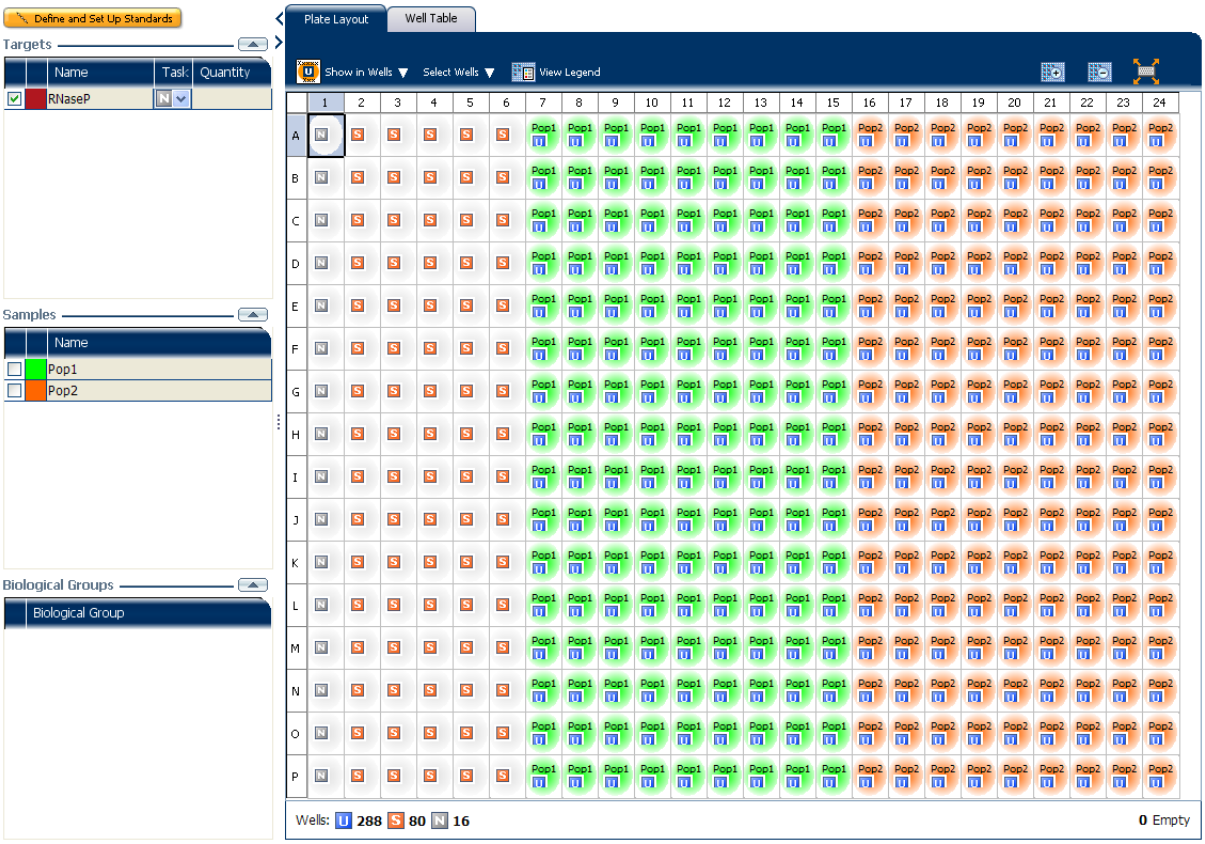


2. Assign targets and samples.

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

Target name	Well number	Task	Quantity	Sample name
RNaseP	A6 - P6 (column 6)	Standard	20000	None
RNaseP	A7 - P15 (columns 7 -15)	Unknown	Determined by run	Pop1
RNaseP	A16 - P24 (columns1 6 - 24)	Unknown	Determined by run	Pop2

Your Assign screen should look like this:



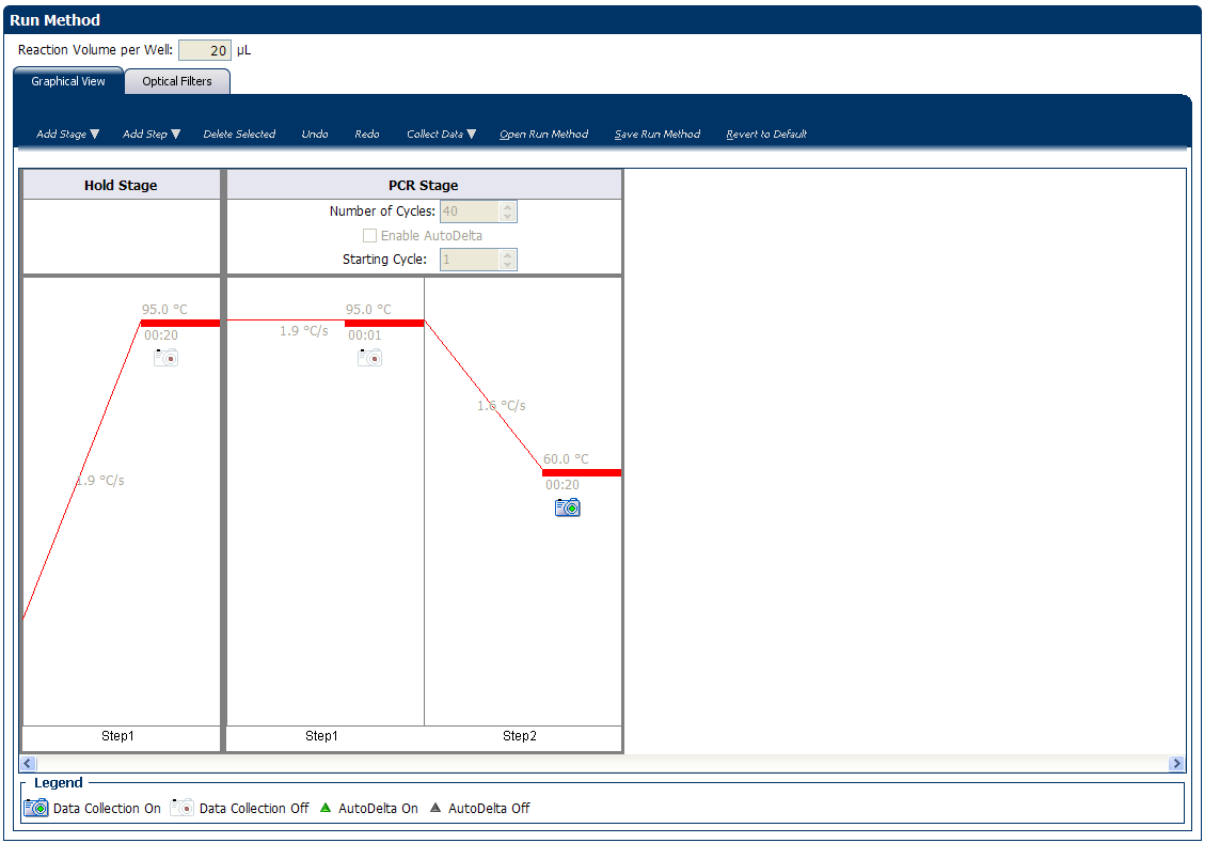
Set up the run method

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

- 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

Your Run Method screen should look like this:



For more information

For more information on...	Refer to...	Part number
Consumables	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* Appendix A in Booklet 7, *QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes*	4470050
Using other quantification methods	Booklet 3, *Running Relative Standard Curve and Comparative C_T Experiments*.	4470050
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*	127AP05-03
Using alternative setup	Chapter 2 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

3 Prepare the Reactions

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

This chapter covers:

- Assemble required materials 17
- Prepare the sample dilutions 17
- Prepare the standard dilution series 18
- Prepare the reaction mix (“cocktail mix”). 19
- Prepare the reaction plate 19
- For more information. 22

Assemble required materials

- Items listed in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.
- Samples - Human Raji cell line-derived cDNA samples (100 ng/µL)
- Example experiment reaction mix components:
  - **TaqMan® Fast Universal PCR Master Mix**
  - RNase P Assay Mix (20×) (PN 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 (ng/µL)	Sample volume (µL)	Diluent volume (µL)	Total volume of diluted sample (µL)
Pop1	100.0	25	355	380
Pop2	100.0	12.5	367.5	380

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 (µL)
1	Pop 1	355
2	Pop 2	367.5

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

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

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

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.

   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 ("cocktail 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 (µL)	Volume for 384 reactions + 10% excess (µL)
TaqMan® Fast Universal PCR Master Mix Kit	5	2112
RNase P Assay (20X)	0.5	211.2
Water	3.5	1478.4
Total reaction mix volume	9	3801.6

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

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

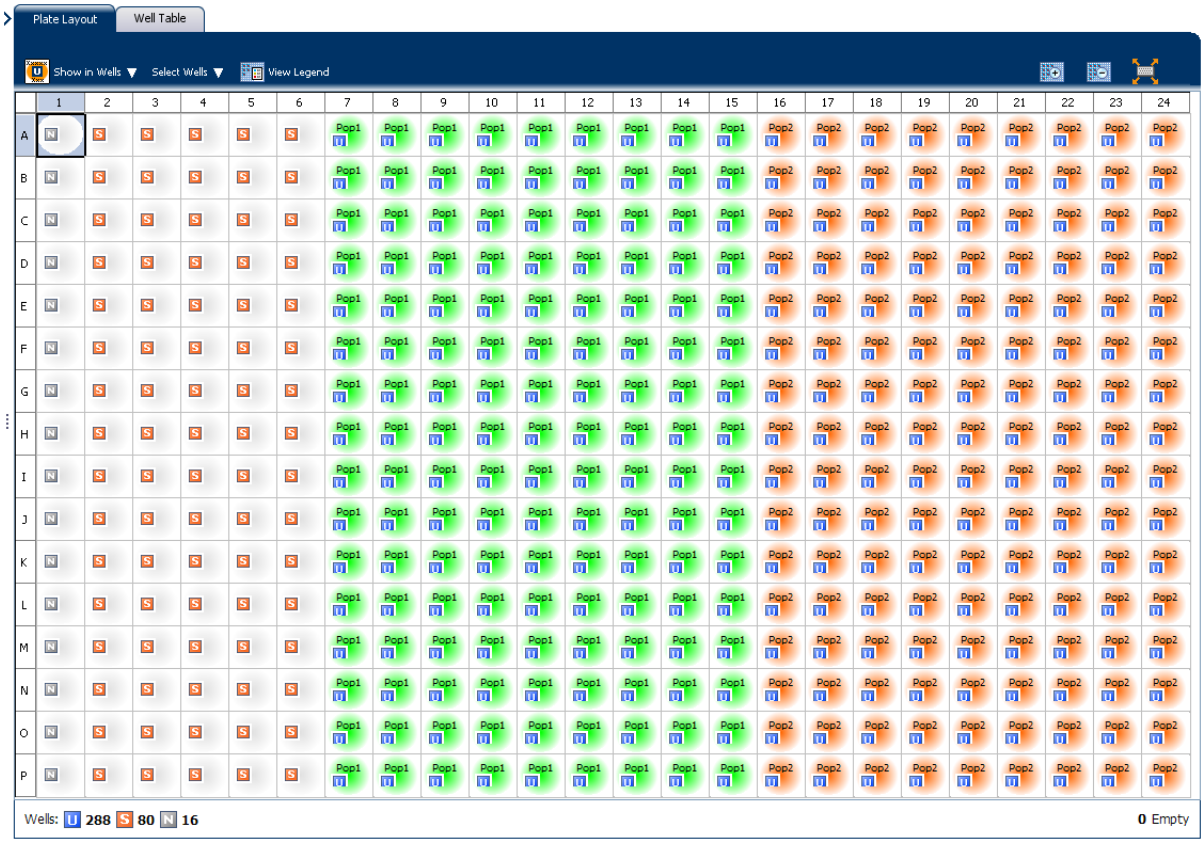
Prepare the reaction plate

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

The plate layout looks like this:



To 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 (µL) (includes 10% excess)	Water volume (µL) (includes 10% excess)
1	RNase P reaction mix	157.5	17.5

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

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 (µL) (includes 10% excess)	Sample	Sample volume (µL)
1	RNase P pop1	RNase P reaction mix	1422	pop1	158
2	RNase P pop2	RNase P reaction mix	1422	pop2	158

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

For more information on...	Refer to...	Part number
Assigning the reaction plate components	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

4 Run the Experiment

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

This chapter covers:

- Start the run 23
- Monitor the run 23

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 QuantStudio™ 12K Flex Instrument is in operation.

Start the run

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

Monitor the run

Monitor the example experiment run:

- From the QuantStudio™ 12K Flex Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio™ 12K Flex Software (to monitor an experiment started from another computer or from the QuantStudio™ 12K Flex Instrument touchscreen).
- From the QuantStudio™ 12K Flex Instrument touchscreen.

From the Instrument Console of the QuantStudio™ 12K Flex Software

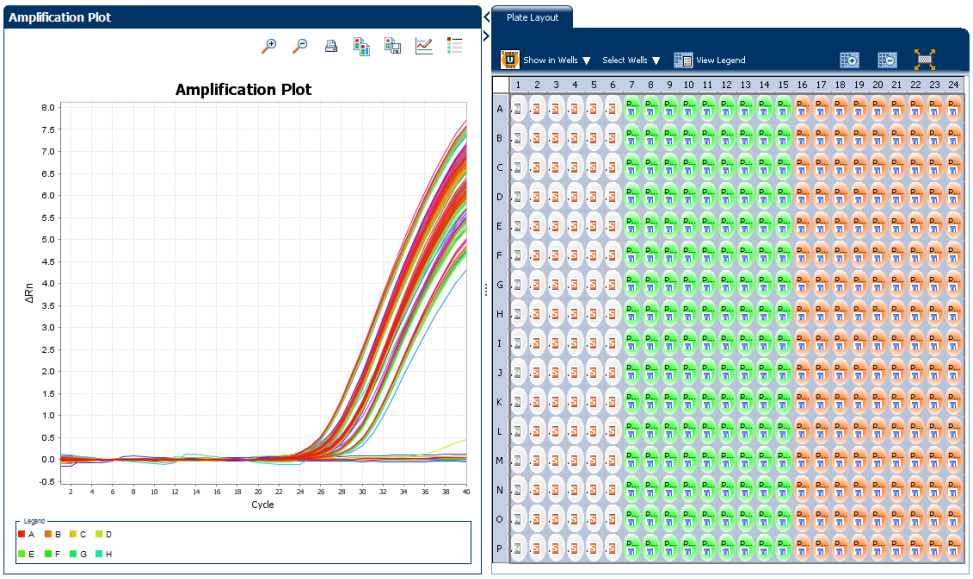
1. In the Instrument Console screen, select the instrument icon.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. On the Manage Instrument screen, click **Monitor Running Experiment** to access the Run screen.

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.

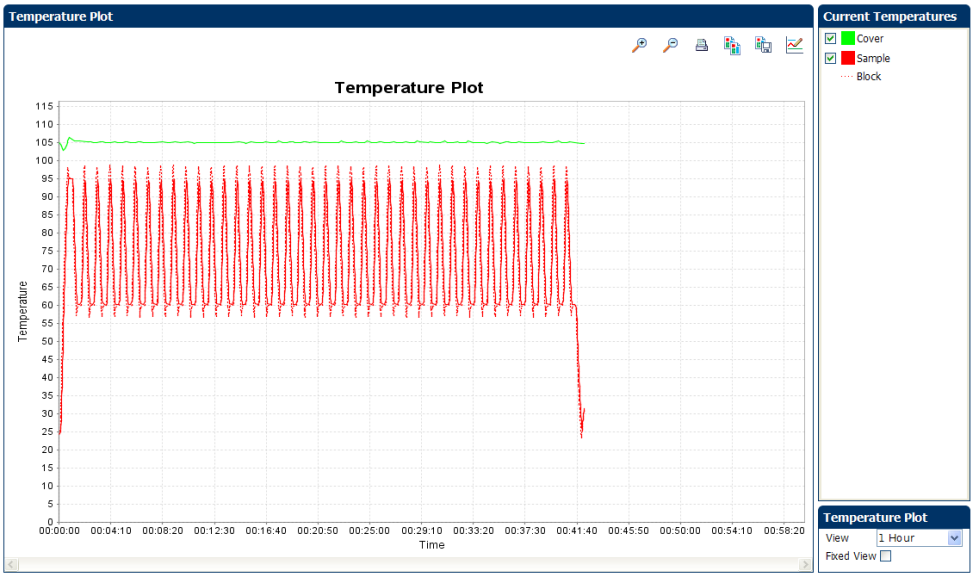
The figure below shows the Amplification Plot screen as it appears at the end of the example experiment.



View the Temperature Plot

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

The figure below shows the Temperature Plot screen as it appears during the example experiment.

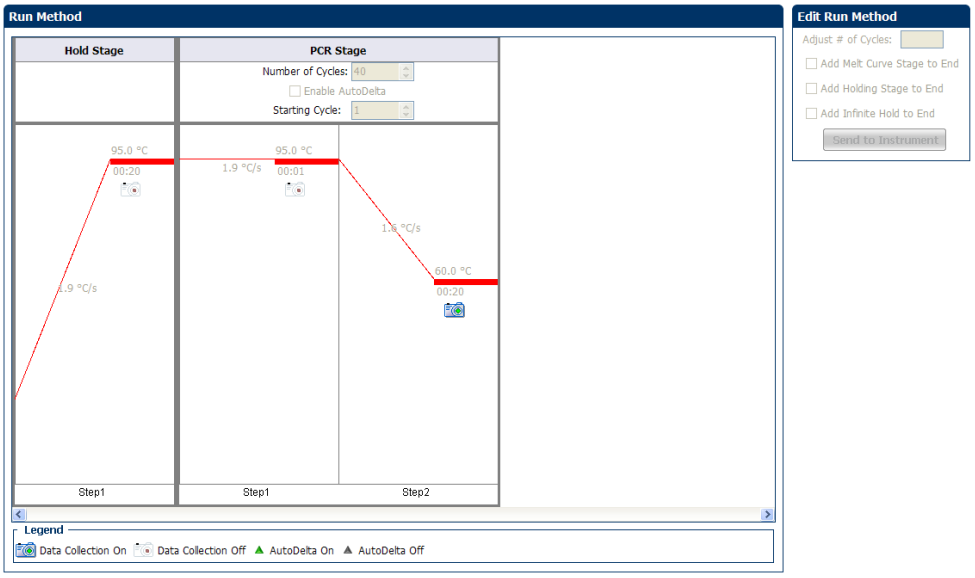


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

View the Run Method

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

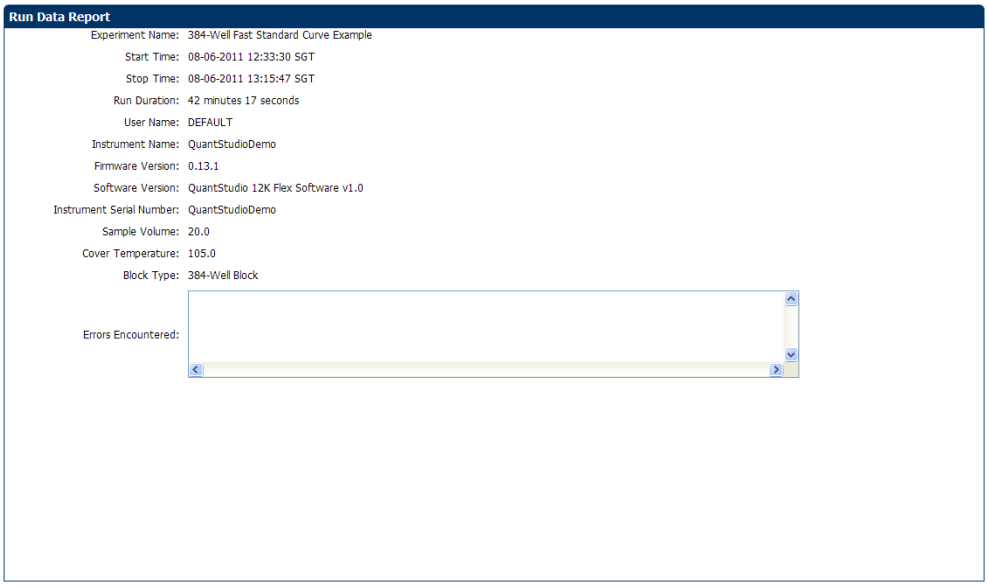
The figure below shows the Run Method screen as it appears in the example experiment.



View the run data

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

The figure below shows the View Run Data screen as it appears in the example experiment.

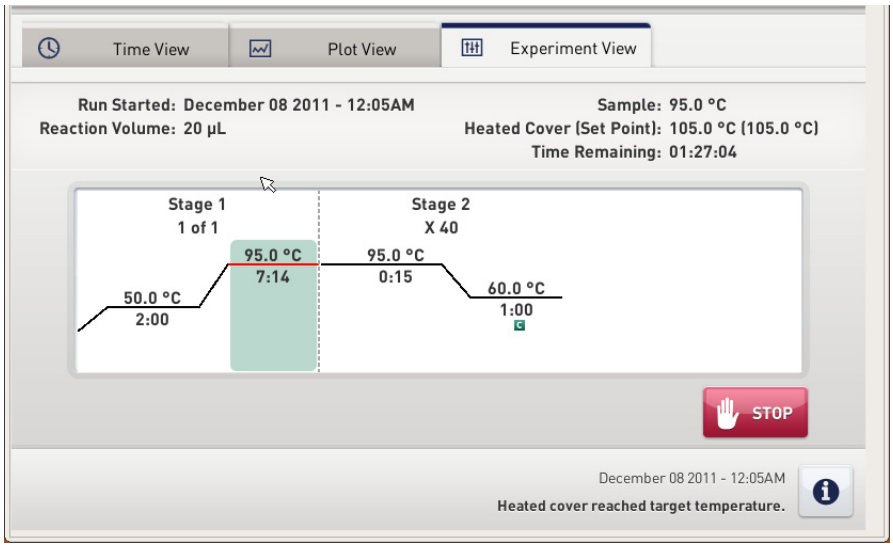


From the QuantStudio™ 12K Flex Instrument touchscreen

You can also view the progress of the run from the touchscreen of the QuantStudio™ 12K Flex Instrument.

The Run Method screen on the QuantStudio™ 12K Flex Instrument touchscreen looks like this:

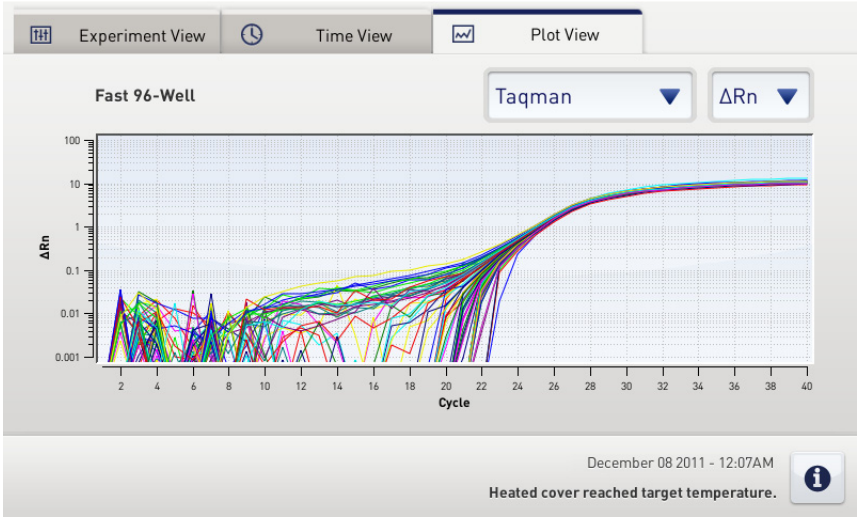
Experiment View



Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

Time View

Plot View


Experiment View
Time View
Plot View
Fast 96-Well
Taqman
ΔRn
ΔRn
100
10
1
0.1
0.01
0.001
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Cycle
December 08 2011 - 12:07AM
Heated cover reached target temperature.

5 Review Results and Adjust Experiment Parameters

In Section 5.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 5.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

Section 5.1 Review Results 31

- Analyze the example experiment 31
- View the Standard Curve Plot 31
- Assess amplification results using the Amplification Plot 33
- Identify well problems using the Well Table 40
- Confirm accurate dye signal using the Multicomponent Plot 43
- Determine signal accuracy using the Raw Data Plot 45
- Review the flags in the QC Summary 47
- For more information 48

Section 5.2 Adjust parameters for re-analysis of your own experiments 49

- Adjust analysis settings 49
- Improve C_T precision by omitting wells 53
- For more information 54

Section 5.1 Review Results

Analyze the example experiment

1. Open the example experiment file that you ran in Chapter 4.
2. Click **Analyze**. The software analyzes the data using the default analysis settings.

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

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:

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

To view and assess the Standard Curve Plot

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

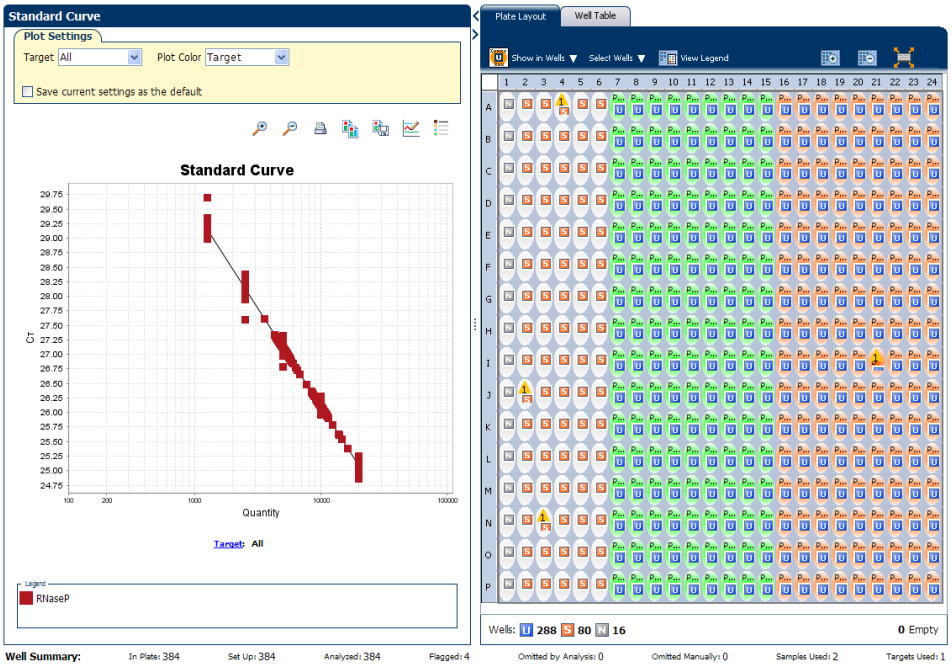
   Note: If no data are displayed, click **Analyze.**
2. Display all 384 wells in the Standard Curve Plot screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.
3. Enter the Plot Settings:

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)

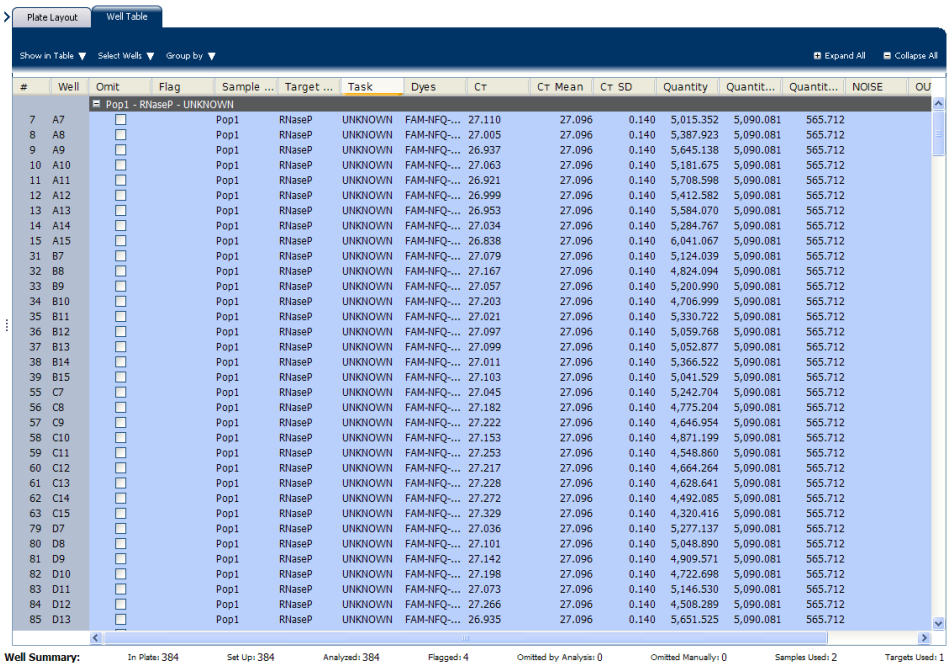
4. View the values displayed below the standard curve.

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).



Tips for analyzing your own experiments

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

- **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. Factors that affect amplification efficiency:
  - Range of standard quantities – For accurate and precise efficiency measurements, use a broad range of standard quantities, 5 to 6 logs (10^5 to 10^6 fold).
  - Number of standard replicates – For accurate efficiency measurements, include replicates to decrease the effects of pipetting inaccuracies.
  - PCR inhibitors – PCR inhibitors in the reaction can reduce amplification efficiency.
- **R^2 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 C_T precision by omitting wells" on page 53).

  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. From the Experiment Menu pane, select **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:

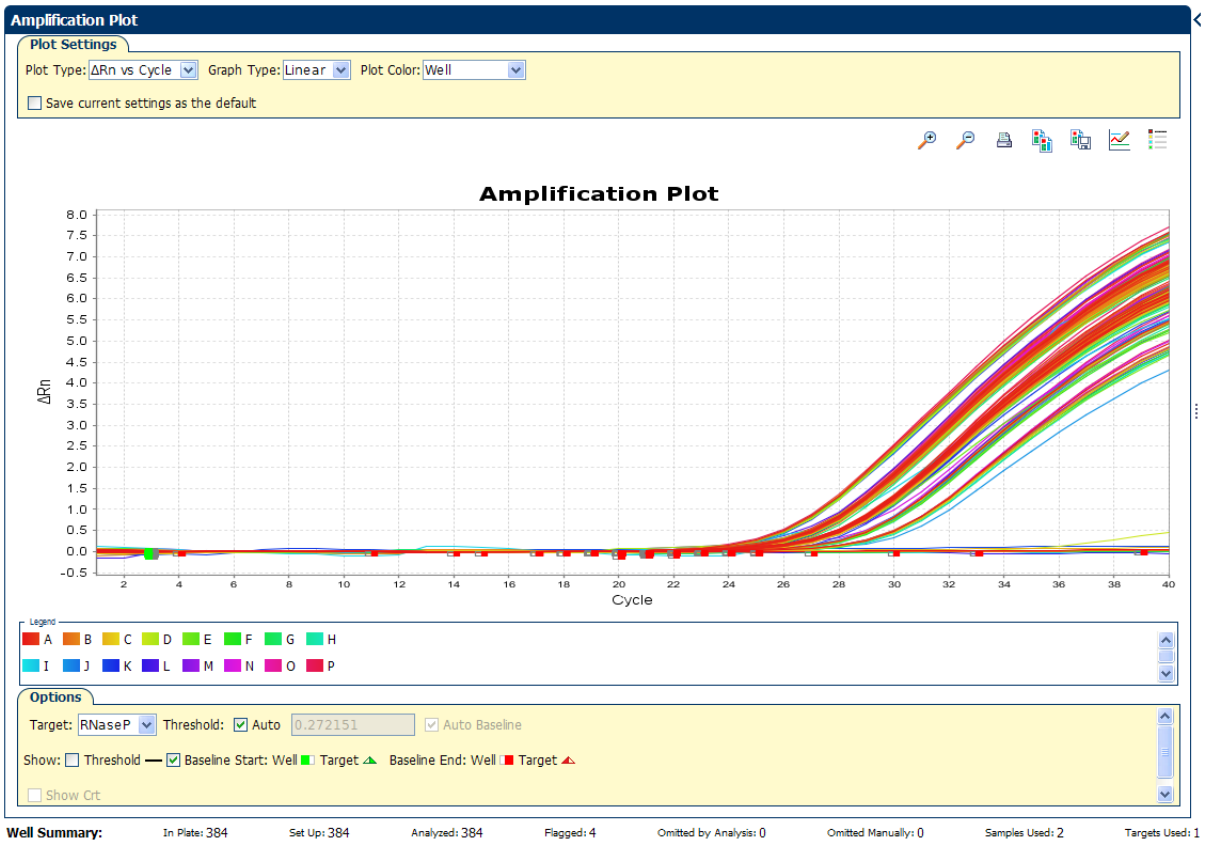
Menu	Selection
Select Wells With	**Target ▸ RNaseP**

3. In the Amplification Plot screen, enter:

Menu	Select
Plot Type	**ΔRn vs Cycle**
Plot Color	**Well** (default)

Menu	Select
 (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 drop-down menu, select **Linear**.
   b. Select the **Baseline** check box to show the start cycle and end cycle.
   c. Verify 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.

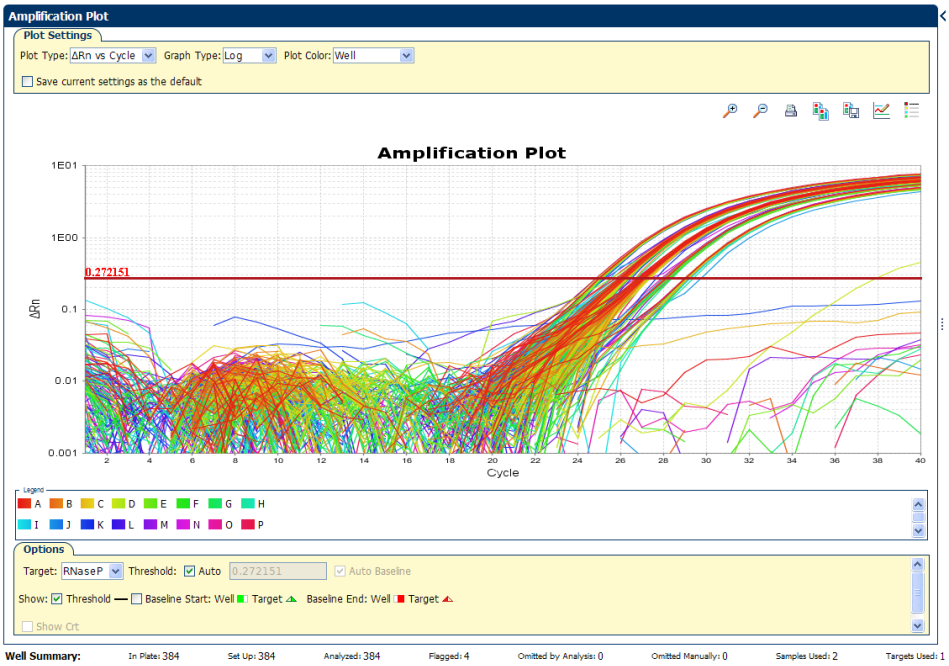


5. View the threshold values.

Menu	Select
Graph Type	Log
Target	RNaseP

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

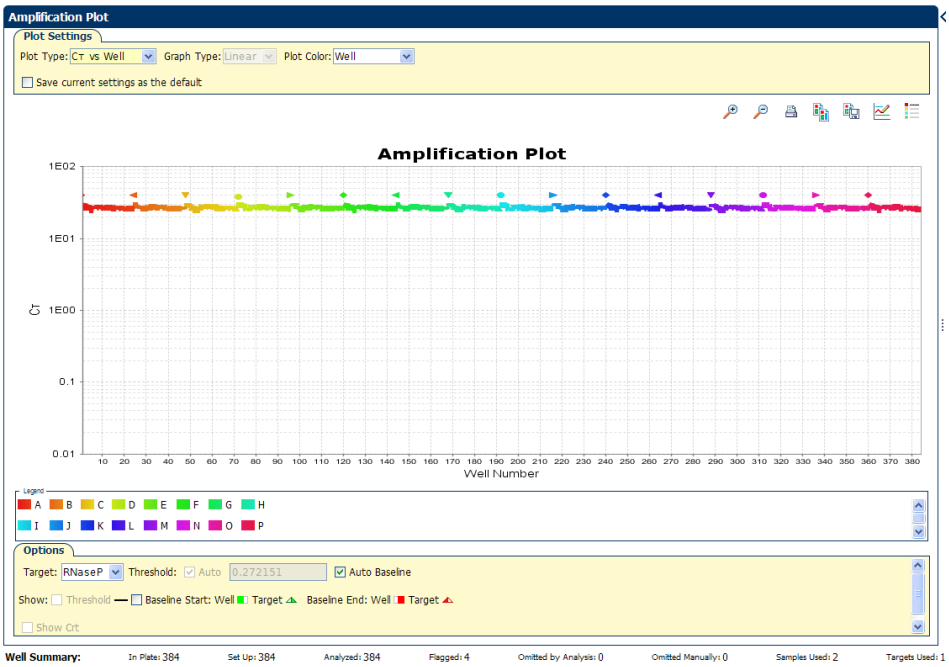
b. Verify that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.



6. Locate outliers:

   a. From the Plot Type drop-down menu, select **C_T vs Well**.

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

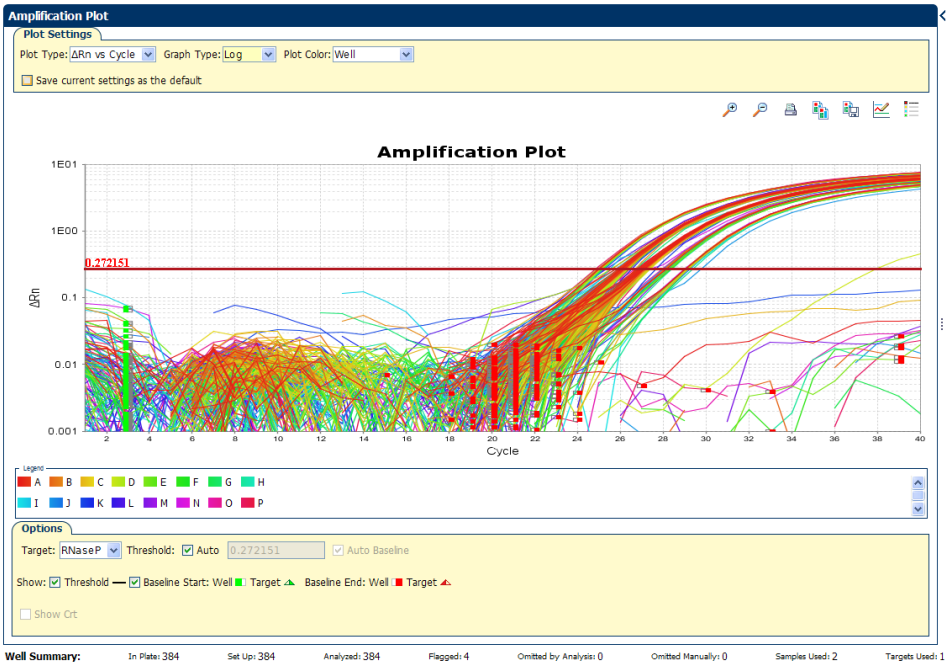


Tips for analyzing your own experiments

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

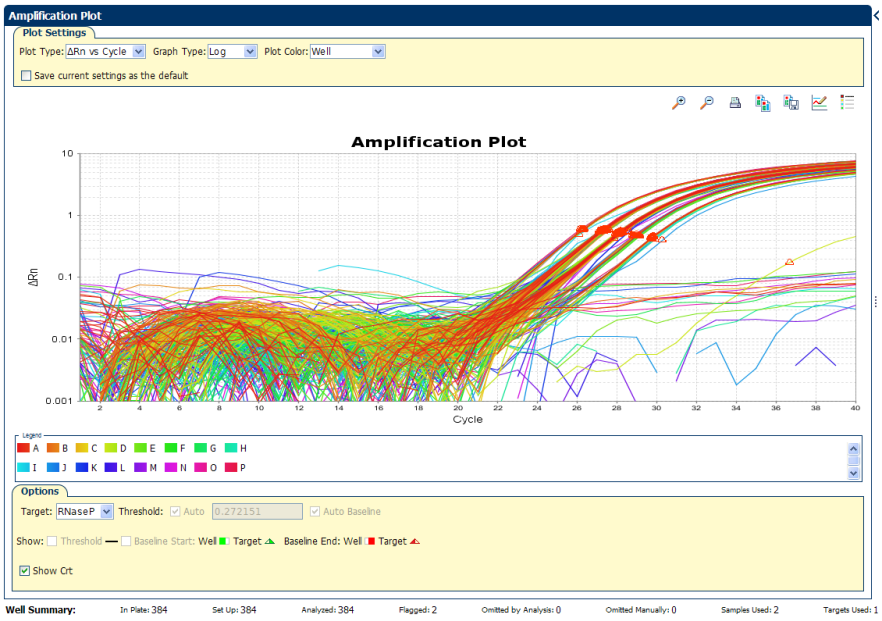
- **Outliers**
- **A typical amplification plot** – The QuantStudio™ 12K Flex Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear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

  A typical amplification plot should look like this:

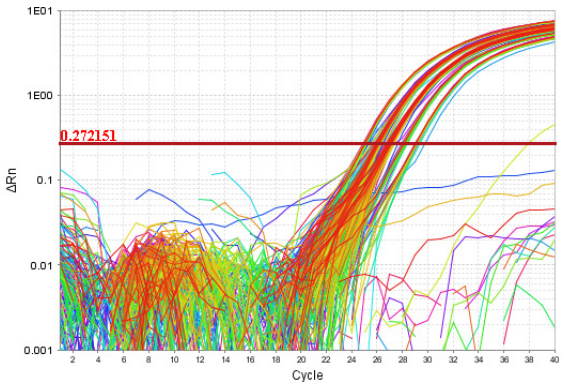
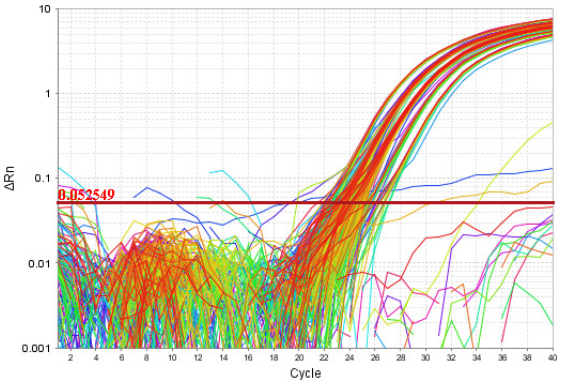


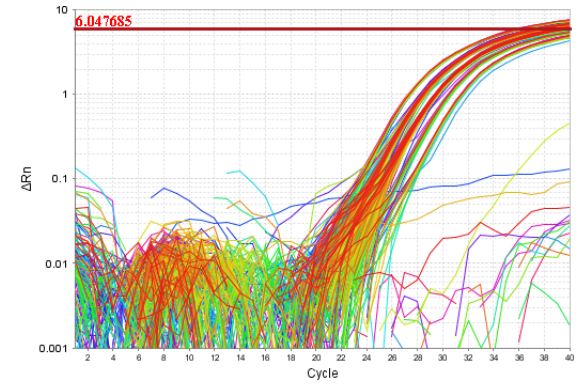
IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudio™ 12K Flex Software. Life Technologies recommends that you examine the Amplification Plot screen 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. Also select the **Show Crt** check box to view the derived fractional cycle on the amplification plot.

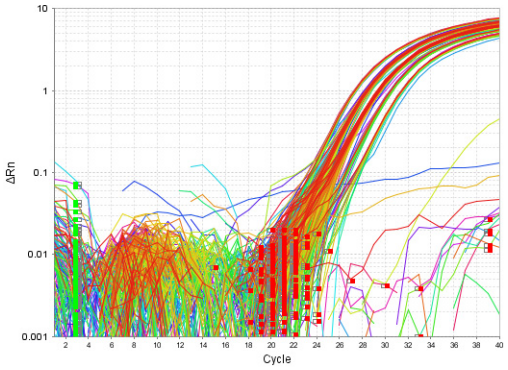
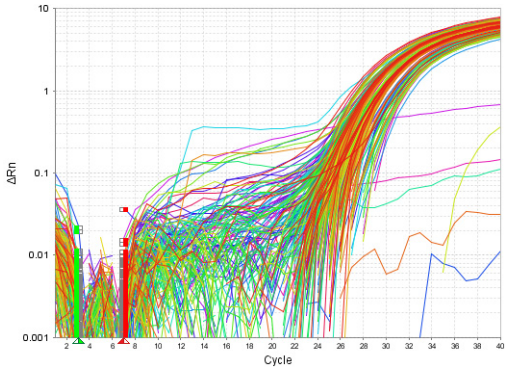
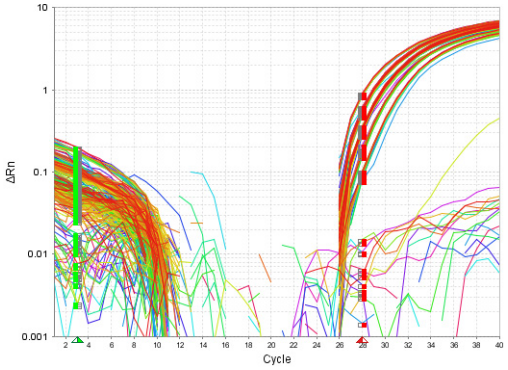


- **Correct threshold values:**

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**:

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 C_T precision by omitting wells" on page 53).

  Or

- Manually adjust the baseline and/or threshold (see "Adjust analysis settings" on page 49).

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
- Comments
- Flags

Purpose

The purpose of viewing the well table is to identify:

- 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**.

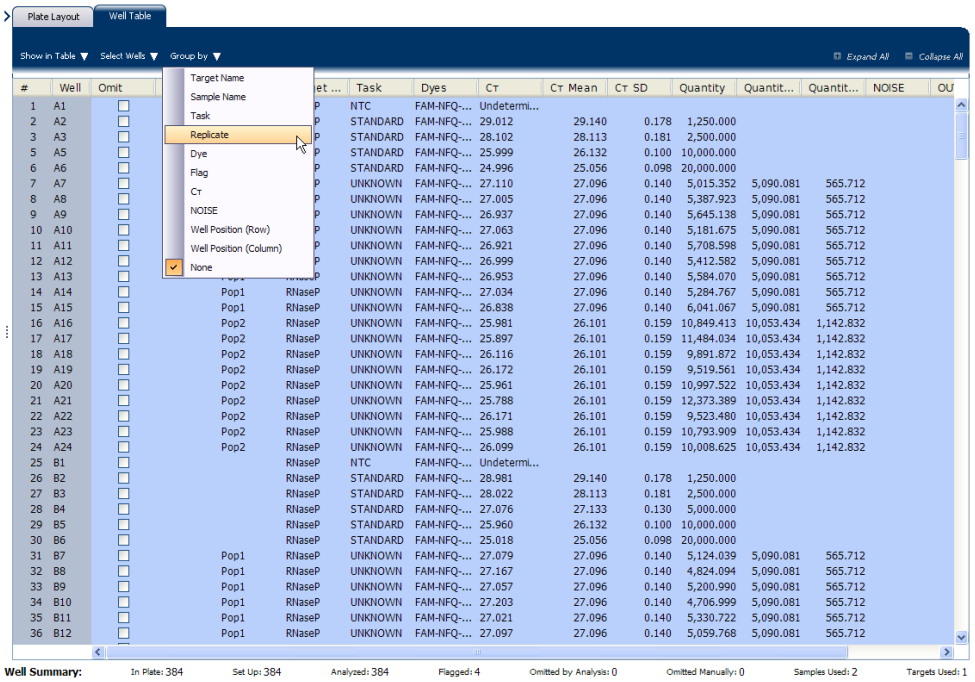
2. Use the Group By drop-down menu to group wells by a specific category. For the example experiment, group the wells by replicate, flag, or C_T value.

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

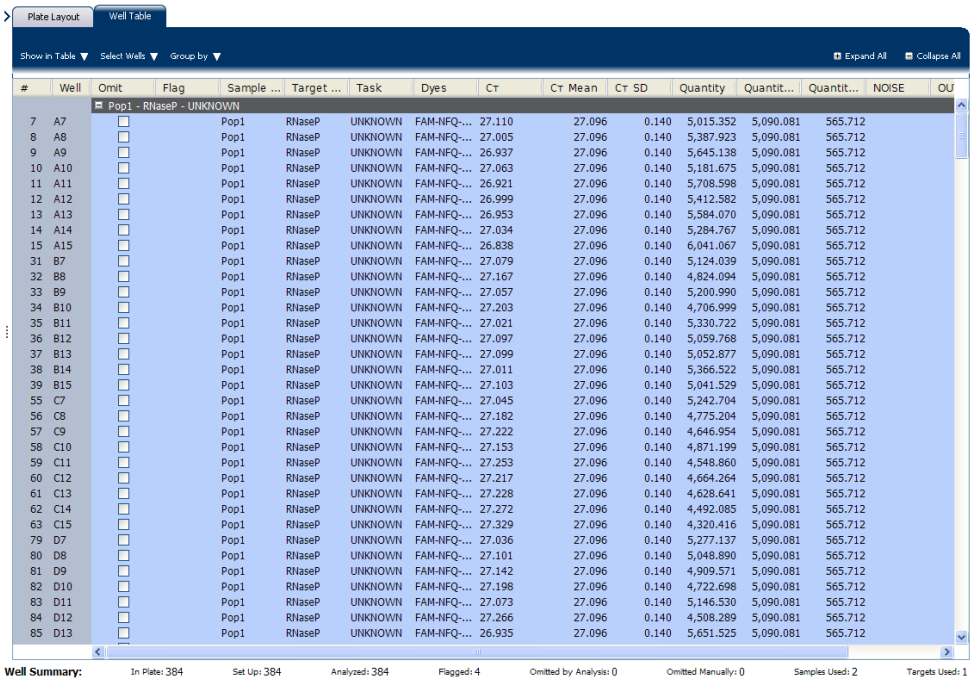
To group by replicate

From the Group By drop-down menu, 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, Quantity Mean, and Quantity SD columns 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.

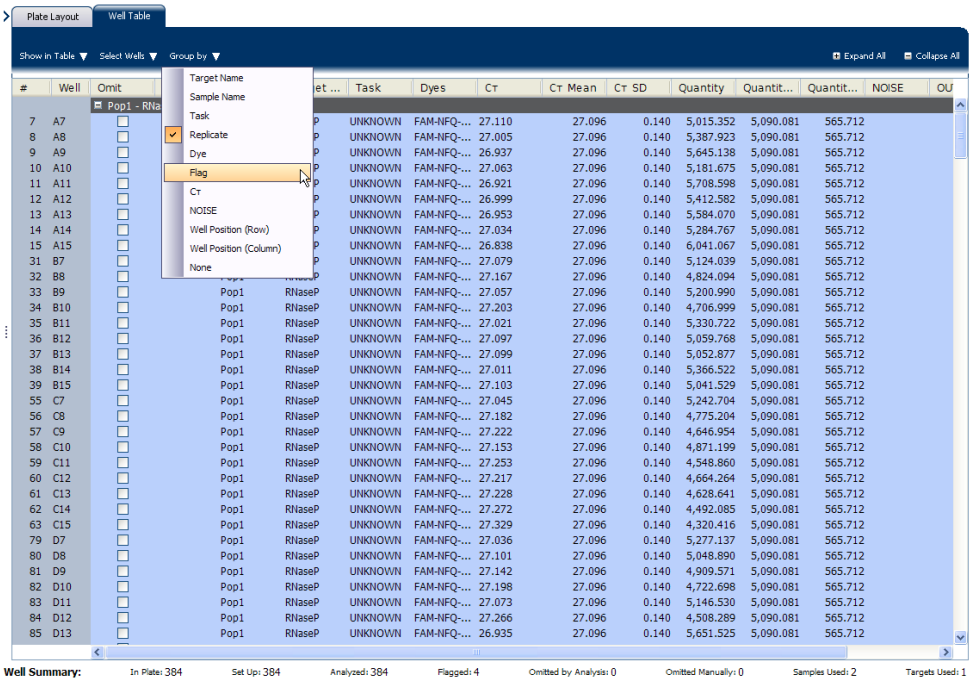


The well table looks like this:



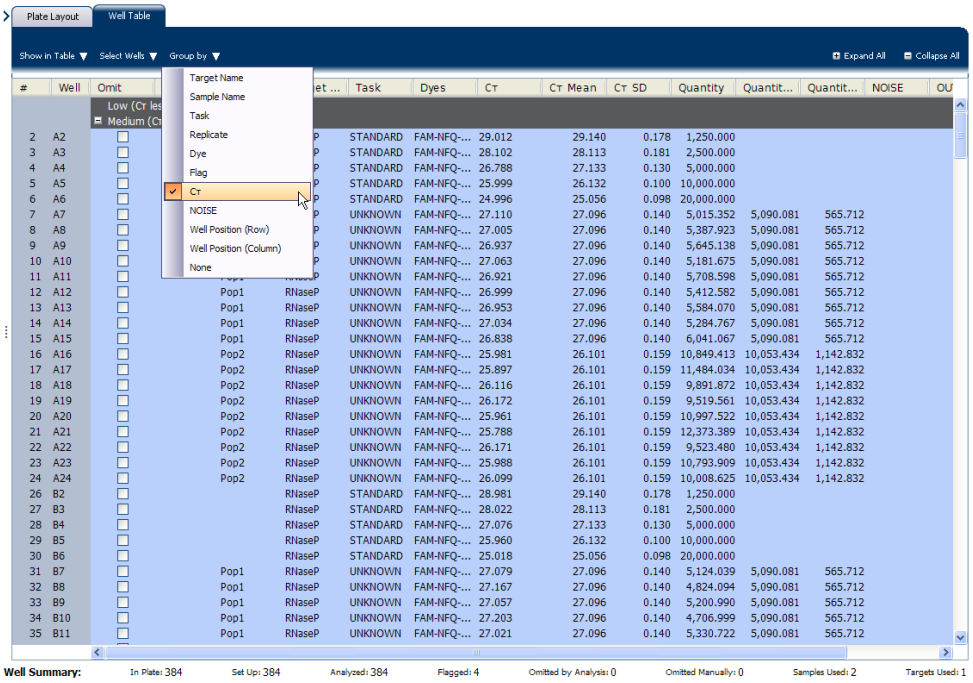
To group by flag

From the Group By drop-down menu, select **Flag**. The software groups the flagged and unflagged wells. In the example experiment, there are four flagged wells.



To group by C_T value

From the Group By drop-down menu, 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 (>8 and <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 QuantStudio™ 12K Flex Software flags, see "Flag Settings" on page 51.
- **C_T** – 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.

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 screen for:

- 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 shown in the Multicomponent Plot screen.

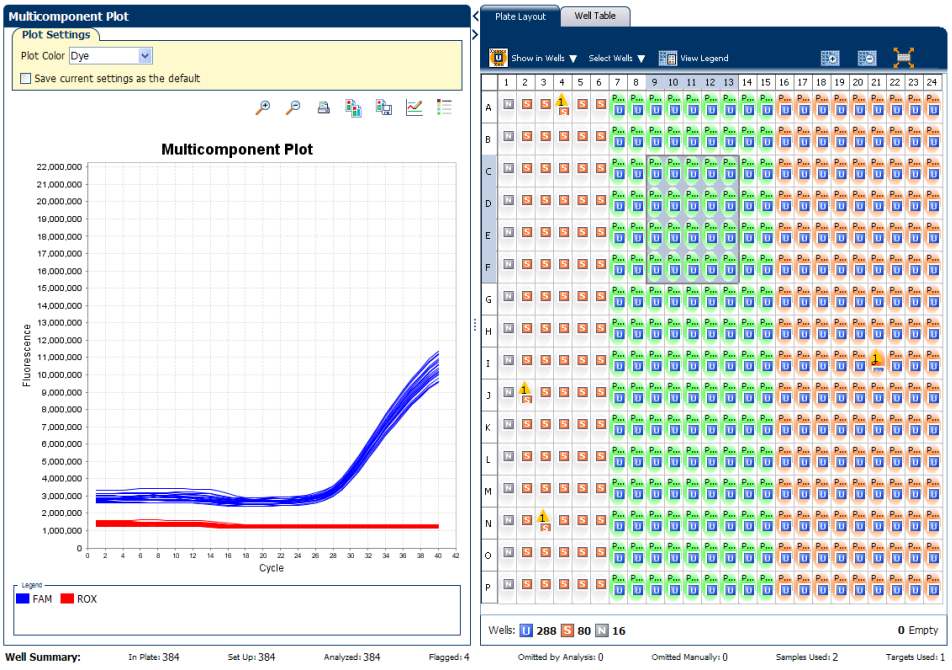
      Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

3. From the Plot Color drop-down menu, select **Dye**.
4. Click  **Show a legend for the plot** (default).

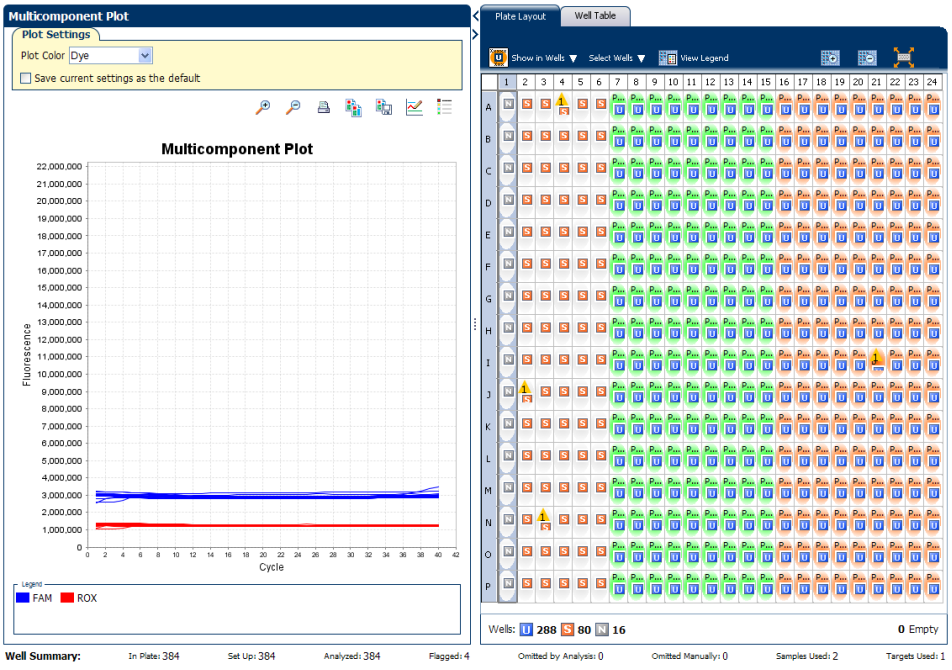
   Note: This is a toggle button. When the legend is displayed, the button changes to **Hide the plot legend**.

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; 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:

- **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 screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

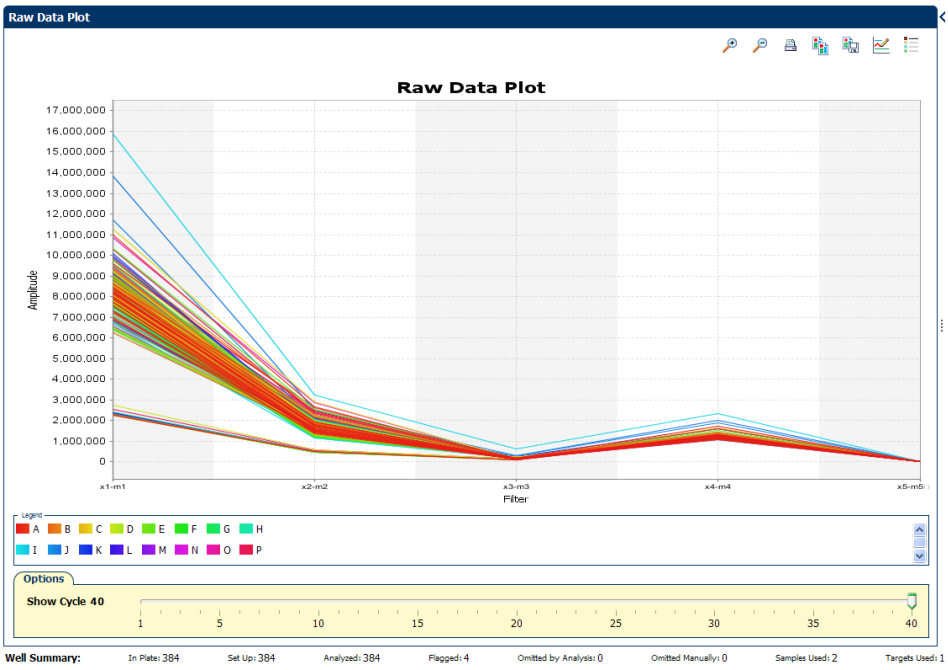
View the Raw Data Plot

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

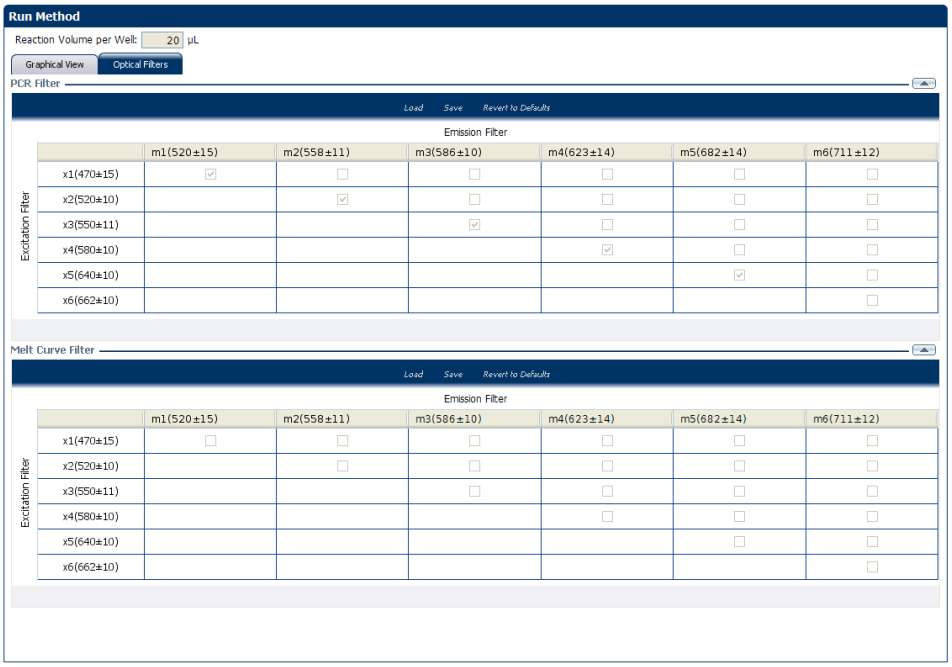
   Note: If no data are displayed, click **Analyze**.

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.



The filters used for the example experiment are:



Tips for determining signal accuracy in your own experiments

When you analyze your own standard 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. From the Experiment Menu pane, select **Analysis ▸ QC Summary**.

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

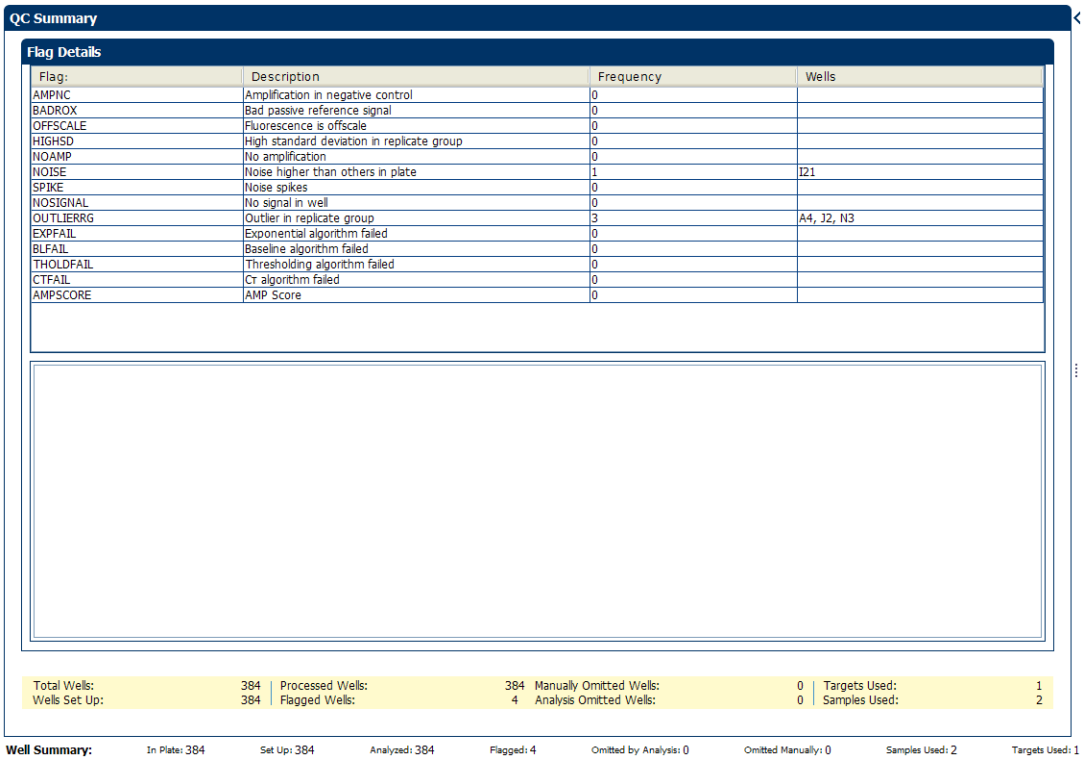
2. Review the Flags Summary.

   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment. If the frequency is >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 >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 >0, click the troubleshooting link to view information on correcting the flag.



Possible flags

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

Note: To change the flag settings, refer to "Flag Settings" on page 51.

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	
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

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

Section 5.2 Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

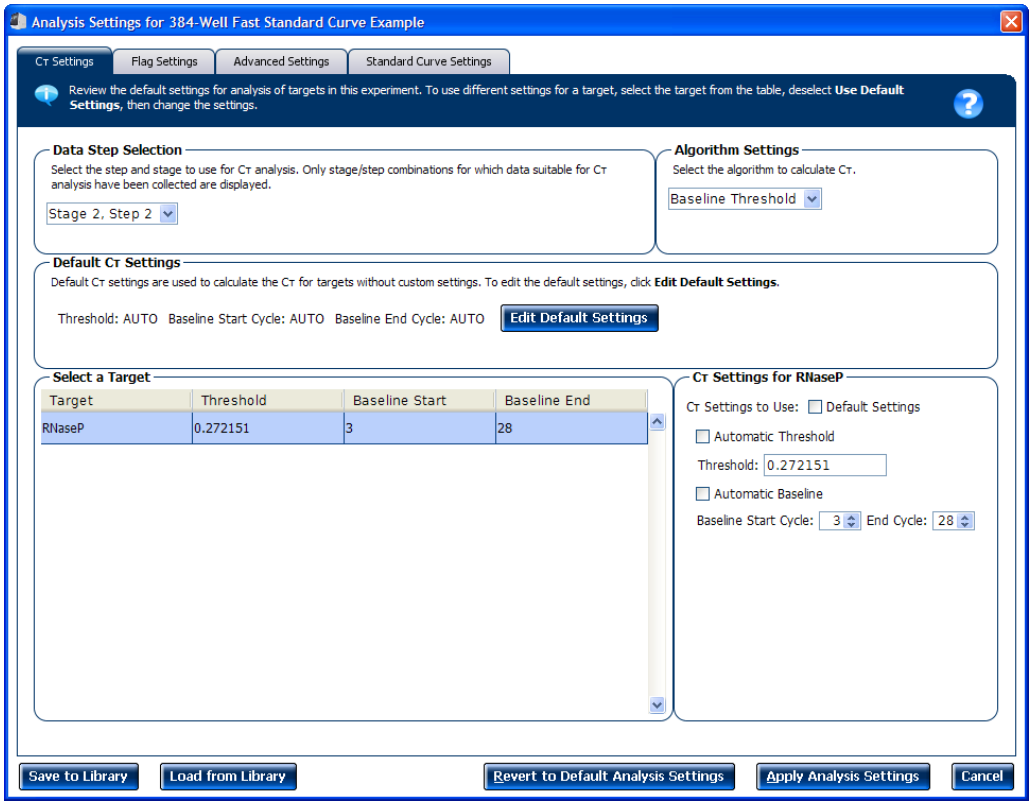
The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, and advanced options.

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

   The Analysis Settings dialog box for a Standard Curve experiment looks like this:



3. View and, if necessary, change the analysis settings (see "Adjust analysis settings" below).

   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 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.

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, Life Technologies recommends:

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: This setting is applicable only to the Baseline Threshold algorithm.

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:

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

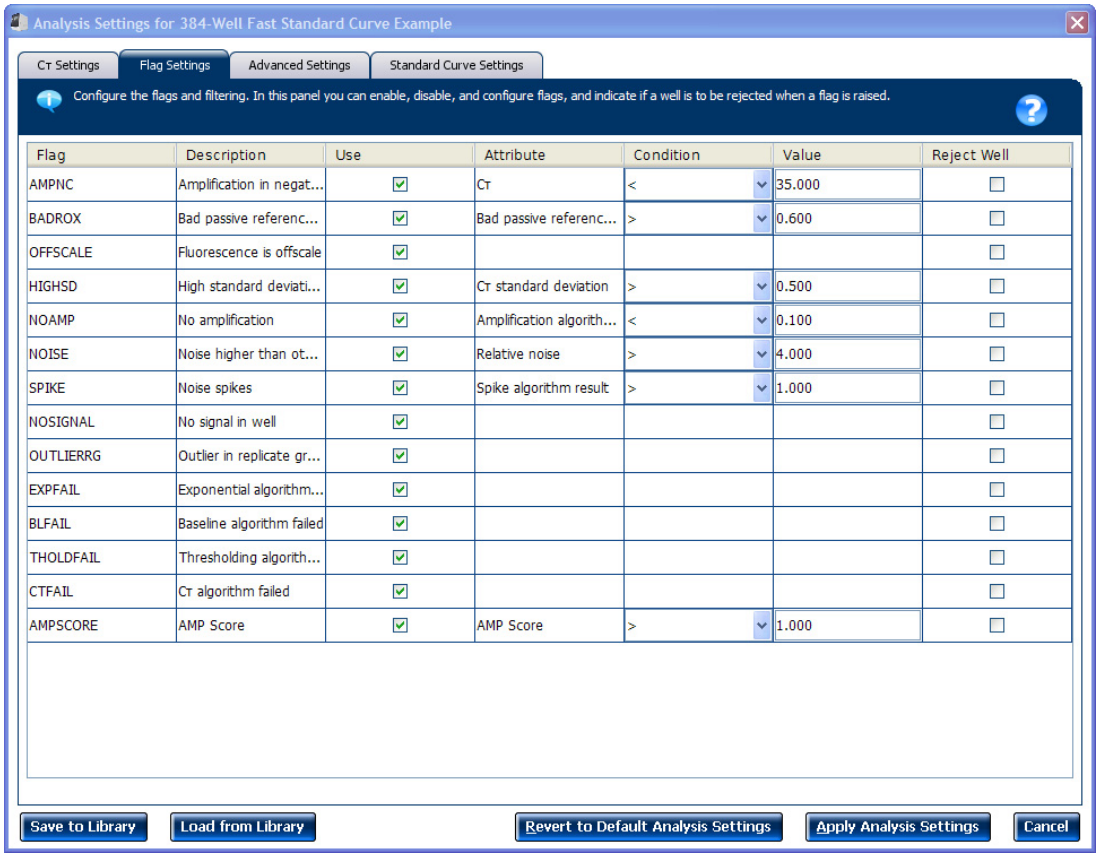
To adjust the flag settings:

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.

   The Flag Settings tab looks like this:



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.

To use custom baseline settings for a well-target combination:

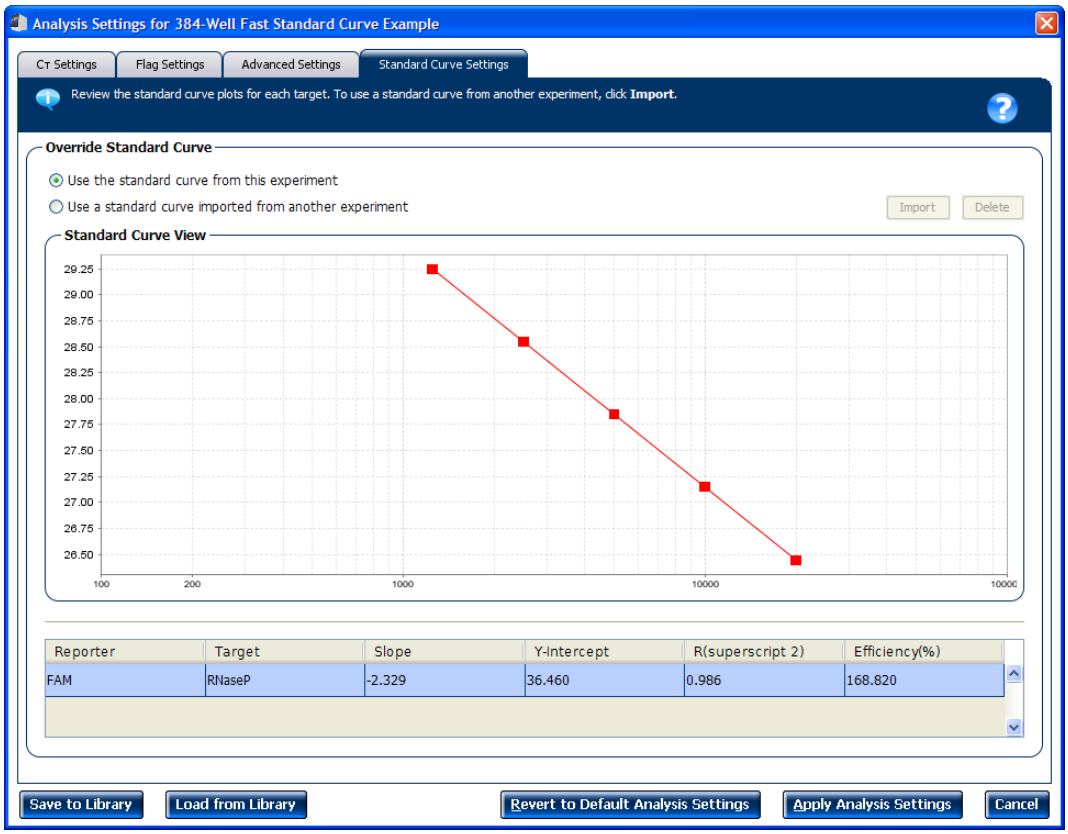
1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** check box.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, 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. Life Technologies recommends 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 ensure 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 drop-down menu.

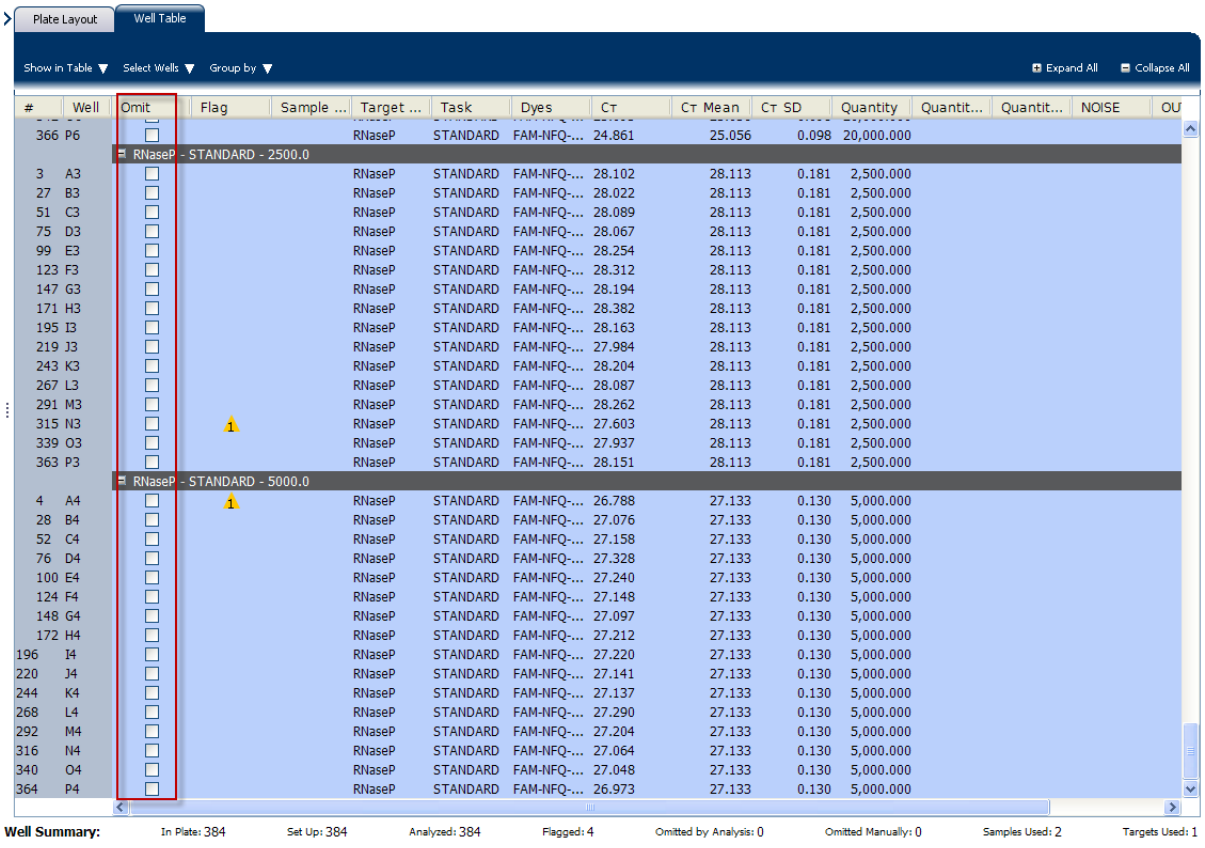
3. Select the **Well Table** tab.

4. In the Well Table, view outliers:

   a. From the Group By drop-down menu, select **Replicate**.

   b. Look for any outliers in the replicate group (make sure they are flagged).

   c. Select the **Omit** check box next to outlying well(s), as shown below.



#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	CT	CT Mean	CT SD	Quantity	Quantit...	Quantit...	NOISE	OU
366	P6	☐			RNaseP	STANDARD	FAM-NFQ-...	24.861	25.056	0.098	20,000.000				
RNaseP - STANDARD - 2500.0															
3	A3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.102	28.113	0.181	2,500.000				
27	B3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.022	28.113	0.181	2,500.000				
51	C3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.089	28.113	0.181	2,500.000				
75	D3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.067	28.113	0.181	2,500.000				
99	E3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.254	28.113	0.181	2,500.000				
123	F3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.312	28.113	0.181	2,500.000				
147	G3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.194	28.113	0.181	2,500.000				
171	H3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.382	28.113	0.181	2,500.000				
195	I3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.163	28.113	0.181	2,500.000				
219	J3	☐			RNaseP	STANDARD	FAM-NFQ-...	27.984	28.113	0.181	2,500.000				
243	K3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.204	28.113	0.181	2,500.000				
267	L3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.087	28.113	0.181	2,500.000				
291	M3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.262	28.113	0.181	2,500.000				
315	N3	☐	!		RNaseP	STANDARD	FAM-NFQ-...	27.603	28.113	0.181	2,500.000				
339	O3	☐			RNaseP	STANDARD	FAM-NFQ-...	27.937	28.113	0.181	2,500.000				
363	P3	☐			RNaseP	STANDARD	FAM-NFQ-...	28.151	28.113	0.181	2,500.000				
RNaseP - STANDARD - 5000.0															
4	A4	☐	!		RNaseP	STANDARD	FAM-NFQ-...	26.788	27.133	0.130	5,000.000				
28	B4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.076	27.133	0.130	5,000.000				
52	C4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.158	27.133	0.130	5,000.000				
76	D4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.328	27.133	0.130	5,000.000				
100	E4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.240	27.133	0.130	5,000.000				
124	F4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.148	27.133	0.130	5,000.000				
148	G4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.097	27.133	0.130	5,000.000				
172	H4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.212	27.133	0.130	5,000.000				
196	I4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.220	27.133	0.130	5,000.000				
220	J4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.141	27.133	0.130	5,000.000				
244	K4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.137	27.133	0.130	5,000.000				
268	L4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.290	27.133	0.130	5,000.000				
292	M4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.204	27.133	0.130	5,000.000				
316	N4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.064	27.133	0.130	5,000.000				
340	O4	☐			RNaseP	STANDARD	FAM-NFQ-...	27.048	27.133	0.130	5,000.000				
364	P4	☐			RNaseP	STANDARD	FAM-NFQ-...	26.973	27.133	0.130	5,000.000				

5. Click **Analyze** to reanalyze the experiment data with the outlying well(s) 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 and select **Omit**.

For more information

For more information on	Refer to	Part number
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*.	127AP05-03

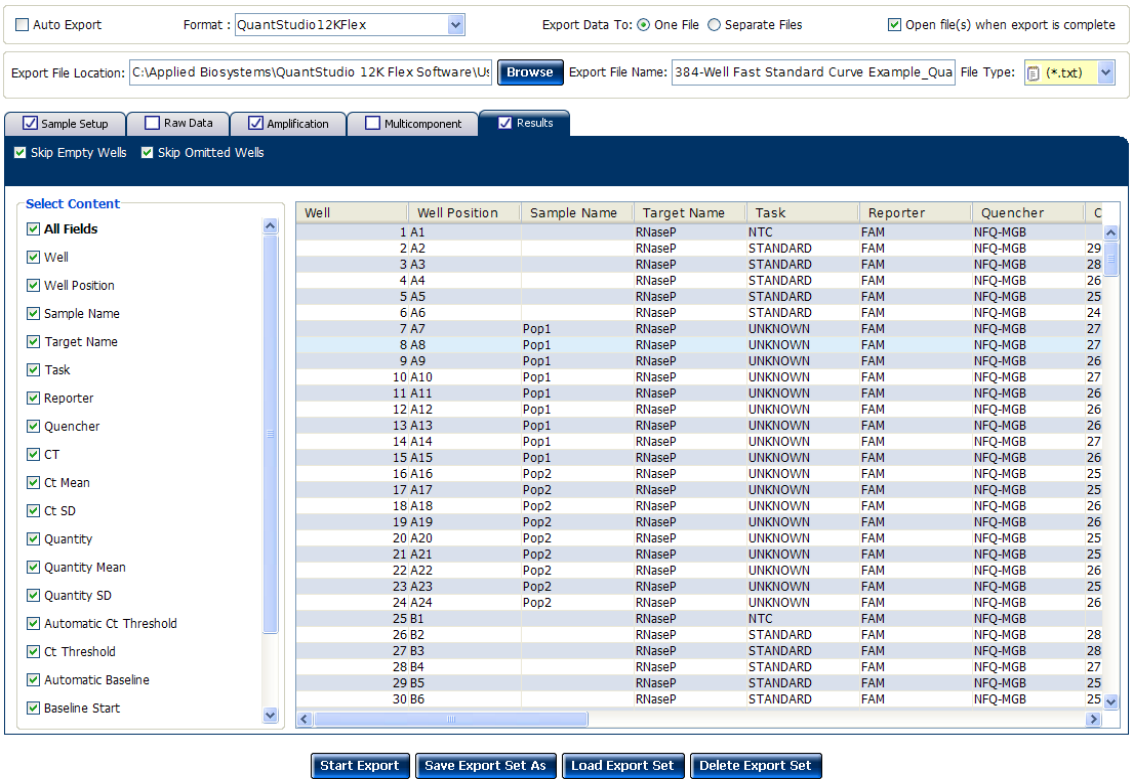
6 Export Analysis Results

1. Open the Standard Curve example experiment file that you analyzed in Chapter 5.
2. In the Experiment Menu, click  **Export.**

   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.
3. Select **QuantStudio™ 12K Flex format**.
4. Complete the Export dialog box as shown below:

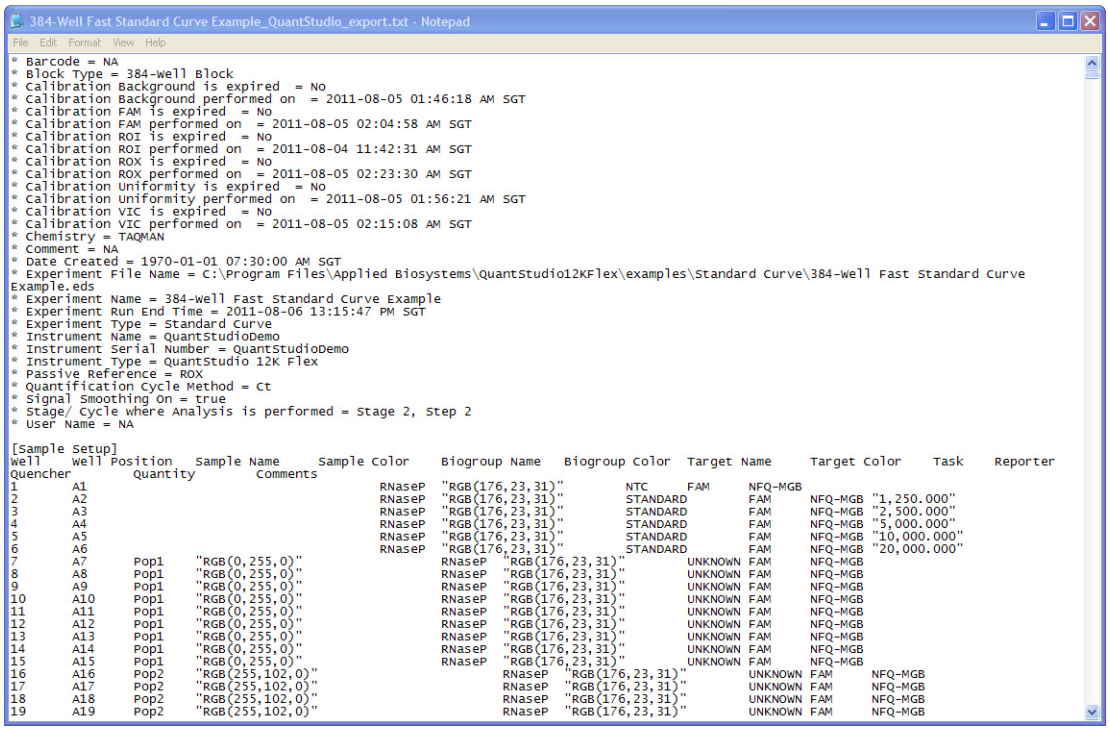
Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	384-Well Fast Standard Curve Example_QuantStudio_export
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\QuantStudio 12K Flex Software\User Files\experiments

Your Export screen should look like this:



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

Your exported file when opened in Notepad should look like this:




```
* 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
```

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GETTING STARTED GUIDE

applied biosystems® by life technologies™

Booklet 3 - Running Relative Standard Curve and Comparative C_T Experiments

Publication Part Number 4470050 Rev. A
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life technologies™

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PART I
Running Relative Standard Curve Experiments

1 About Relative Standard Curve Experiments

This chapter covers:

- About Relative Standard Curve experiments. 9
- About the example experiment 11

IMPORTANT! First-time users of the QuantStudio™ 12K Flex System, please read Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* and Booklet 7, *QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes* of this binder thoroughly. The booklets provide information and general instructions that are applicable to all the experiments described in this binder.

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 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:

- 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.

- **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 and multiplex PCR (page 10)

  and
- 1-step and 2-step RT-PCR (page 11)

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. Typically, a probe labeled with FAM™ dye detects the target and a probe 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 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 Invitrogen VILO™ Kit is used for reverse transcription; the TaqMan® Gene Expression Master Mix (2✕) is used for PCR.
- Select primer and probe sets from the Life Technologies TaqMan® Gene Expression Assays product line:
  - For the target assay (HPRT), select assay ID Hs99999909_m1.
  - For the endogenous control assay (FAS), select assay ID Hs00907759_m1.

2 Design the Experiment

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

This chapter covers:

- Define the experiment properties. 13
- Define targets, samples, and biological replicates 14
- Assign targets, samples, and biological groups 15
- Set up the run method 17
- Tips for designing your own experiment 18
- For more information 19

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 on Auto Export, refer to Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.

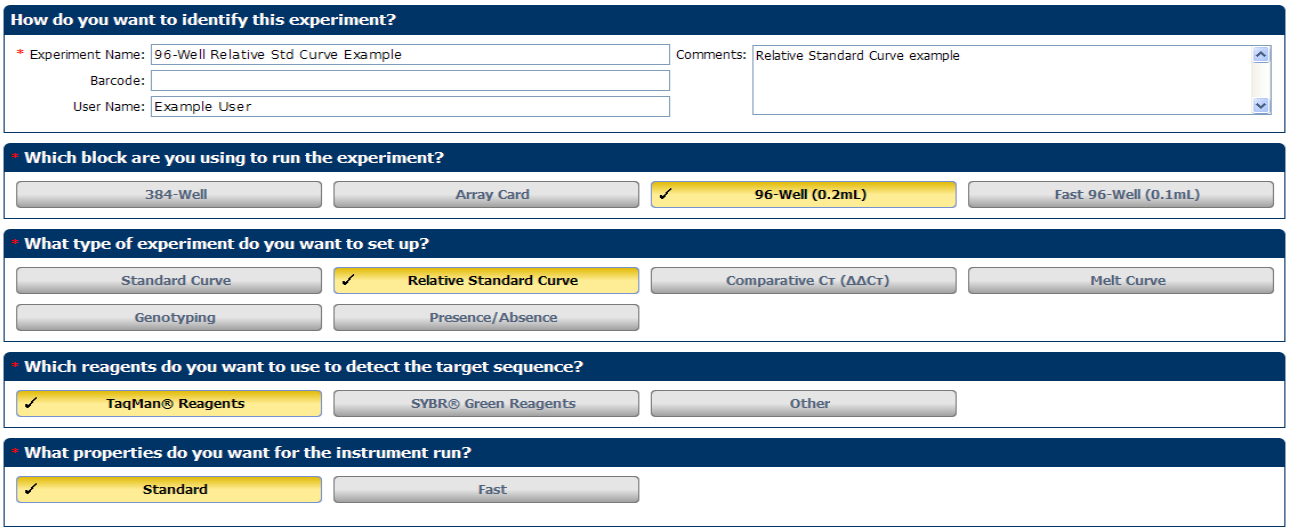
Define the experiment properties

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

Field or Selection	Entry
Experiment Name	96-Well Relative Std Curve Example
Barcode	Leave field empty
User Name	Example User
Comments	Relative Standard Curve example
Block	96-Well (0.2 mL)
Experiment Type	Relative Standard Curve
Reagents	TaqMan® Reagents
Ramp speed	Standard

Save the experiment.

Your Experiment Properties screen should look like this:



Define targets, samples, and biological replicates

Click **Define** to access the Define screen. Enter:

1. Targets

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

2. Samples

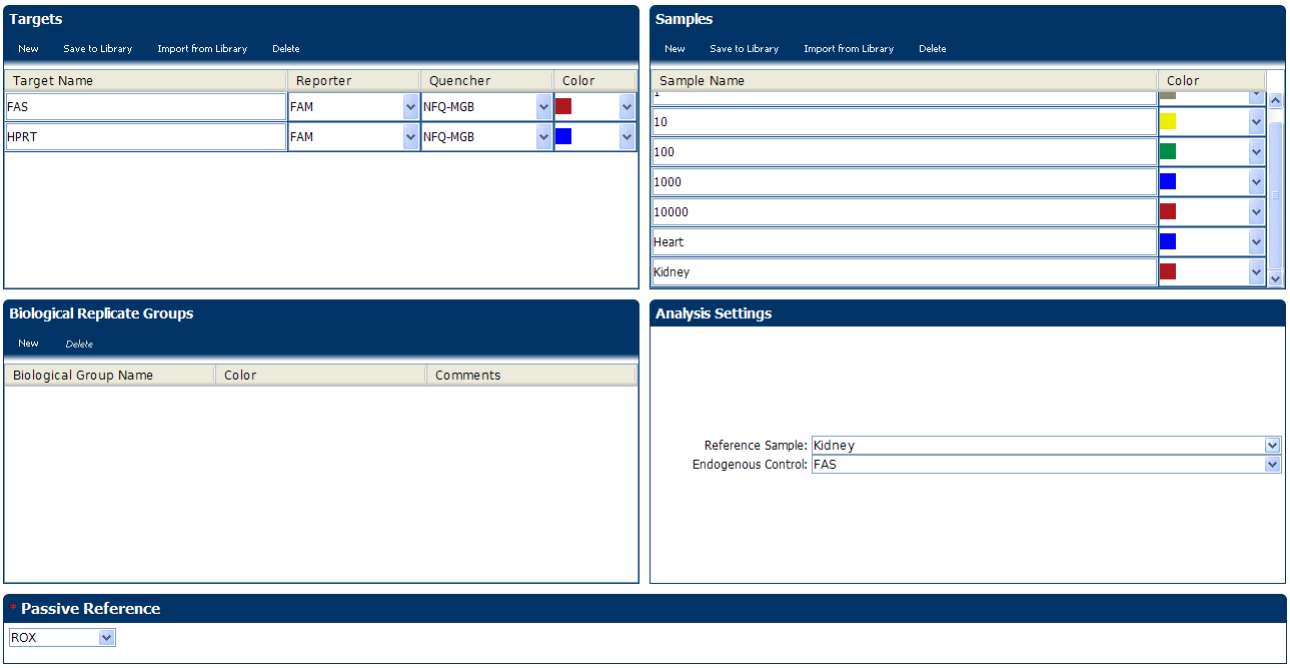
Sample name	Color
1	
10	
100	
1000	
10000	
Heart	
Kidney	

3. Dye to be used as a Passive Reference
   ROX

4. Analysis Settings

Field	Select
Reference Sample	Kidney
Endogenous Control	FAS

Your Define screen should look like this:



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

Assign targets, samples, and biological groups

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

Note: To automatically set up and assign standards, click 

to open the Define and Set Up Standards dialog box.

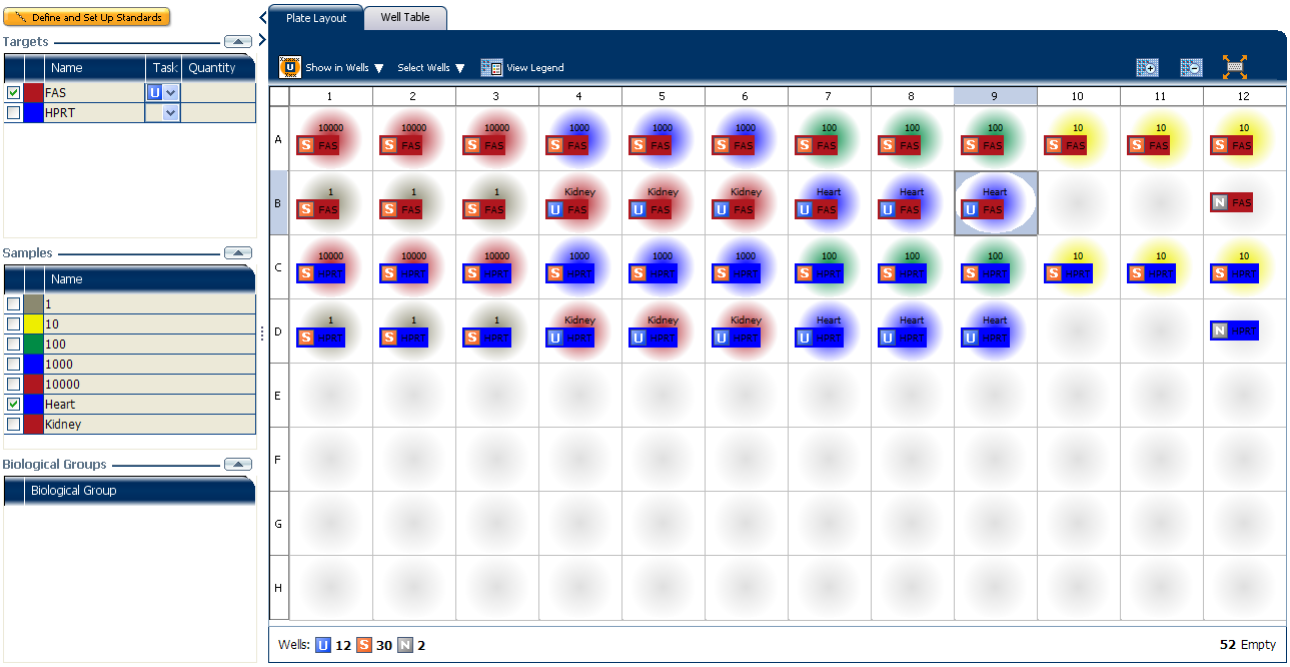
To assign the targets and samples:

1. Set up the standards.
2. For the first standard for the FAS target:
   a. Click-drag to select wells A1-A3.
   b. Check check box next to FAS in the Targets list.
   c. Select S in the Task drop-down menu.
   d. Enter 10,000 in the Quantity column.
   e. Repeat steps a through c for each of the standards for the FAS target, selecting the wells listed in the table below, and entering the corresponding quantity.

3. Repeat step 2 for each standard for the HPRT target.

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

Your Assign screen should look like this:



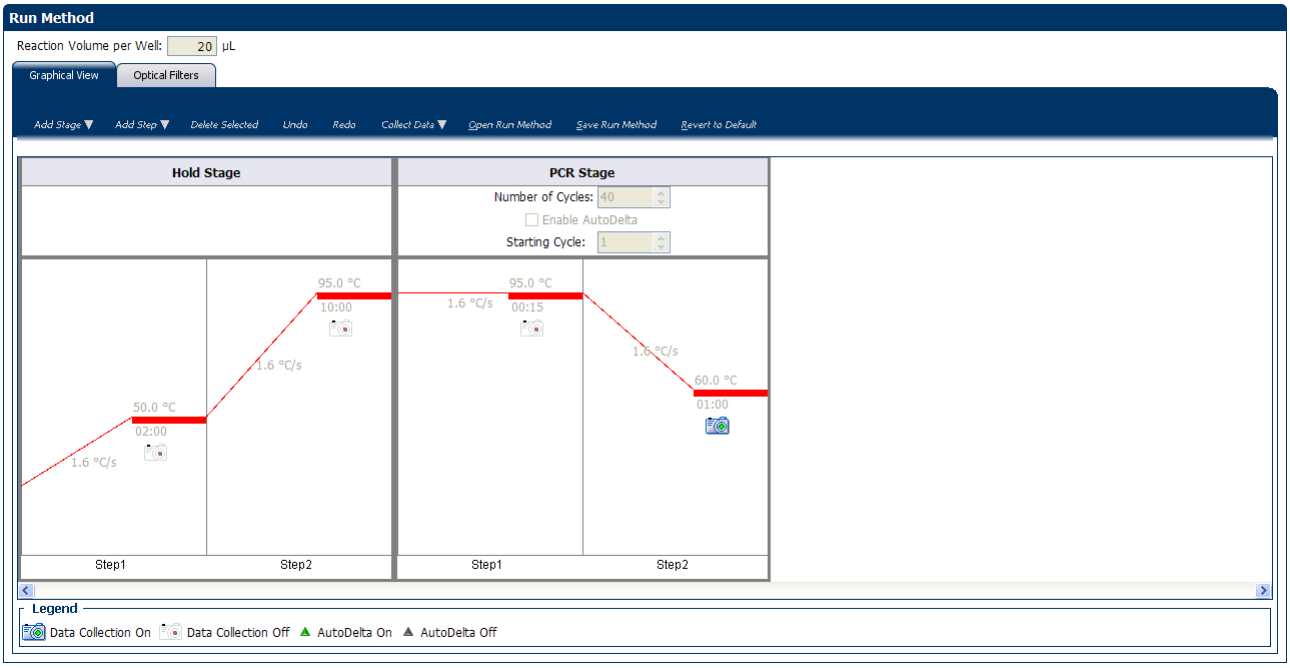
Set up the run method

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

- 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	Step 1	1.6°C/s	95°C	15 seconds
Number of Cycles: 40 Enable AutoDelta: Unchecked (default) Starting Cycle: Disabled when Enable AutoDelta is unchecked	Step 2	1.6°C/s	60°C	1 minute

Your Run Method screen should look like this:



Tips for designing your own experiment

Life Technologies recommends that you:

- 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 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: 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.

For more information

For more information on...	Refer to...	Part number
Consumables	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* Appendix A in Booklet 7, *QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes*	4470050
Using other quantification methods	Booklet 2, *Running Standard Curve Experiments* and Part 2 of Booklet 3, *Running Relative Standard Curve and Comparative C_T Experiments*.	4470050
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*	127AP05-03
Using alternative setup	Chapter 2 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

3 Prepare the Reactions

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

This chapter covers:

- Assemble required materials 21
- Prepare the template 21
- Prepare the sample dilutions 22
- Prepare the standard dilution series for FAS and HPRT assays 22
- Prepare the reaction mix (“cocktail mix”). 23
- Prepare the reaction plate 24
- Tips for preparing reactions for your own experiments. 26
- For more information. 27

Assemble required materials

- Items listed in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.
- 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 one of the Invitrogen VILO™ kits, SuperScript® VILO™ cDNA Synthesis Kit (PN 4453650).

Example experiment settings

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

To prepare the template

Use the Invitrogen VILO™ kits to reverse-transcribe cDNA from the total RNA samples. Follow the procedures in the *Invitrogen VILO Kits Protocol* (PN 100002284) to:

1. Isolate total RNA from cells using an Ambion® 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 via 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 (µL)
1	Kidney	76
2	Heart	76

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

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

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 (µL)	Diluent volume (µL)	Total volume (µL)	Standard concentration (ng/µL)
Std. 1	1	Stock	20	20	40	100
Std. 2	2	Dilution 1	20	20	40	50
Std. 3	3	Dilution	20	20	40	25
Std. 4	4	Dilution 3	20	20	40	12.5

Standard name (labeled tube)	Dilution point	Source	Source volume (µL)	Diluent volume (µL)	Total volume (µL)	Standard concentration (ng/µL)
Std. 5	5	Dilution 4	20	20	40	6.25

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 ("cocktail 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 (µL) for 1 reaction	Volume (µL) for 30 reactions
TaqMan® Gene Expression Master Mix (2X)	10	300
FAS Assay Mix (20X)	1	30
Water	8	240
Total Reaction Mix Volume	19	570

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

Component	Volume (µL) for 1 reaction	Volume (µL) for 30 reactions
TaqMan® Gene Expression Master Mix (2X.)	10	300

Component	Volume (µL) for 1 reaction	Volume (µL) for 30 reactions
HPRT Assay Mix (20X	1	30
Water	8	240
Total Reaction Mix Volume	19	570

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.

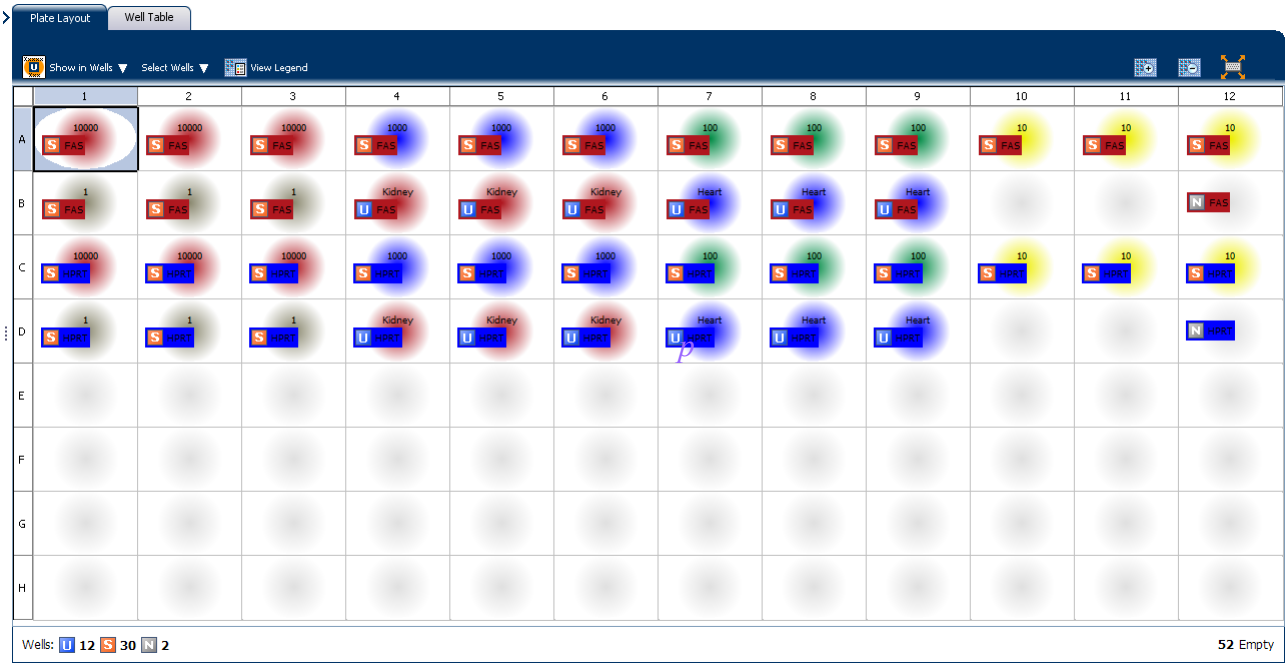
Prepare the reaction plate

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

The plate layout for the example experiment looks like this:



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 (µL)	Water volume (µL)
1	FAS Reaction Mix	19	1
2	HPRT Reaction Mix	19	1

   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 voulme (µL)	Standard	Standard volume (µL)
1	FAS Std. 1	FAS Reaction Mix	76	FAS Std. 1	4
2	FAS Std. 2	FAS Reaction Mix	76	FAS Std. 2	4
3	FAS Std. 3	FAS Reaction Mix	76	FAS Std. 3	4
4	FAS Std. 4	FAS Reaction Mix	76	FAS Std. 4	4
5	FAS Std. 5	FAS Reaction Mix	76	FAS Std. 5	4
6	HPRT Std. 1	HPRT Reaction Mix	76	HPRT Std. 1	4
7	HPRT Std. 2	HPRT Reaction Mix	76	HPRT Std. 2	4
8	HPRT Std. 3	HPRT Reaction Mix	76	HPRT Std. 3	4
9	HPRT Std. 4	HPRT Reaction Mix	76	HPRT Std. 4	4
10	HPRT Std. 5	HPRT Reaction Mix	76	HPRT Std. 5	4

   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 (µL)	Sample	Sample volume (µL)
1	FAS Kidney	FAS Reaction Mix	76	Kidney	4
2	FAS Heart	FAS Reaction Mix	76	Heart	4
3	HPRT Kidney	HPRT Reaction Mix	76	Kidney	4
4	HPRT Heart	HPRT Reaction Mix	76	Heart	4

   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, Life Technologies recommends 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 TaqMan® Gene Expression Assays or Custom TaqMan® Gene Expression Assays, use 10 to 100 ng of cDNA template per 10µL 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.

For more information

For more information on...	Refer to...	Part number
Assigning the reaction plate components	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

4 Run the Experiment

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

This chapter covers:

- Start the run 29
- Monitor the run 29

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 QuantStudio™ 12K Flex Instrument is in operation.

Start the run

1. Open the Relative Standard Curve example file that you created using instructions in Chapter 2.
2. Load the reaction plate into the instrument.
3. Start the run.

Monitor the run

Monitor the example experiment run:

- From the QuantStudio™ 12K Flex Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio™ 12K Flex Software (to monitor an experiment started from another computer or from the QuantStudio™ 12K Flex Instrument touchscreen).
- From the QuantStudio™ 12K Flex Instrument touchscreen.

From the Instrument Console of the QuantStudio™ 12K Flex Software

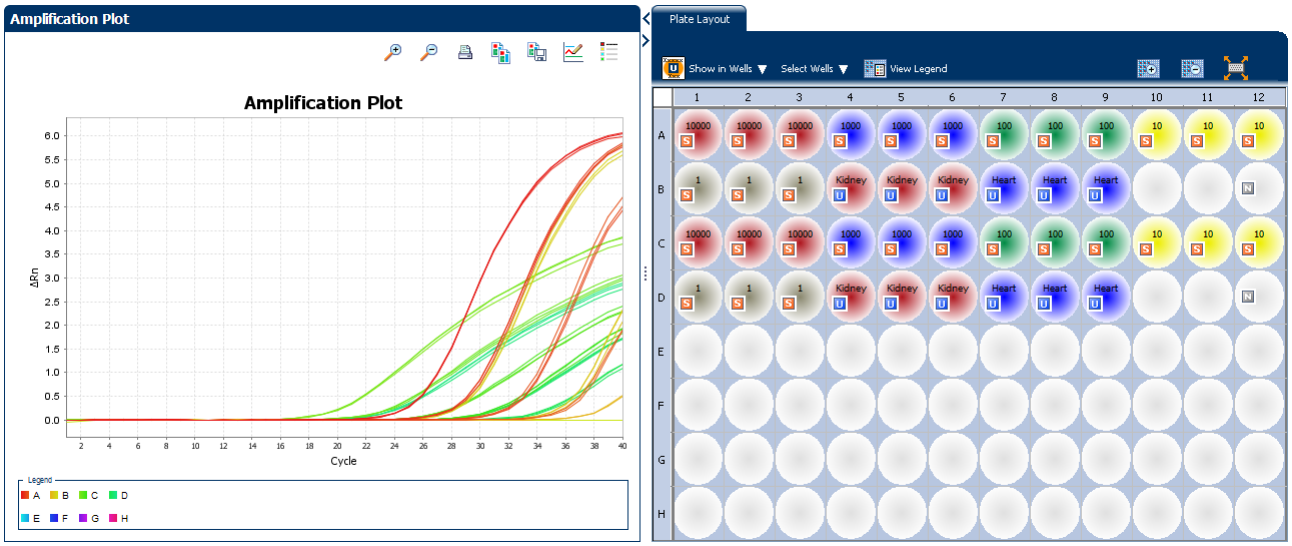
1. In the Instrument Console screen, select the instrument icon.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. In the Manage Instrument screen, click **Monitor Running Experiment** to access the Run screen.

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.

To view data in the Amplification Plot screen, click **Amplification Plot** from the Run Experiment Menu, and select the Plate Layout tab, then select the wells that you want to view.

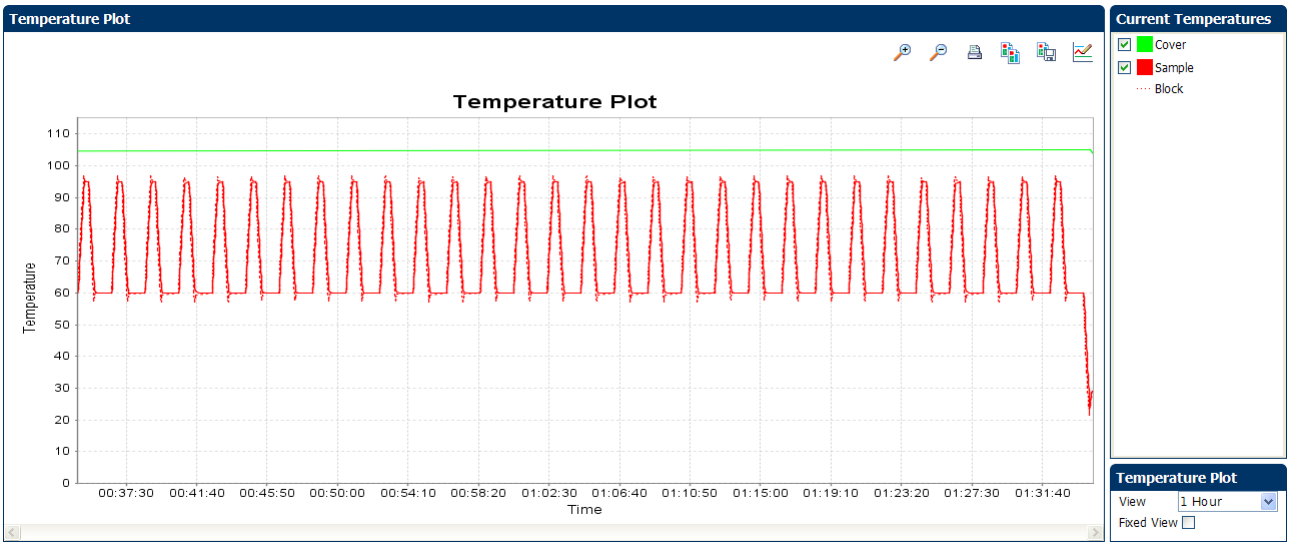
The figure below shows the Amplification Plot screen as it appears at the end of the example experiment.



View the Temperature Plot

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

The figure below shows the Temperature Plot screen as it appears during the example experiment.

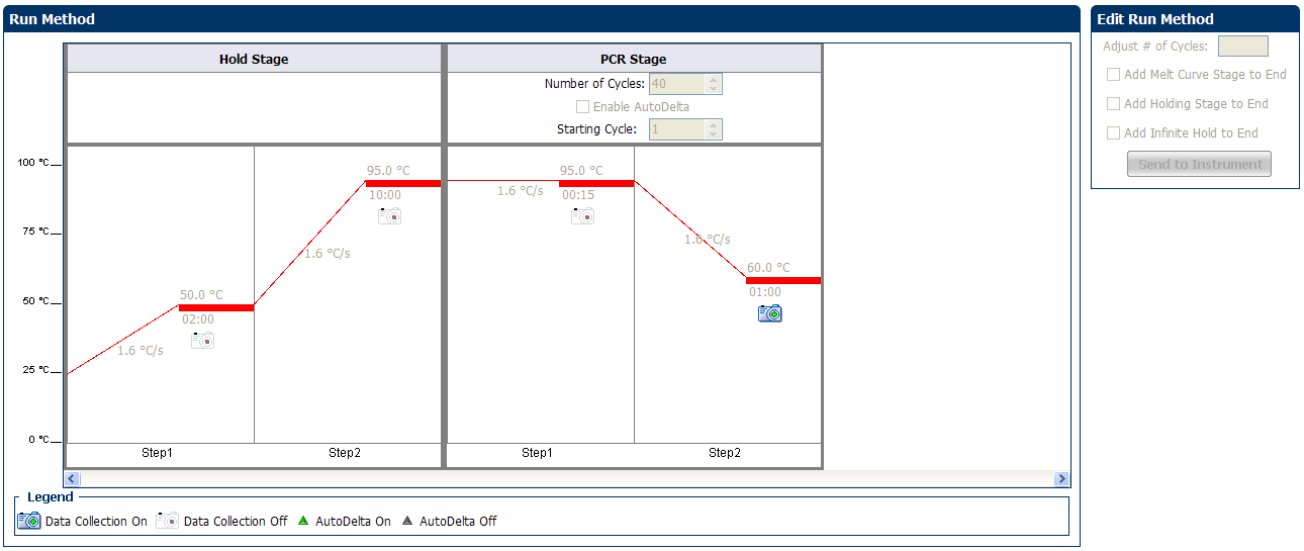


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

View the Run Method

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

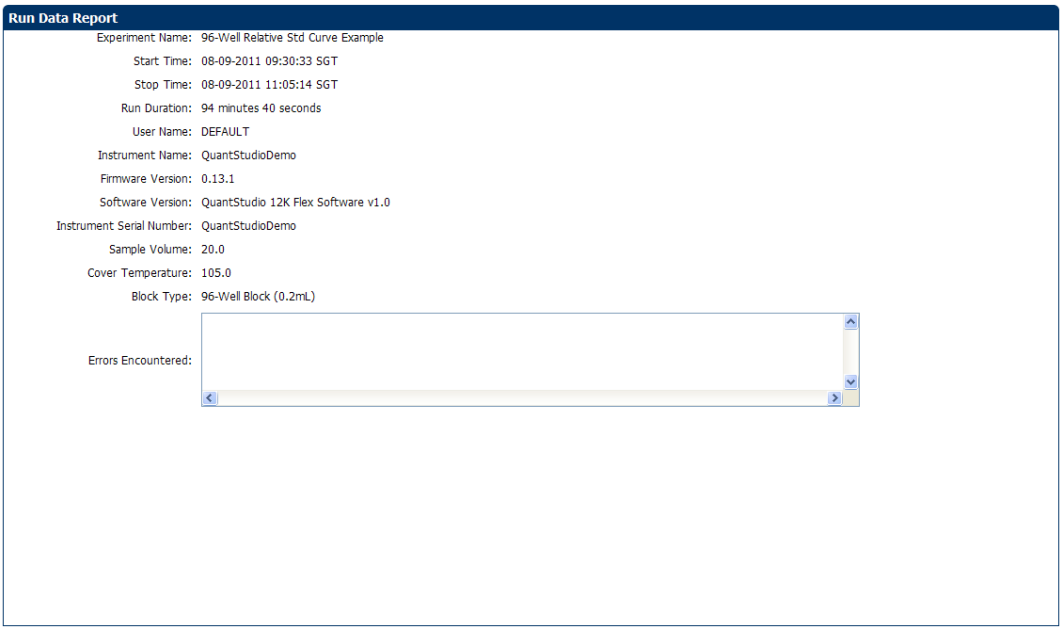
The figure below shows the Run Method screen as it appears in the example experiment.



View run data

To view the run data, click **View Run Data** from the Run Experiment Menu.

Your View Run Data screen should look like this:

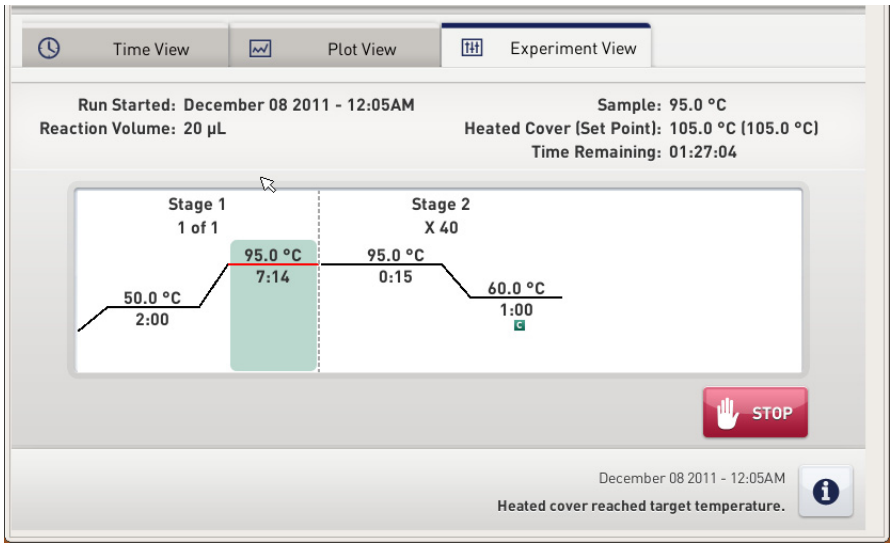


From the QuantStudio™ 12K Flex Instrument touchscreen

You can also view the progress of the run from the touchscreen of the QuantStudio™ 12K Flex Instrument.

The Run Method screen on the **QuantStudio™ 12K Flex Instrument** touchscreen looks like this:

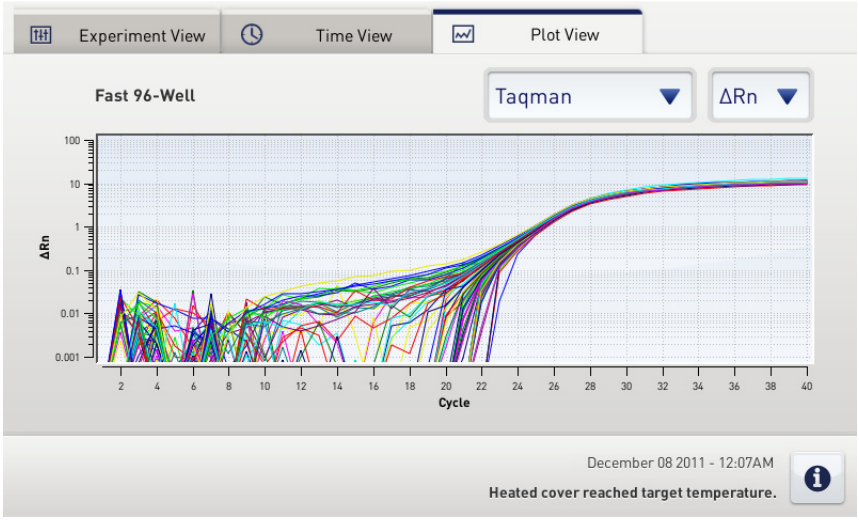
Experiment view



Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

Time view

Plot view


Experiment View
Time View
Plot View
Fast 96-Well
Taqman
ΔRn
100
10
1
0.1
0.01
0.001
ΔRn
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Cycle
December 08 2011 - 12:07AM
Heated cover reached target temperature.

5 Review Results and Adjust Experiment Parameters

In Section 5.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 5.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

Section 5.1 Review Results 37

- Analyze the example experiment 37
- View the Standard Curve Plot 37
- Assess amplification results using the Amplification Plot 39
- Assess the gene expression profile using the Gene Expression Plot 46
- Identify well problems using the Well Table 48
- Confirm accurate dye signal using the Multicomponent Plot 50
- Determine signal accuracy using the Raw Data Plot 51
- View the endogenous control profile using the QC Plot 53
- Review the QC flags in the QC Summary 54
- For more information 55

Section 5.2 Adjust parameters for re-analysis of your own experiments 57

- Adjust analysis settings 57
- Improve C_T precision by omitting wells 61
- For more information 62

Section 5.1 Review Results

Analyze the example experiment

1. Open the example experiment file that you ran in Chapter 4.
2. Click **Analyze**. The software analyzes the data using the default analysis settings. You can also access the experiment to analyze from the Home screen.

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

In the standard curve example experiment, you review the Standard Curve Plot screen for the following regression coefficient values:

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

To view and assess the Standard Curve plot

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

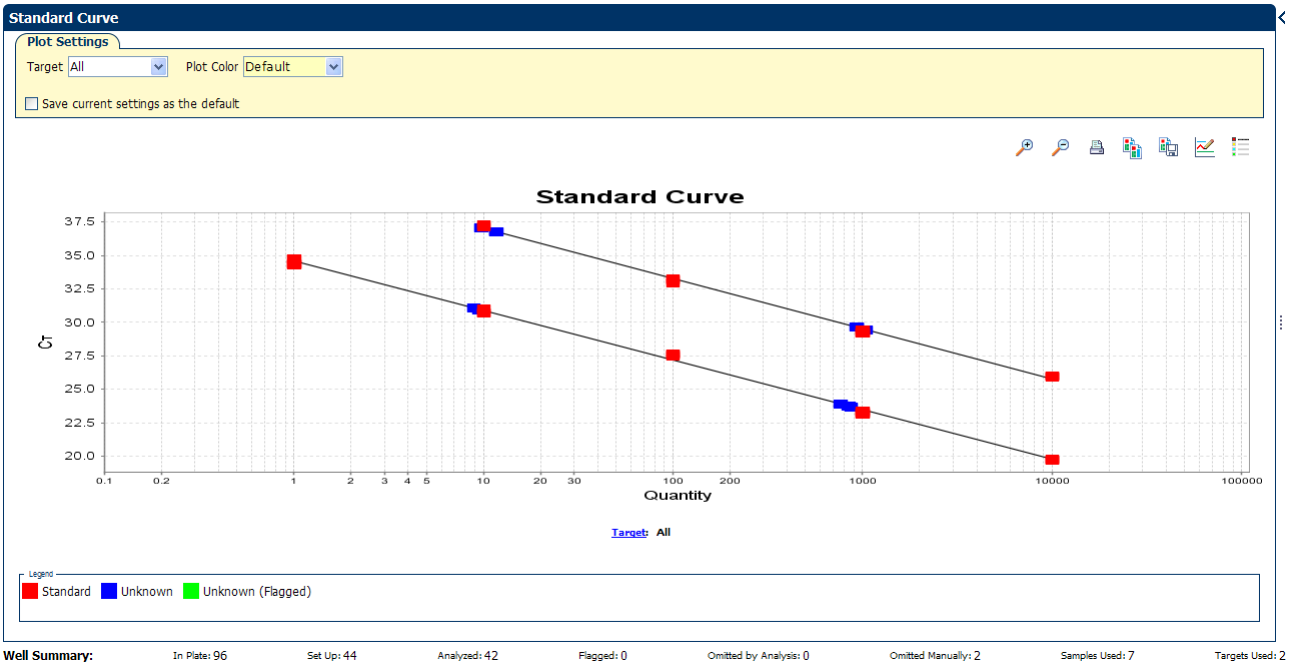
   Note: If no data are displayed, click **Analyze**.
2. Display all 96 wells in the Standard Curve Plot screen by clicking the upper left corner of the plate layout in the Plate Layout tab.
3. Enter the Plot Settings:

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)

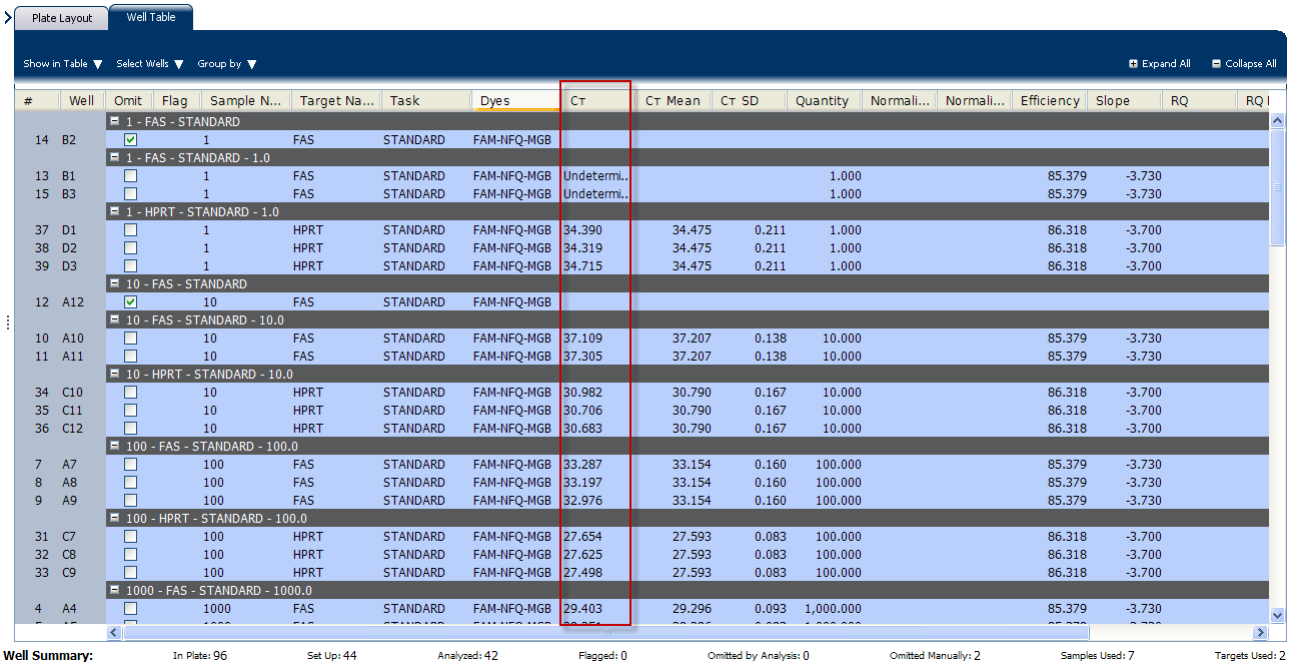
4. View the values displayed below the standard curve. In the example experiment, the values for each target fall within the acceptable ranges:

Target	Slope	R^2 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:
7. Click the **Well Table** tab.
8. From the Group By drop-down menu, select **Replicate**.
9. 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).



Tips for analyzing your own experiments

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

- **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. Factors that 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 C_T precision by omitting wells" on page 61).

  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:

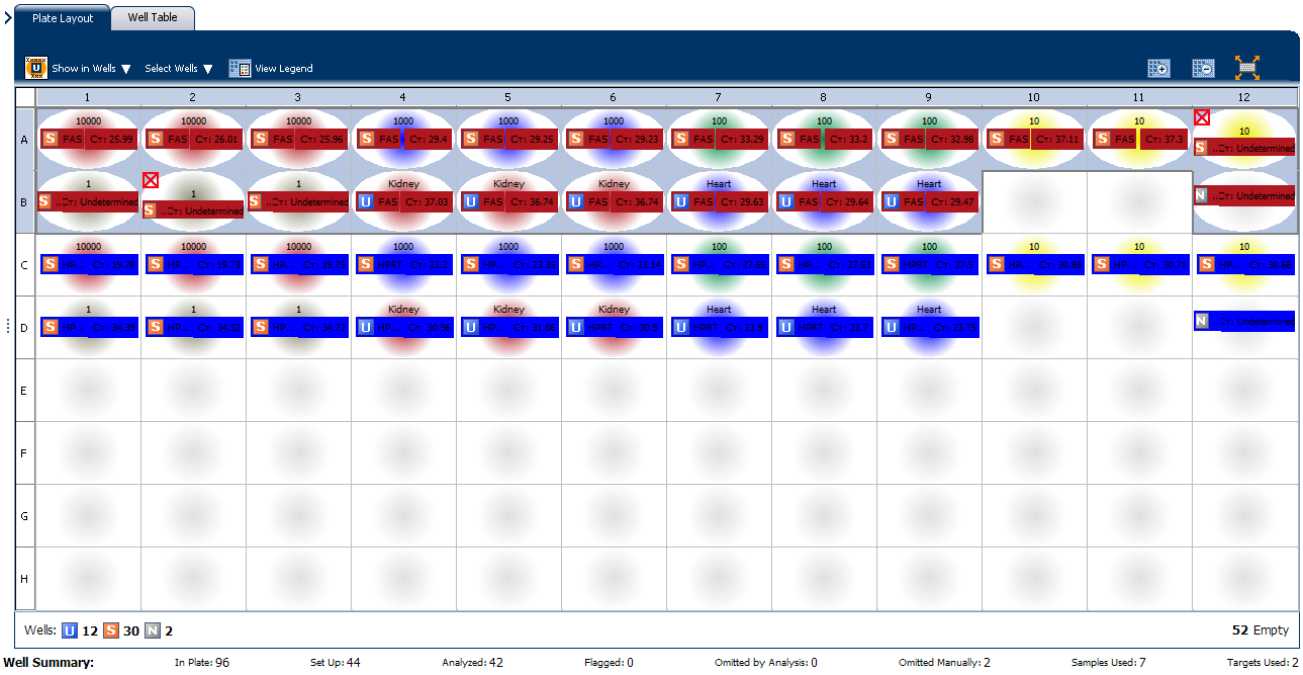
- Correct baseline and threshold values
- Outliers

View the Amplification Plot

1. From the Experiment Menu pane, select **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. From the Select Wells drop-down menu, select **Target**, then **FAS**.
   The Plate Layout screen should look like this:

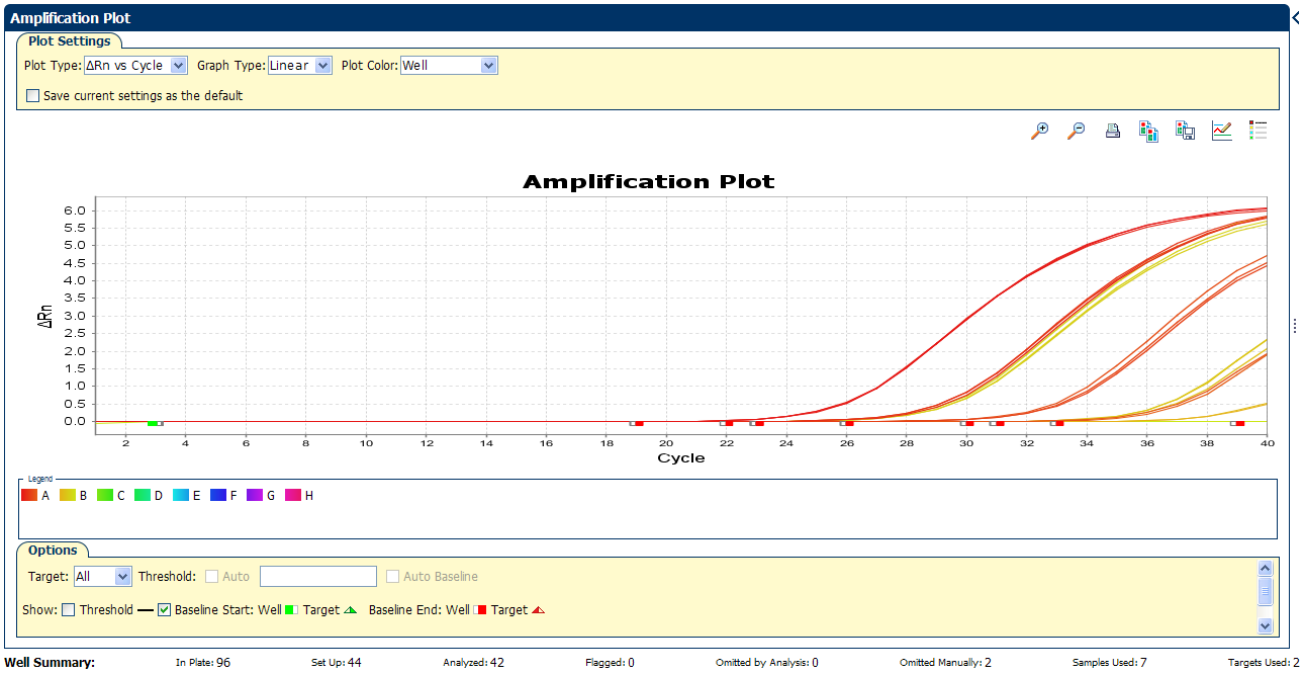


3. In the Amplification Plot screen, enter:

Menu	Selection
Plot Type	ΔRn vs Cycle (default)
Plot Color	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 drop-down menu, select **Linear**.
   b. Select the **Baseline** check box to show the start cycle and end cycle.

c. Verify 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.

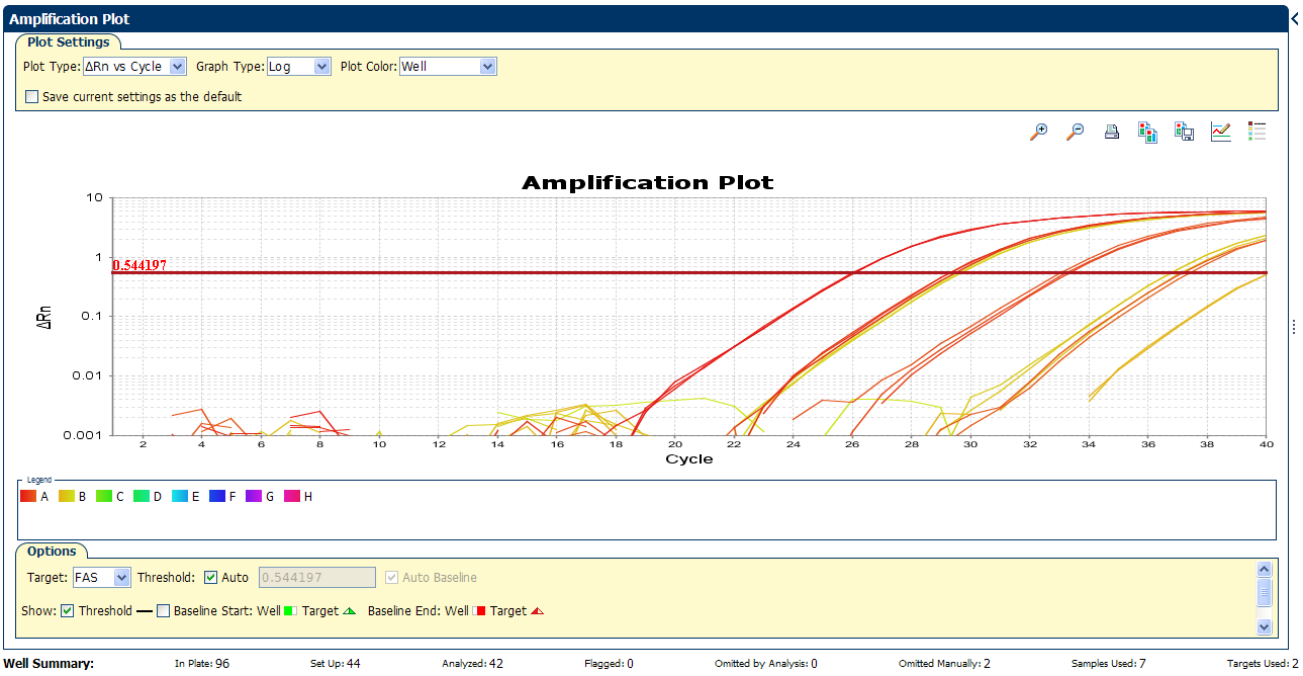


5. View the threshold values:

Menu	Select
Graph Type	Log
Target	FAS

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

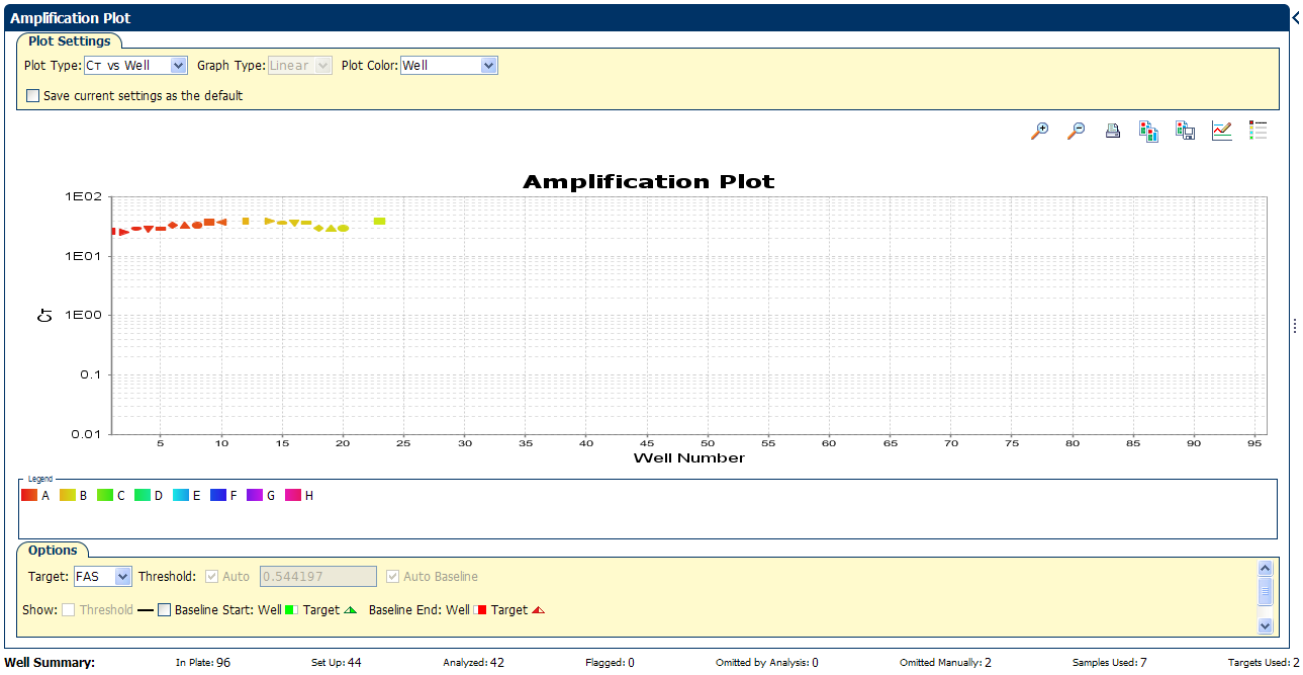
b. Verify that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.



6. Locate outliers:

   a. From the Plot Type drop-down menu, select **C_T vs Well**.

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



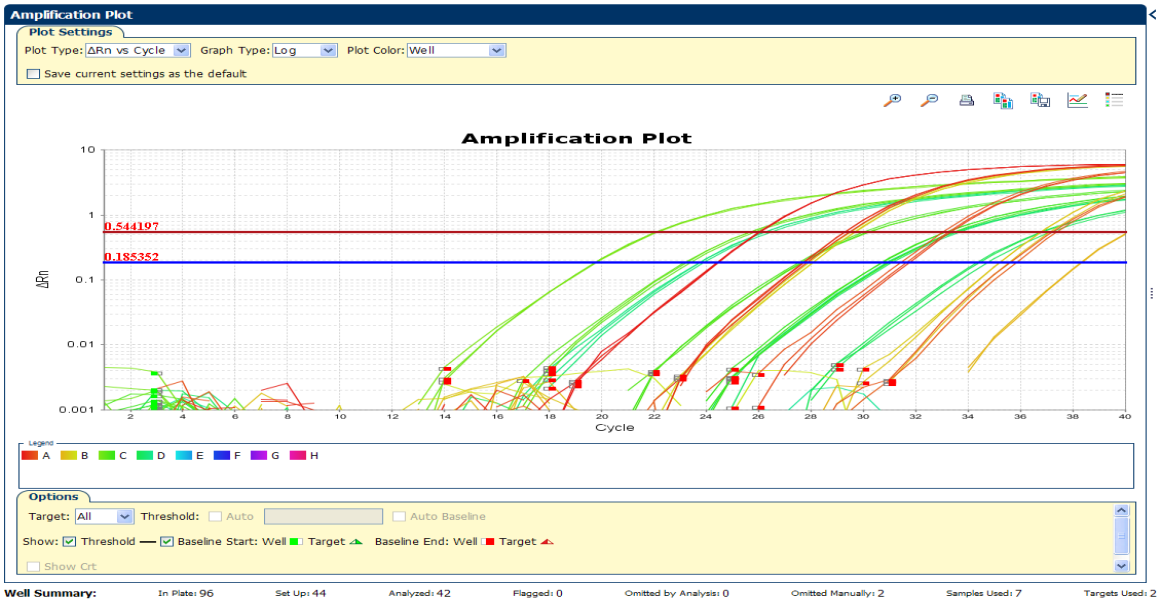
7. Repeat steps 2 through 6 for the HPRT wells.

Tips for analyzing your own experiments

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

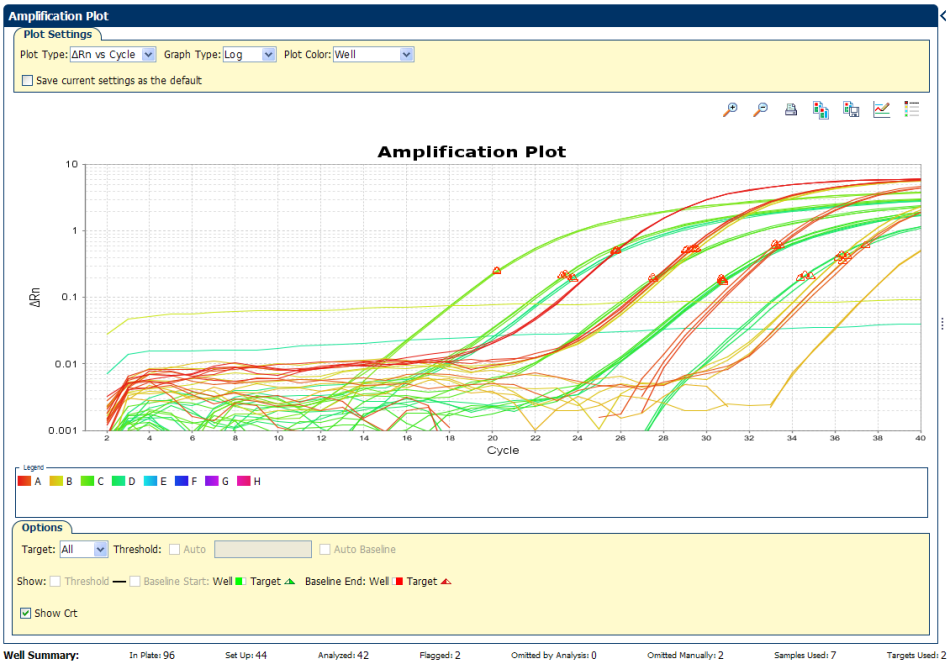
- **Outliers**
- **A typical amplification plot** – The QuantStudio™ 12K Flex Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear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

  A typical amplification plot should look like this:

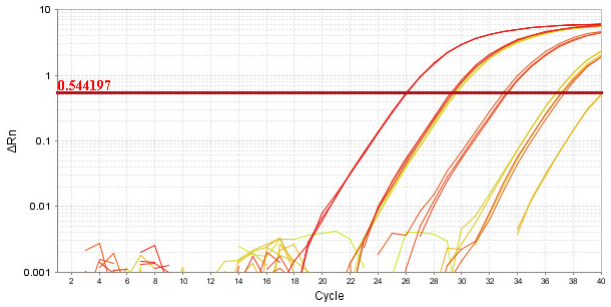
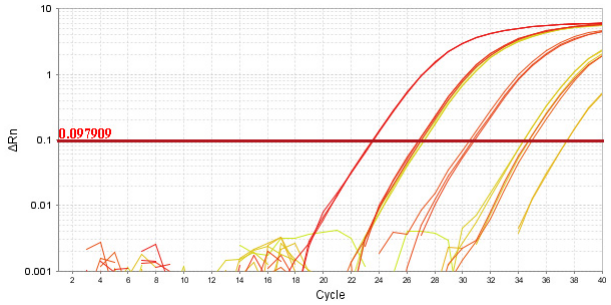


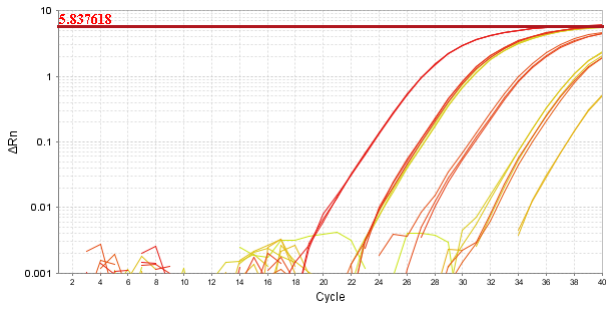
IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudio™ 12K Flex Software. Therefore, Life Technologies recommends that you examine the Amplification 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.

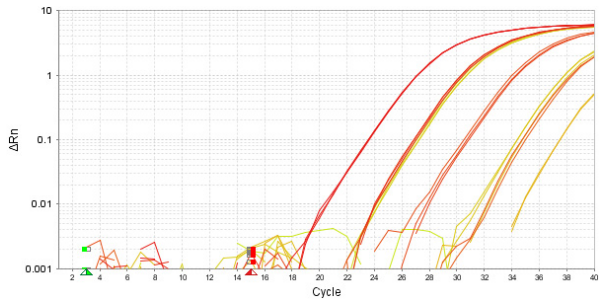
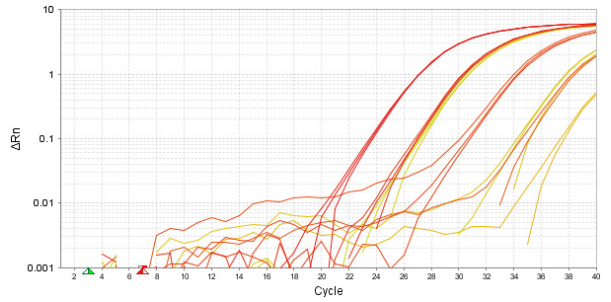
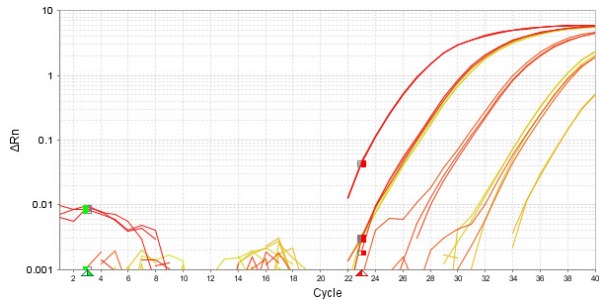


- **Correct threshold values.**

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**

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 C_T precision by omitting wells" on page 61).

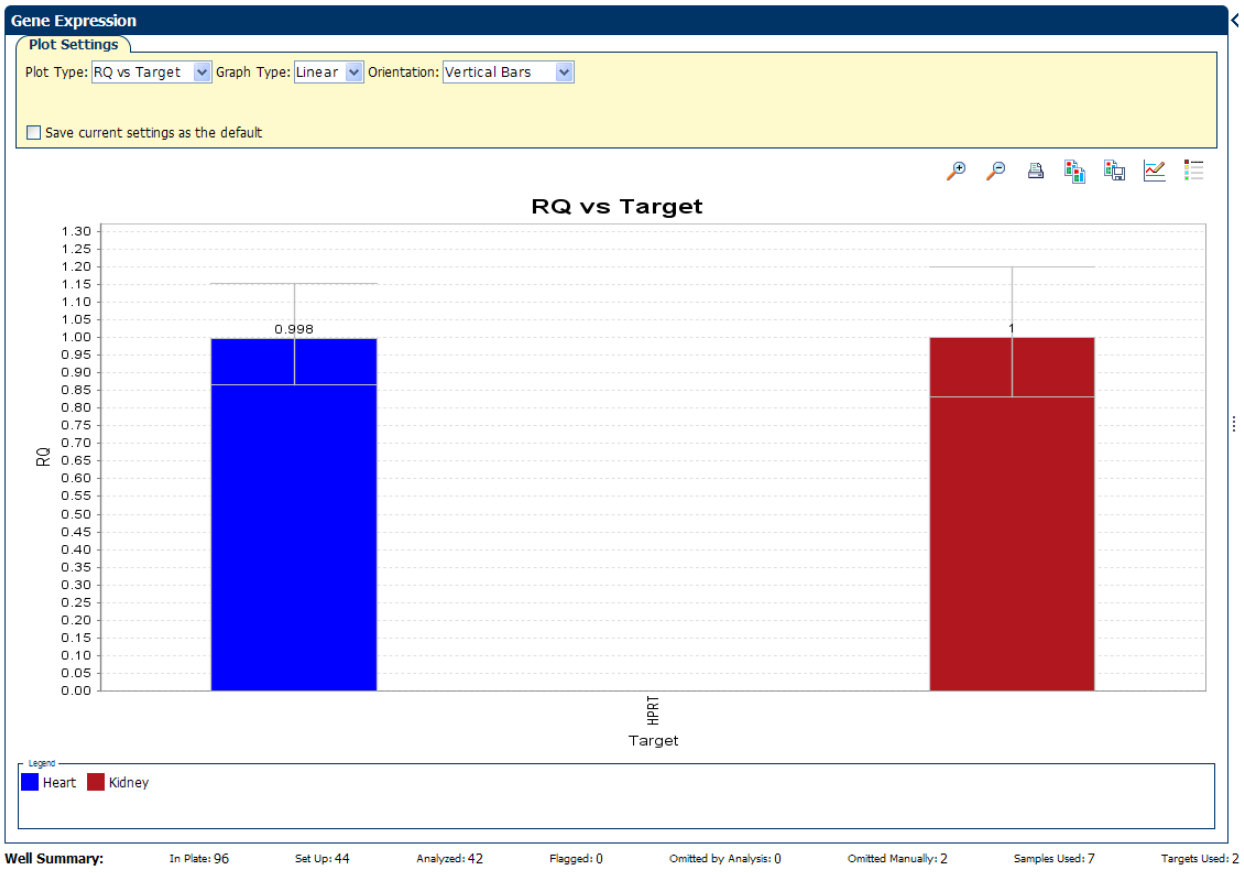
  Or

- Manually adjust the baseline and/or threshold (see "Adjust analysis settings" on page 57).

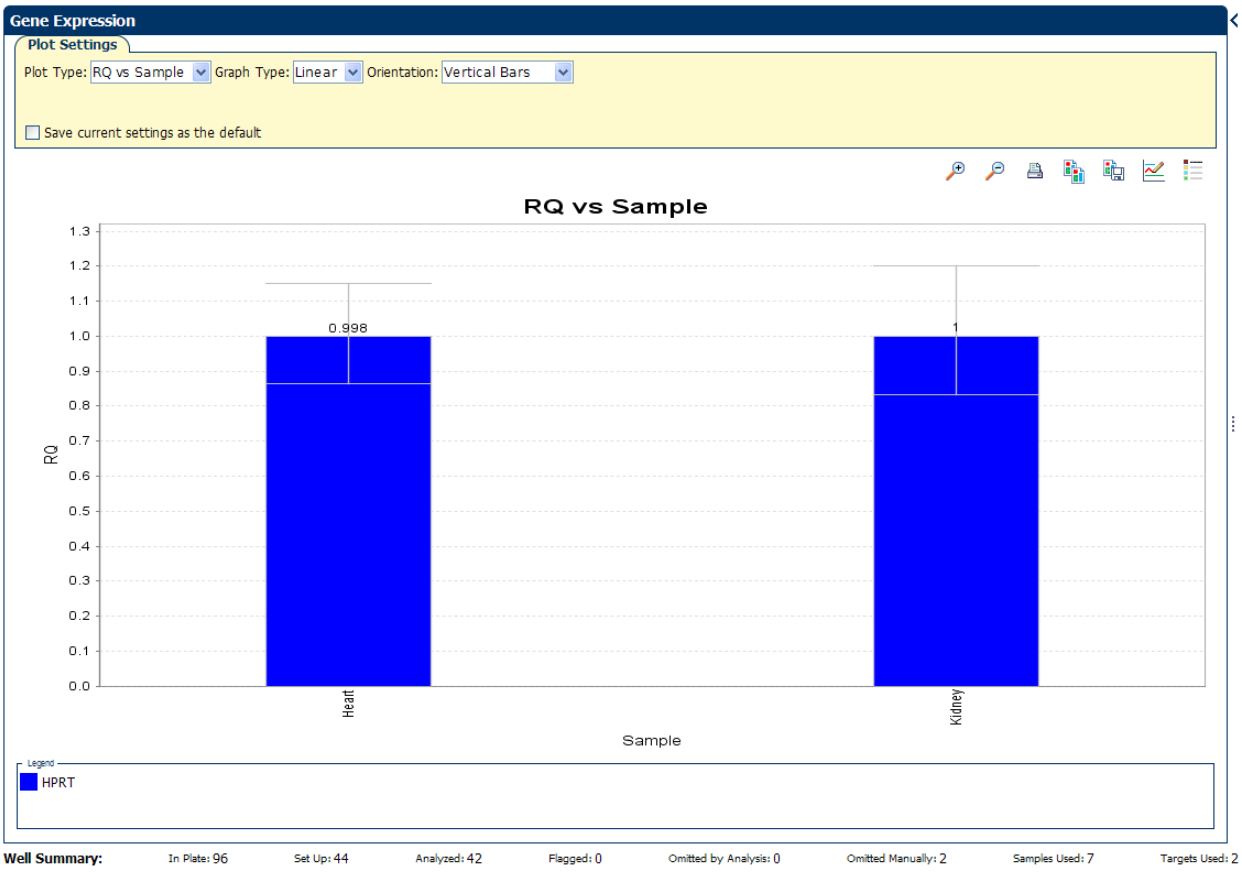
Assess the gene expression profile using the Gene Expression Plot

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. The Gene Expression plot when viewed as a linear graph type looks like this:



- **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. The Gene Expression plot when viewed as a linear graph type looks like this:



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

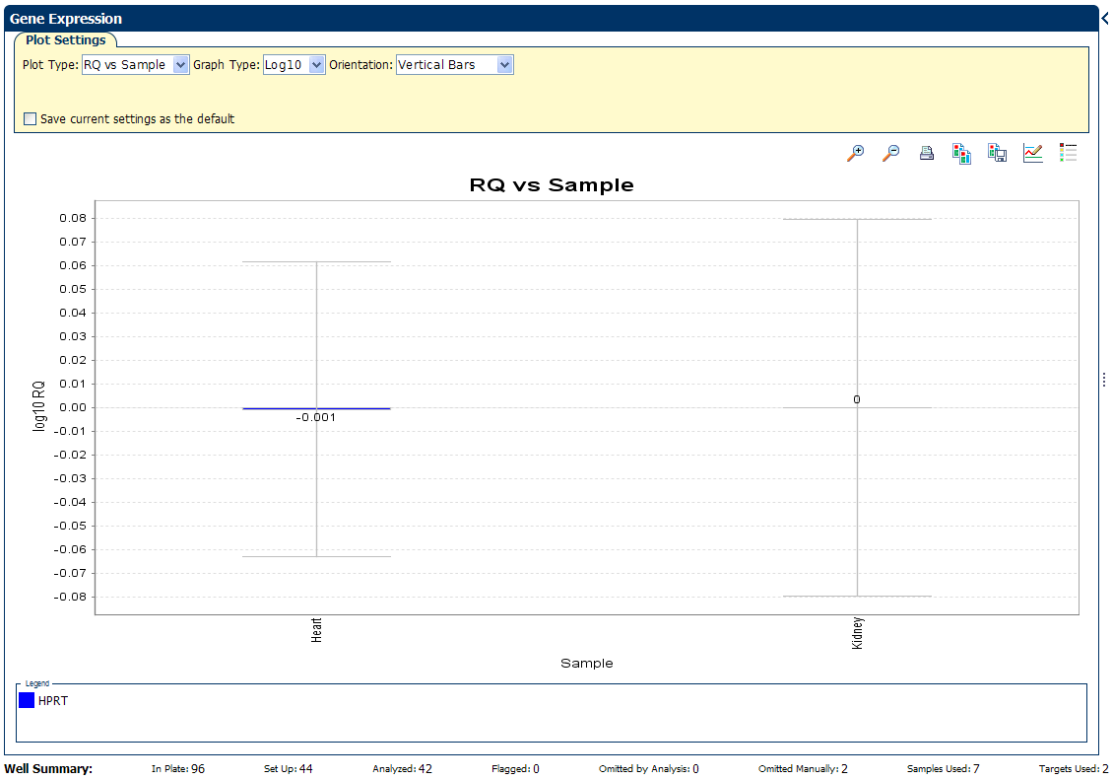
1. From the Experiment Menu pane, select **Analysis** ▸ **Gene Expression**.

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

2. In the Gene Expression Plot screen:
   a. From the Plot Type drop-down menu, select **RQ vs Sample**.
   b. From the Graph Type drop-down menu, select **Log10**.
   c. From the Orientation drop-down menu, 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:

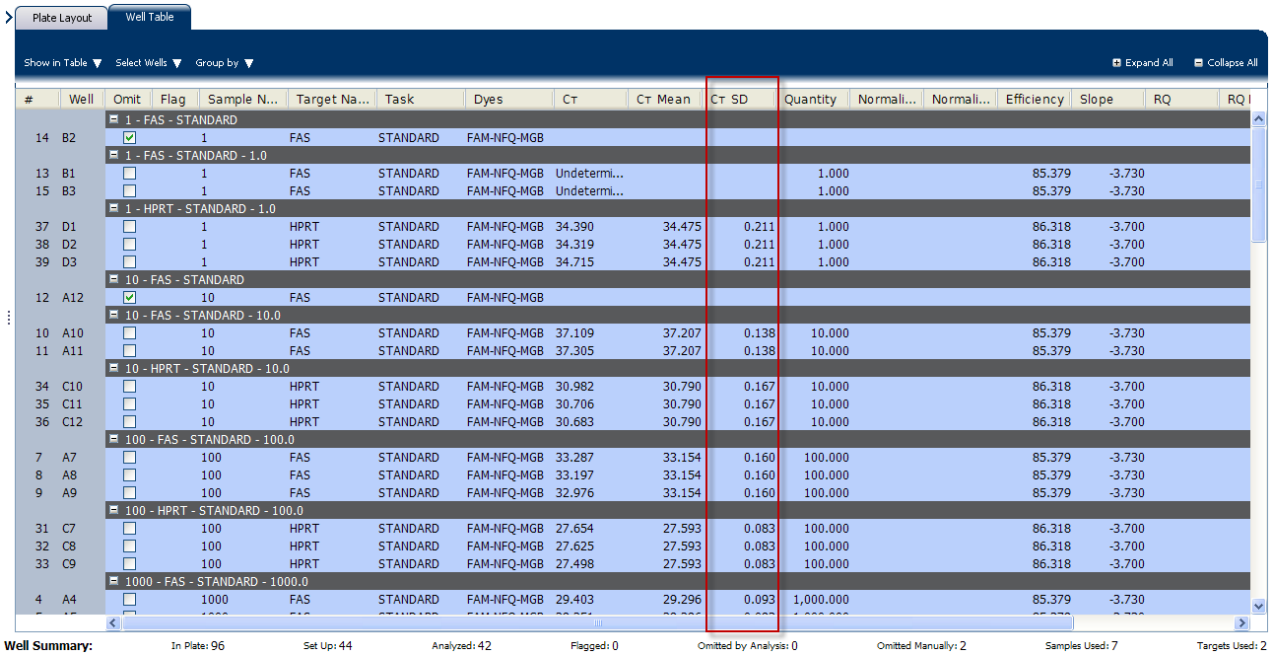
- The sample name, target name, task, and dyes
- The calculated threshold cycle (C_T), normalized fluorescence (Rn), and quantity values
- Flags

Example experiment values and flags

Review the Well Table to evaluate the C_T precision of the replicate groups.

View the well table

1. From the Experiment Menu pane, select **Analysis ▸ Amplification Plot**, then click the **Well Table** tab.
2. From the Group By drop-down menu, 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 drop-down menu.

Assessing 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 C_T precision by omitting wells" on page 61).

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:

- 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. 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 shown in the Multicomponent Plot screen.

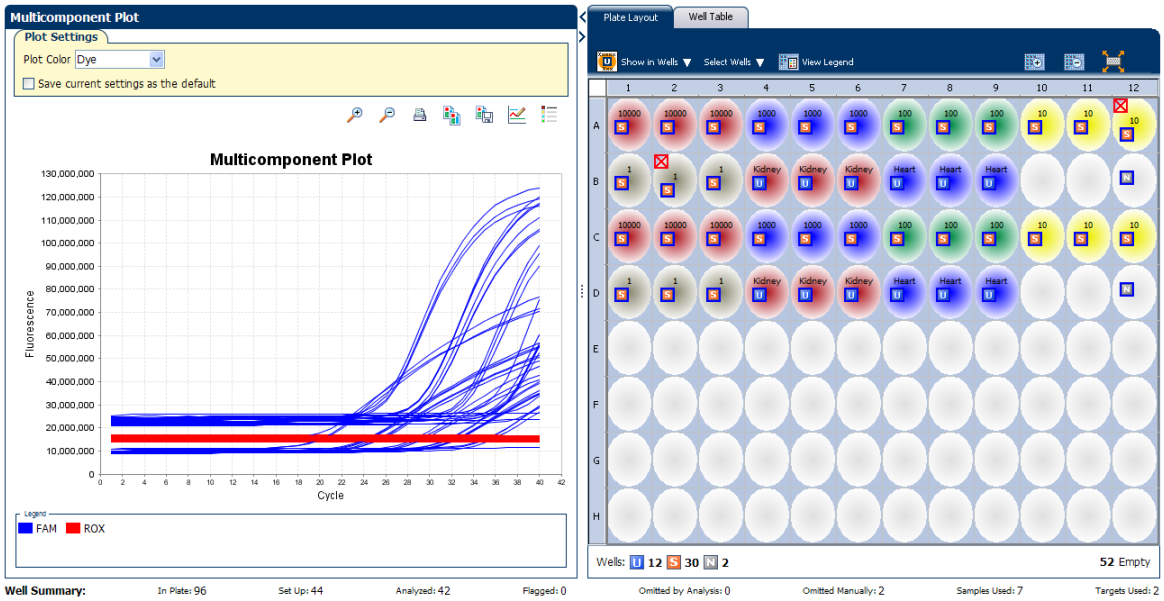
   Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

3. From the Plot Color drop-down menu, select **Dye**.

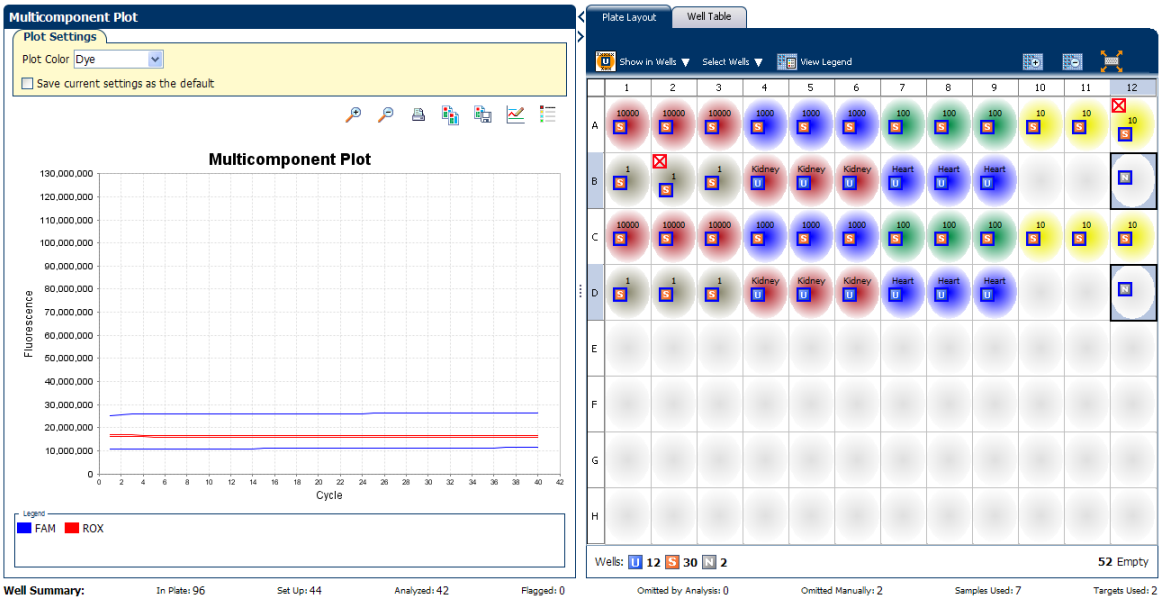
4. Click  **Show a legend for the plot** (default).

   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:

- **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.

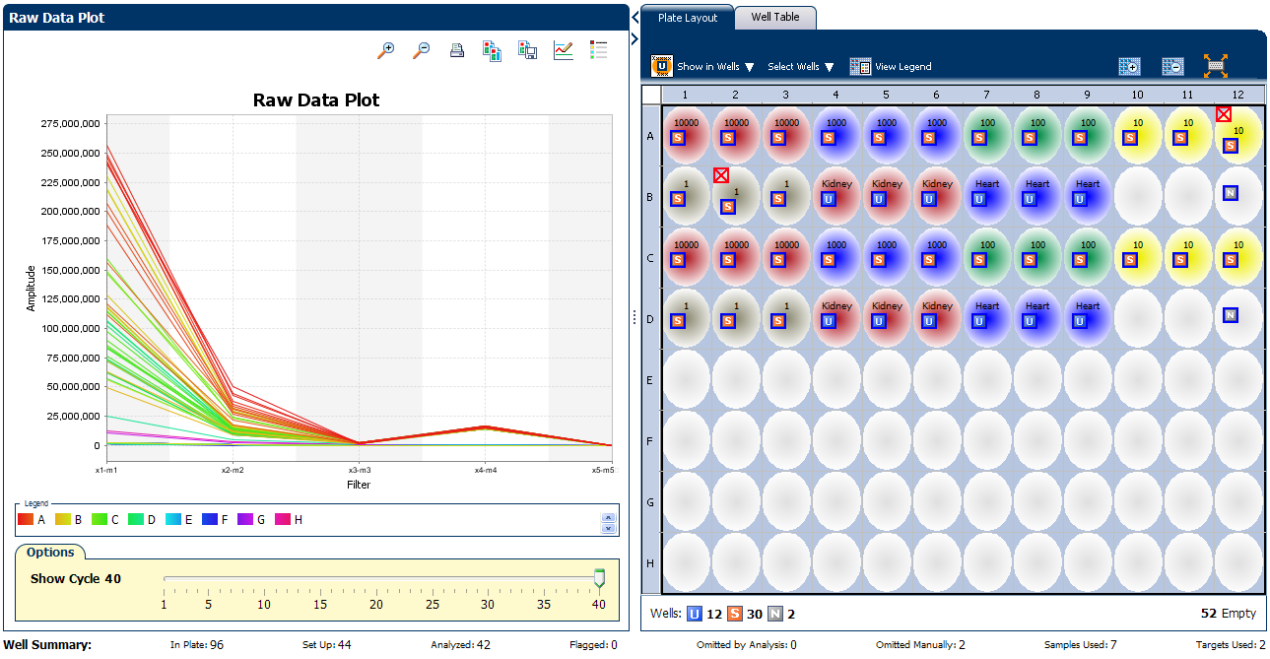
View the Raw Data Plot

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

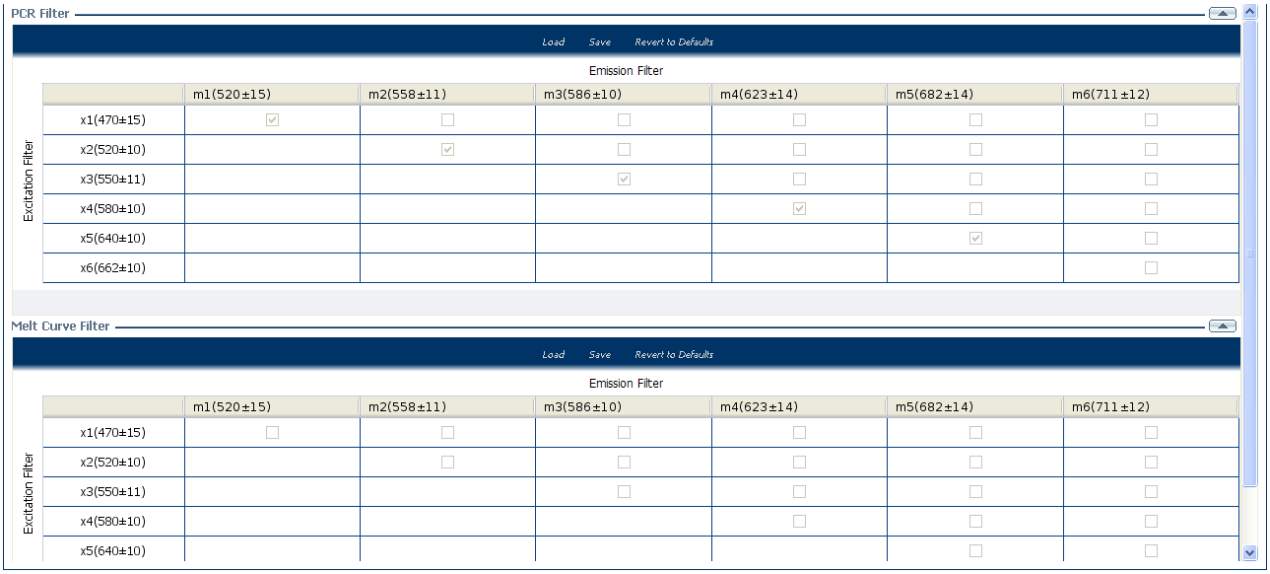
   Note: If no data are displayed, click **Analyze**.
2. Display all 384 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the Plate Layout tab.

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.



The filters used for the example experiment are:



Tips for determining signal accuracy in your own experiment

When you analyze your own Relative Standard Curve 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 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.

To view the QC Plot:

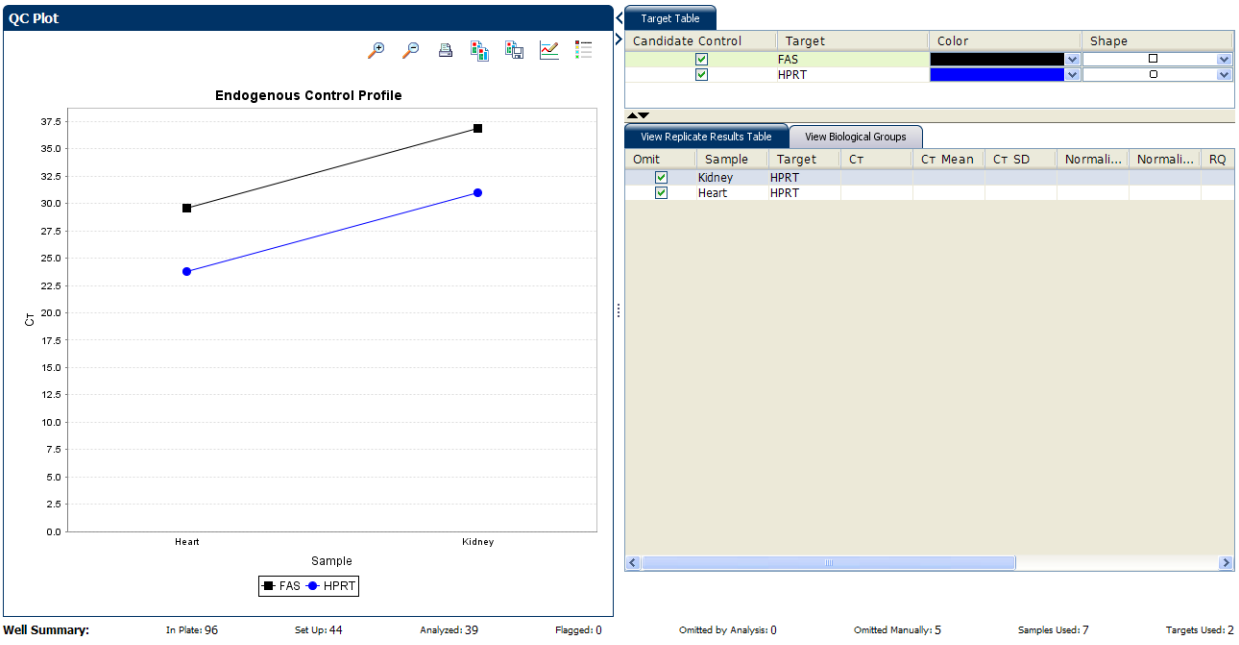
1. From the Experiment Menu pane, select **Analysis ▸ QC Plot**.

   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 drop-down menu.
   c. Select a shape from the Shape drop-down menu.
3. Click the **View Replicate Results Table**.
4. Select the check box of 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.

The QC Plot in the Relative Standard Curve example experiment looks like this.



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

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

2. Review the Flags Summary.

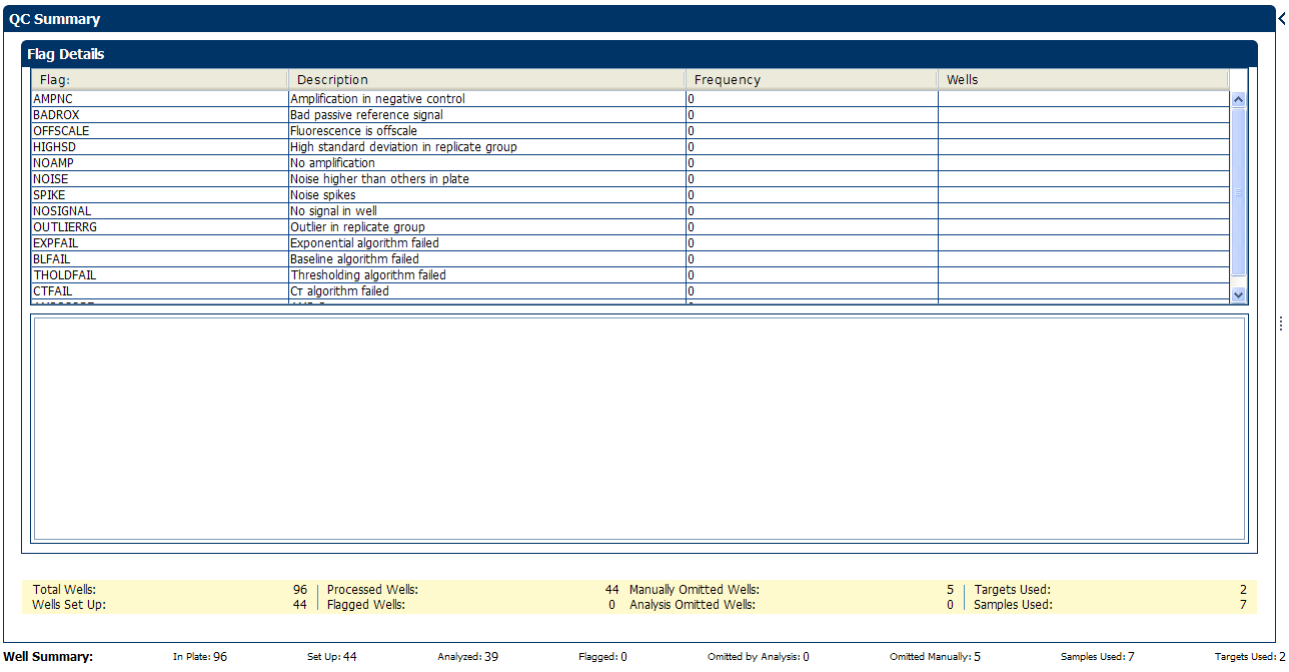
   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment. If the frequency is > 0, the flag appears somewhere in the experiment; the well position is listed in the Wells column.

   In the example experiment, there are no flagged wells.

3. In the Flag Details table, click each flag with a frequency > 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 > 0, click the troubleshooting link to view information on correcting the flag.

The QC Summary screen for the example experiment looks like this:



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
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

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.	4470050

Section 5.2 Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

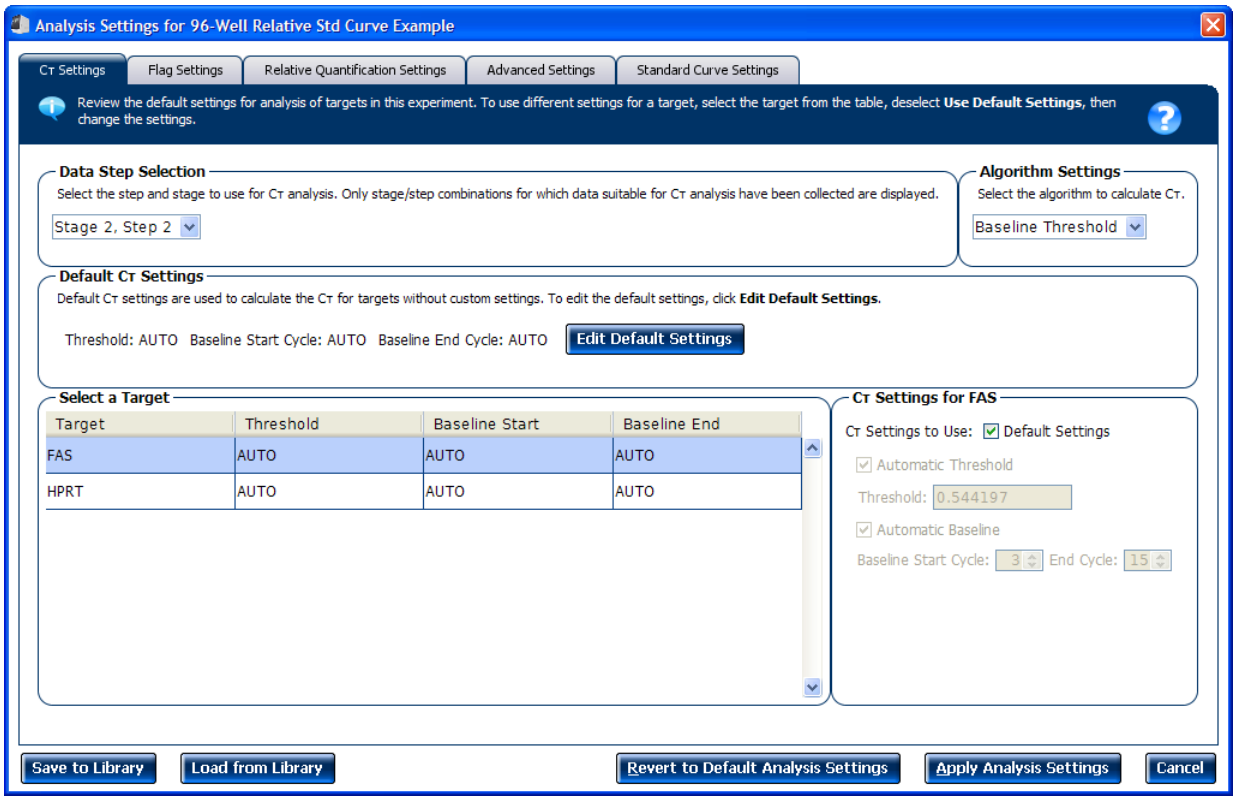
The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, and advanced options.

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
   - Relative Quantification Settings
   - Advanced Settings
   - Standard Curve Settings

   The Analysis Settings dialog box for a Relative Standard Curve experiment looks like this:



3. View and, if necessary, change the analysis settings (see "Adjust analysis settings" below).

   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 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.

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, Life Technologies recommends:

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: This setting is applicable only to the Baseline Threshold algorithm.

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:

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

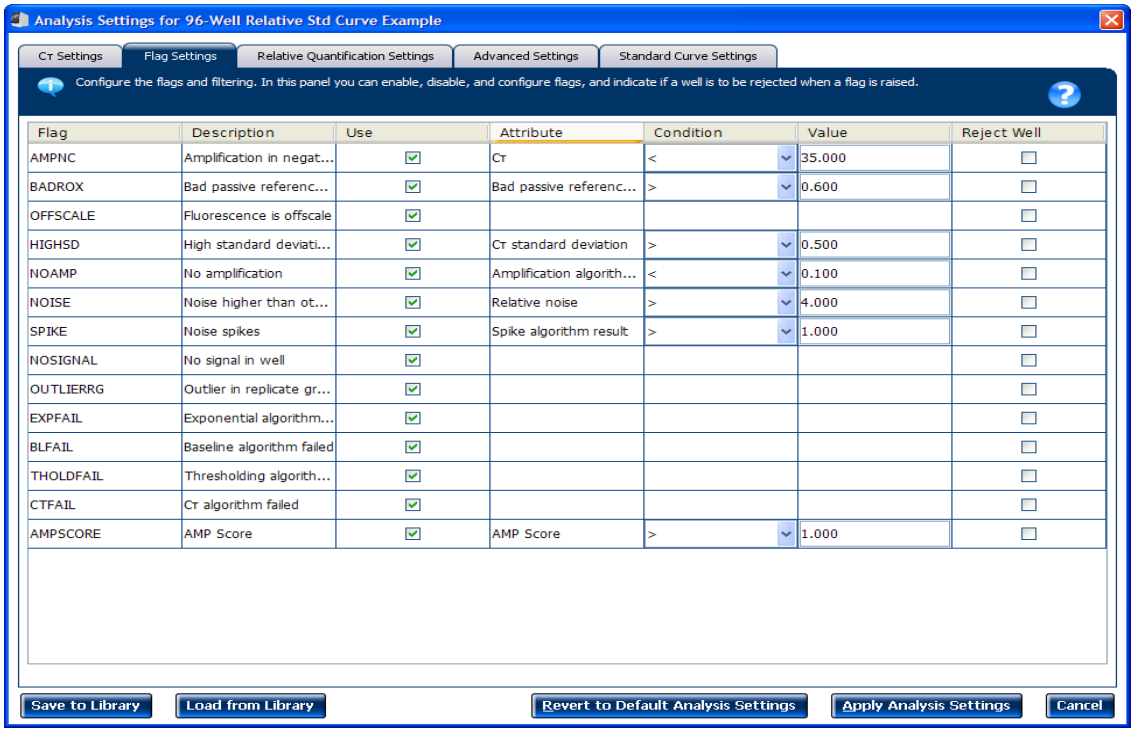
To adjust the flag settings

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.

The Flag Settings tab looks like this:



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.

To use custom baseline settings for a well-target combination:

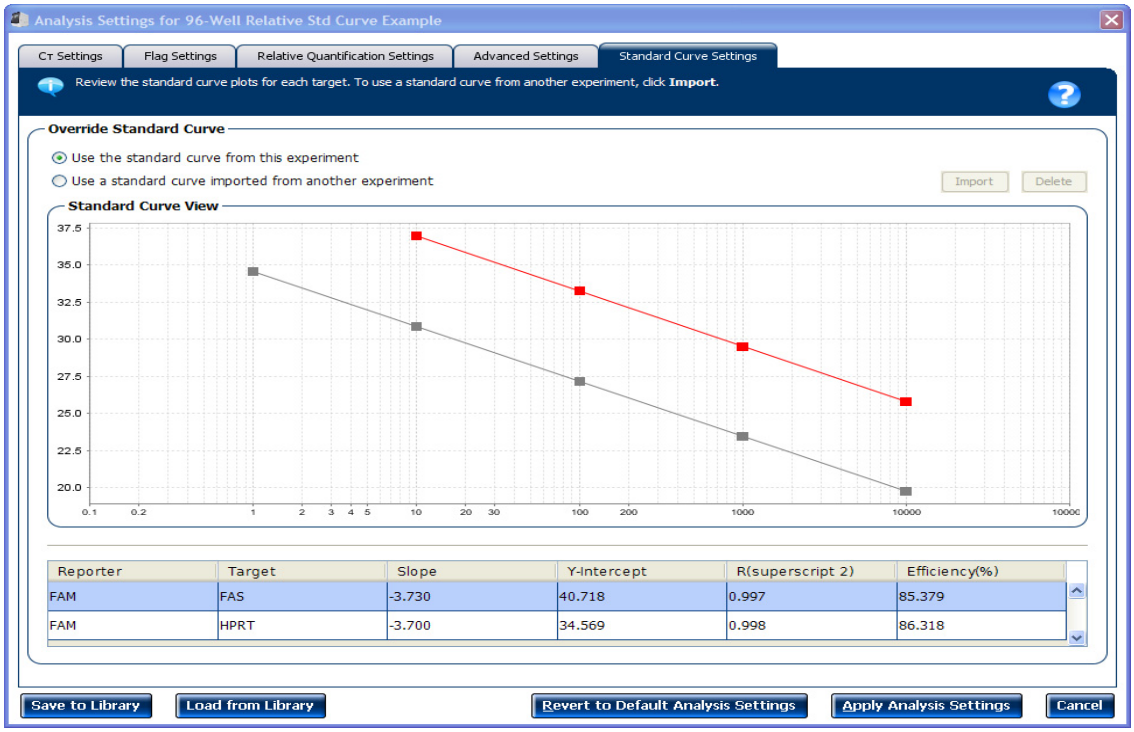
1. Select one or more well-target combinations in the table.
2. Deselect the **Use C_T Settings Defined for Target** check box.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, 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. Life Technologies recommends 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 ensure 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. 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 drop-down menu.

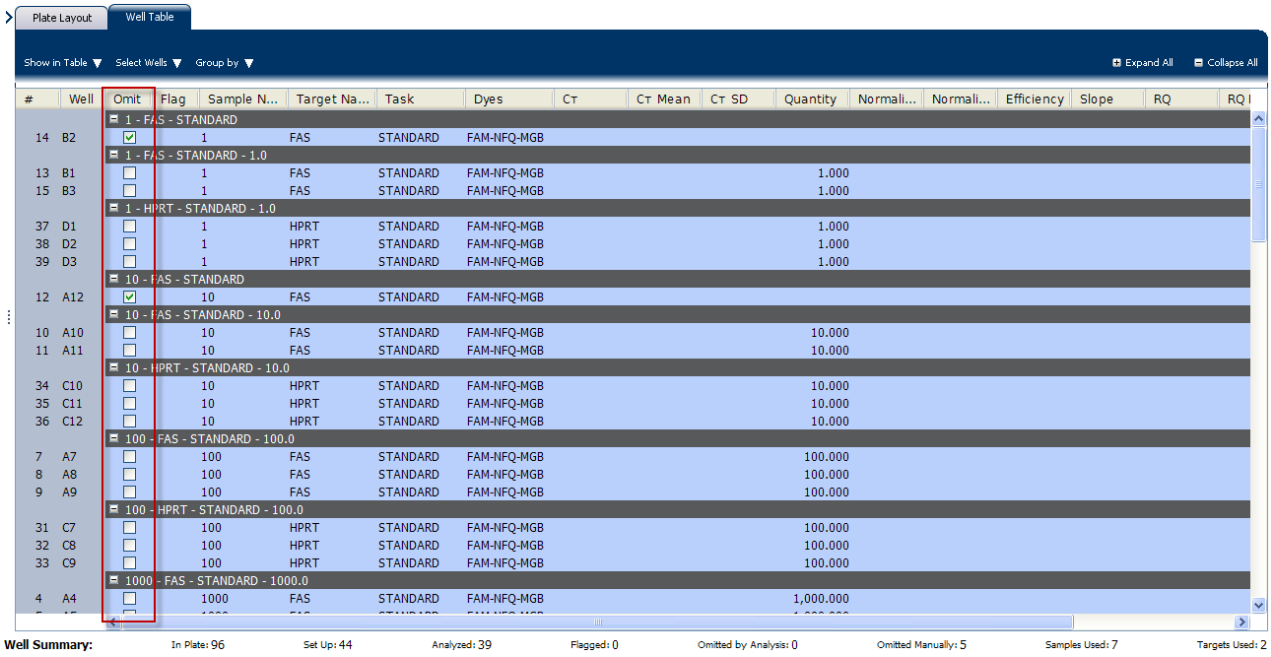
3. Select the **Well Table** tab, select replicates to omit:

4. In the Well Table:

   a. From the Group By drop-down menu, select **Replicate**.

   b. Look for outliers in the replicate group (make sure they are flagged).

c. Select the **Omit** check box next to outlying well(s), as shown below.



#	Well	Omit	Flag	Sample N...	Target Na...	Task	Dyes	CT	CT Mean	CT SD	Quantity	Normali...	Normali...	Efficiency	Slope	RQ	RQ
				1 - FAS - STANDARD													
14	B2	☑		1	FAS	STANDARD	FAM-NFQ-MGB										
				1 - FAS - STANDARD - 1.0													
13	B1	☐		1	FAS	STANDARD	FAM-NFQ-MGB				1.000						
15	B3	☐		1	FAS	STANDARD	FAM-NFQ-MGB				1.000						
				1 - HPRT - STANDARD - 1.0													
37	D1	☐		1	HPRT	STANDARD	FAM-NFQ-MGB				1.000						
38	D2	☐		1	HPRT	STANDARD	FAM-NFQ-MGB				1.000						
39	D3	☐		1	HPRT	STANDARD	FAM-NFQ-MGB				1.000						
				10 - FAS - STANDARD													
12	A12	☑		10	FAS	STANDARD	FAM-NFQ-MGB										
				10 - FAS - STANDARD - 10.0													
10	A10	☐		10	FAS	STANDARD	FAM-NFQ-MGB				10.000						
11	A11	☐		10	FAS	STANDARD	FAM-NFQ-MGB				10.000						
				10 - HPRT - STANDARD - 10.0													
34	C10	☐		10	HPRT	STANDARD	FAM-NFQ-MGB				10.000						
35	C11	☐		10	HPRT	STANDARD	FAM-NFQ-MGB				10.000						
36	C12	☐		10	HPRT	STANDARD	FAM-NFQ-MGB				10.000						
				100 - FAS - STANDARD - 100.0													
7	A7	☐		100	FAS	STANDARD	FAM-NFQ-MGB				100.000						
8	A8	☐		100	FAS	STANDARD	FAM-NFQ-MGB				100.000						
9	A9	☐		100	FAS	STANDARD	FAM-NFQ-MGB				100.000						
				100 - HPRT - STANDARD - 100.0													
31	C7	☐		100	HPRT	STANDARD	FAM-NFQ-MGB				100.000						
32	C8	☐		100	HPRT	STANDARD	FAM-NFQ-MGB				100.000						
33	C9	☐		100	HPRT	STANDARD	FAM-NFQ-MGB				100.000						
				1000 - FAS - STANDARD - 1000.0													
4	A4	☐		1000	FAS	STANDARD	FAM-NFQ-MGB				1,000.000						

5. Click **Analyze** to reanalyze the experiment data with the outlying well(s) 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 and select **Omit**.

For more information

For more information on	Refer to	Part number
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*	127AP05-03

6 Export Analysis Results

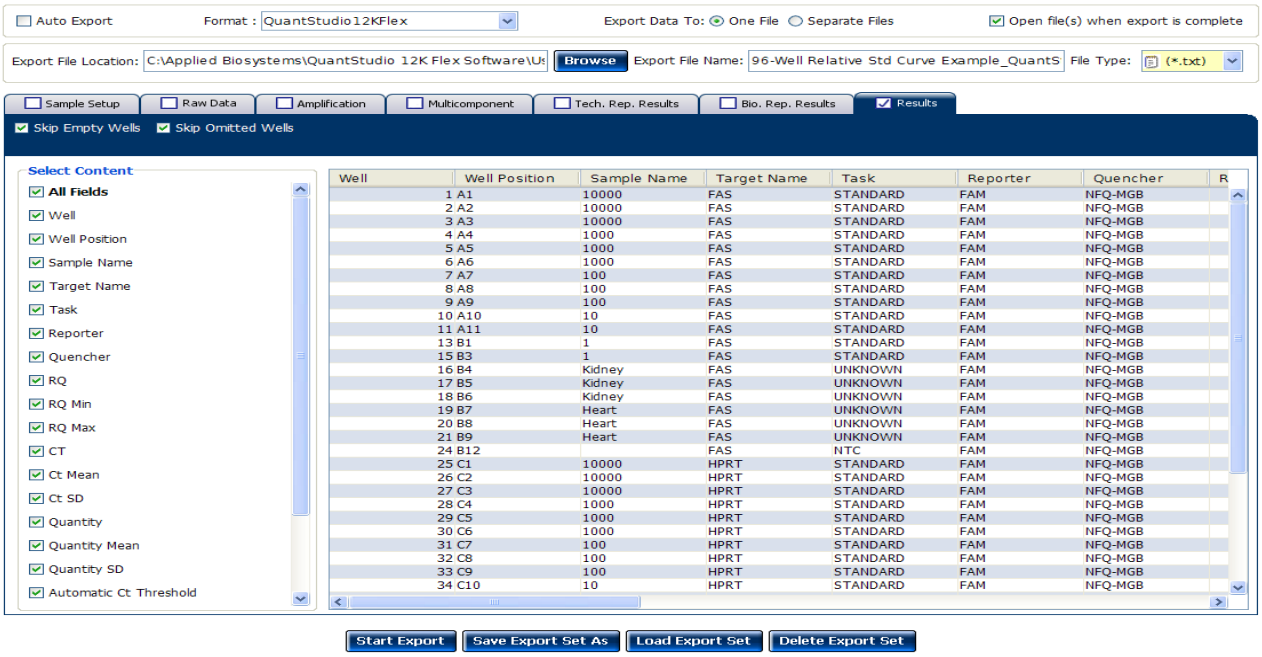
1. Open the Relative Standard Curve example experiment file that you analyzed in Chapter 5.
2. In the Experiment Menu, click  **Export.**

   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.
3. Select **QuantStudio™ 12K Flex format**.

   Note: Select **7900** Format if you want to export the Clipped Data.
4. Complete the Export dialog box as shown below:

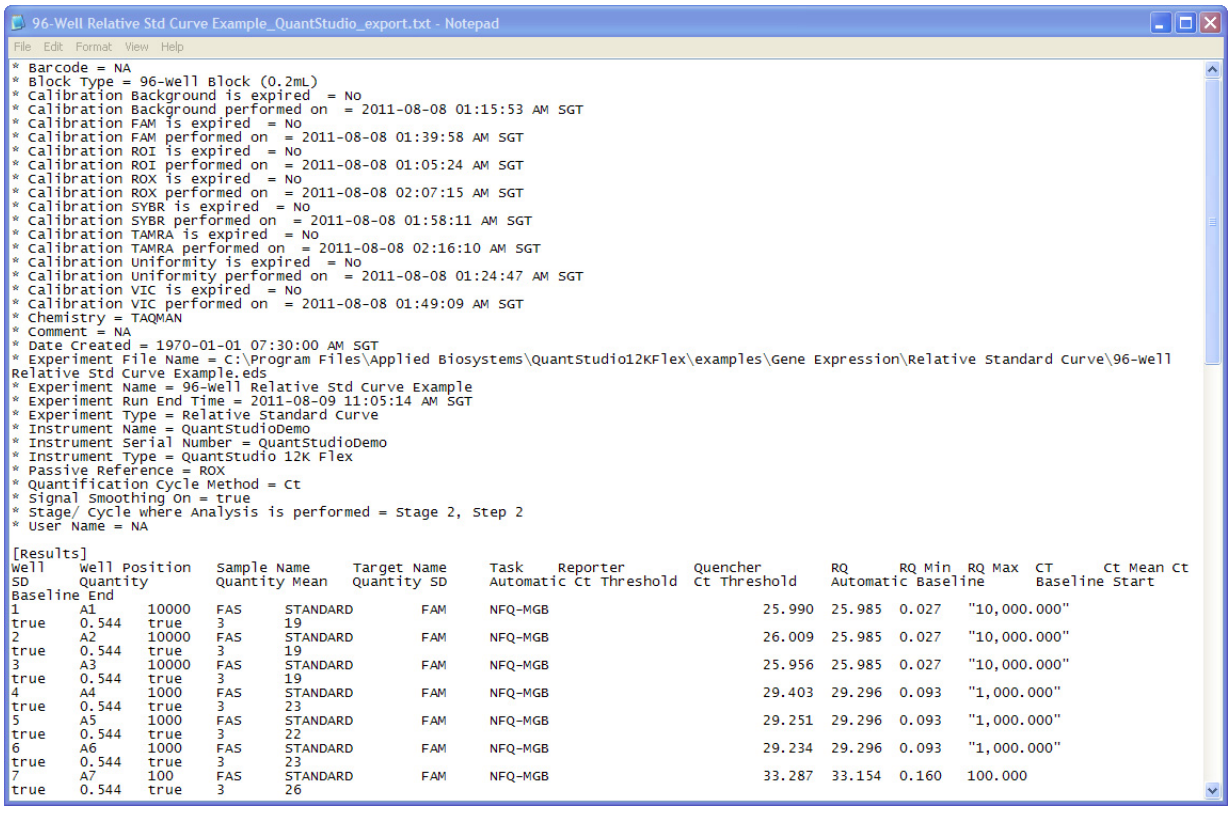
Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	96-Well Relative Std Curve Example_QuantStudio_export
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments

Your Export screen should look like this:



Well	Well Position	Sample Name	Target Name	Task	Reporter	Quencher
1	A1	10000	FAS	STANDARD	FAM	NFQ-MGB
2	A2	10000	FAS	STANDARD	FAM	NFQ-MGB
3	A3	10000	FAS	STANDARD	FAM	NFQ-MGB
4	A4	1000	FAS	STANDARD	FAM	NFQ-MGB
5	A5	1000	FAS	STANDARD	FAM	NFQ-MGB
6	A6	1000	FAS	STANDARD	FAM	NFQ-MGB
7	A7	100	FAS	STANDARD	FAM	NFQ-MGB
8	A8	100	FAS	STANDARD	FAM	NFQ-MGB
9	A9	100	FAS	STANDARD	FAM	NFQ-MGB
10	A10	10	FAS	STANDARD	FAM	NFQ-MGB
11	A11	10	FAS	STANDARD	FAM	NFQ-MGB
13	B1	1	FAS	STANDARD	FAM	NFQ-MGB
15	B3	1	FAS	STANDARD	FAM	NFQ-MGB
16	B4	Kidney	FAS	UNKNOWN	FAM	NFQ-MGB
17	B5	Kidney	FAS	UNKNOWN	FAM	NFQ-MGB
18	B6	Kidney	FAS	UNKNOWN	FAM	NFQ-MGB
19	B7	Heart	FAS	UNKNOWN	FAM	NFQ-MGB
20	B8	Heart	FAS	UNKNOWN	FAM	NFQ-MGB
21	B9	Heart	FAS	UNKNOWN	FAM	NFQ-MGB
24	B12		FAS	NTC	FAM	NFQ-MGB
25	C1	10000	HPRT	STANDARD	FAM	NFQ-MGB
26	C2	10000	HPRT	STANDARD	FAM	NFQ-MGB
27	C3	10000	HPRT	STANDARD	FAM	NFQ-MGB
28	C4	1000	HPRT	STANDARD	FAM	NFQ-MGB
29	C5	1000	HPRT	STANDARD	FAM	NFQ-MGB
30	C6	1000	HPRT	STANDARD	FAM	NFQ-MGB
31	C7	100	HPRT	STANDARD	FAM	NFQ-MGB
32	C8	100	HPRT	STANDARD	FAM	NFQ-MGB
33	C9	100	HPRT	STANDARD	FAM	NFQ-MGB
34	C10	10	HPRT	STANDARD	FAM	NFQ-MGB

Your exported file when opened in Notepad should look like this:




```
* 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\Gene Expression\Relative Standard Curve\96-Well
Relative Std Curve Example.eds
* Experiment Name = 96-Well Relative Std Curve Example
* Experiment Run End Time = 2011-08-09 11:05:14 AM SGT
* Experiment Type = Relative 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

[Results]
Well    Well Position   Sample Name     Target Name     Task    Reporter        Quencher        RQ      RQ Min  RQ Max  CT      Ct Mean Ct
SD      Quantity        Quantity Mean   Quantity SD     Automatic Ct Threshold  Ct Threshold    Automatic Baseline      Baseline Start
Baseline End
1       A1      10000   FAS     STANDARD        FAM     NFQ-MGB                         25.990  25.985  0.027   "10,000.000"
true    0.544   true    3       19
2       A2      10000   FAS     STANDARD        FAM     NFQ-MGB                         26.009  25.985  0.027   "10,000.000"
true    0.544   true    3       19
3       A3      10000   FAS     STANDARD        FAM     NFQ-MGB                         25.956  25.985  0.027   "10,000.000"
true    0.544   true    3       19
4       A4      1000    FAS     STANDARD        FAM     NFQ-MGB                         29.403  29.296  0.093   "1,000.000"
true    0.544   true    3       23
5       A5      1000    FAS     STANDARD        FAM     NFQ-MGB                         29.251  29.296  0.093   "1,000.000"
true    0.544   true    3       22
6       A6      1000    FAS     STANDARD        FAM     NFQ-MGB                         29.234  29.296  0.093   "1,000.000"
true    0.544   true    3       23
7       A7      100     FAS     STANDARD        FAM     NFQ-MGB                         33.287  33.154  0.160   100.000
true    0.544   true    3       26
```

PART II
Running Comparative C_T Experiments

7 About Comparative C_T Experiments

This chapter covers:

- About Comparative C_T experiments 69
- About the example experiment 71

IMPORTANT! First-time users of the QuantStudio™ 12K Flex System, please read Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* and Booklet 7, *QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes* of this binder thoroughly. The booklets provide information and general instructions that are applicable to all the experiments described in this binder.

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 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:

- 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 and multiplex PCR (below)

  and

- 1-step and 2-step RT-PCR (page 70)

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. Typically, a probe labeled with FAM™ dye detects the target and a probe 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 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 Invitrogen VILO™ 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 Life Technologies TaqMan® Gene Expression Assays 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

8 Design the Experiment

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

This chapter covers:

- Define the experiment properties. 73
- Define targets, samples and biological replicates 74
- Assign targets, samples and biological groups 75
- Set up the run method 77
- Tips for designing your own experiment 78
- For more information. 79

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 on Auto Export, refer to Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.

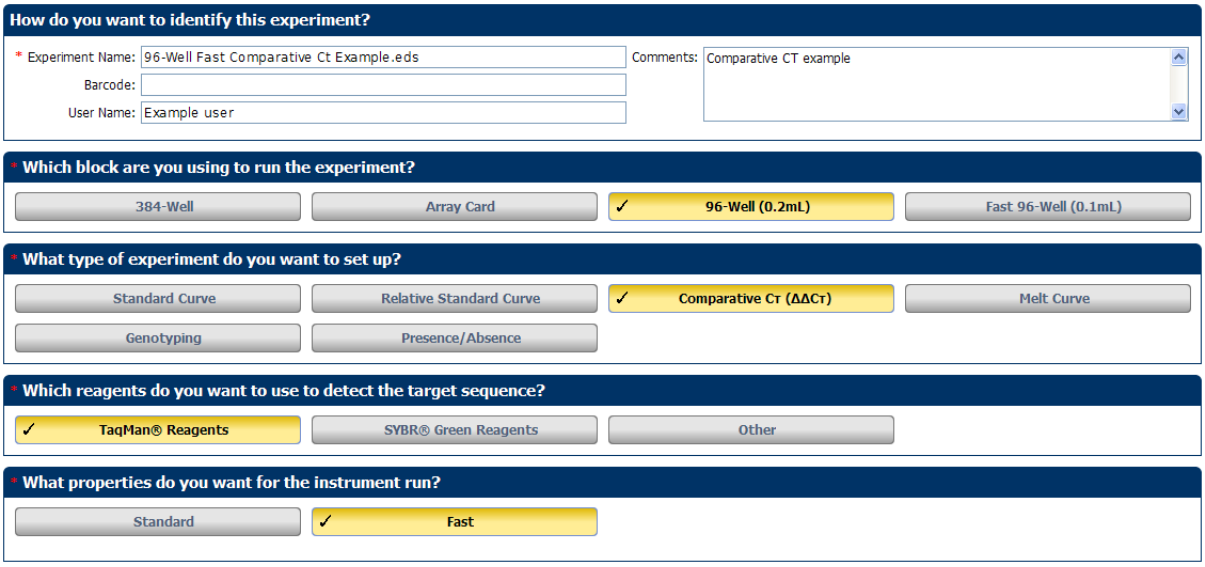
Define the experiment properties

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

Field or Selection	Entry
Experiment Name	96-Well Fast Comparative Ct Example.eds
Barcode	Leave field empty
User Name	Example User
Comments	Comparative C_T example
Block	96-Well (0.2mL)
Experiment Type	Comparative C_T ($\Delta\Delta C_T$)
Reagents	TaqMan® Reagents
Ramp speed	Fast

Save the experiment.

Your Experiment Properties screen look like this:



Define targets, samples and biological replicates

Click **Define** to access the Define screen. Enter:

1. Targets

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	

2. Samples

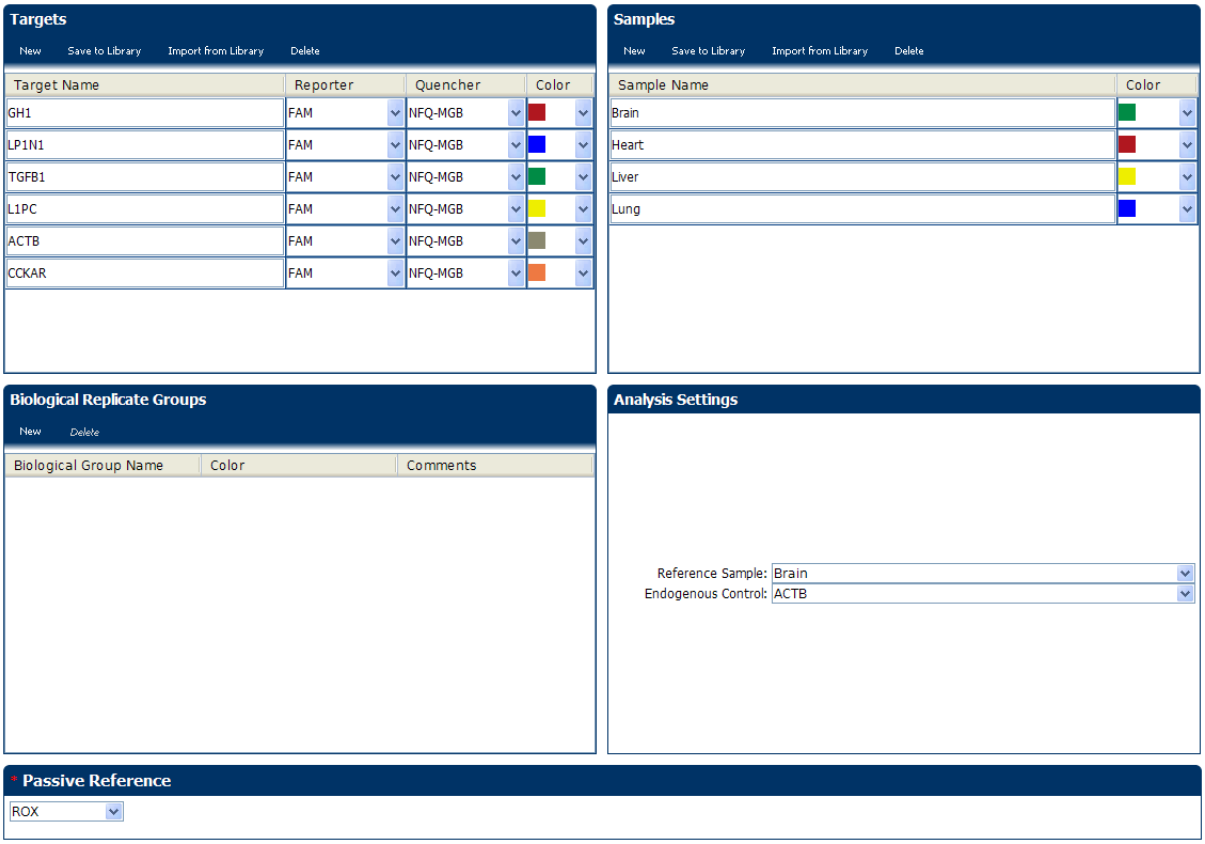
Sample Name	Color
Brain	
Lung	
Liver	
Heart	

3. Dye to be used as a Passive Reference
ROX

4. Analysis Settings

Field	Select
Reference Sample	Brain
Endogenous Control	ACTB

Your Define screen should look like this:



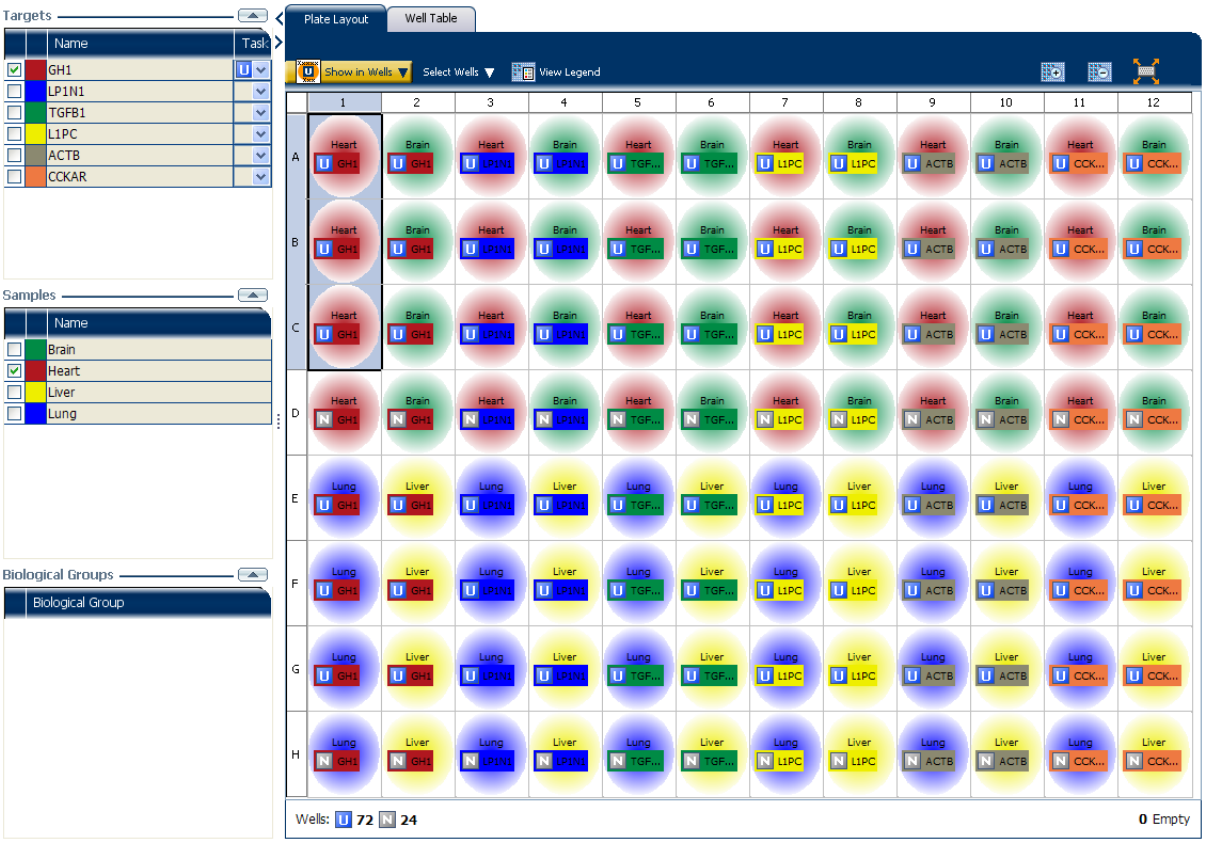
Note: This example experiment does not define biological replicate groups. Leave Biological Replicate Groups blank.

Assign targets, samples and biological groups

Click **Assign** to access the Assign screen. Enter the 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
	A12, B12, C12	Unknown	Brain
	E11, F11, G11	Unknown	Lung
	E12, F12, G12	Unknown	Liver
	D11, D12, H11, H12	Negative	Heart, Brain, Lung, Liver

Your Assign screen should look like this:



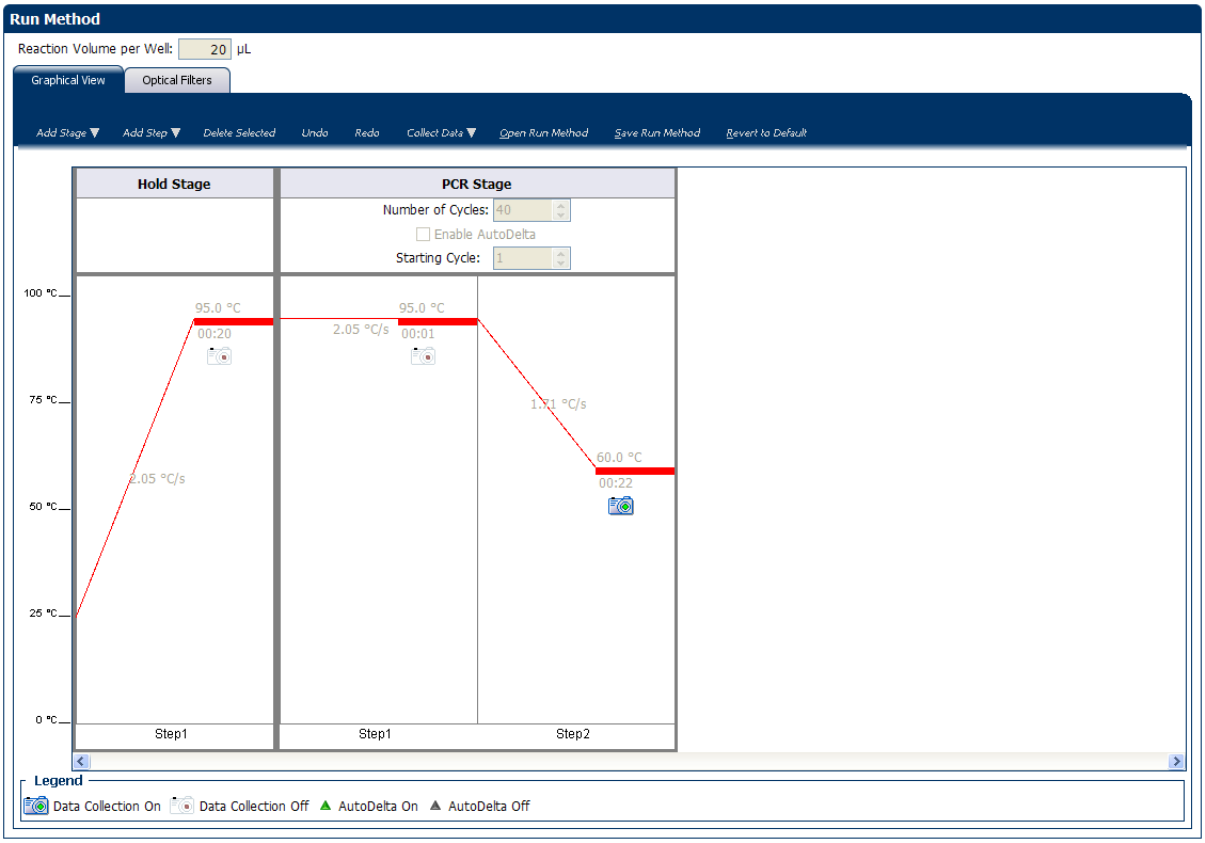
Set up the run method

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

- 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

Your Run Method screen should look like this:



Tips for designing your own experiment

Life Technologies recommends that you:

- 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

For more information on	Refer to	Part number
Consumables	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* *Appendix A in Booklet 7, QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes*	4470050
Using the Standard Curve quantification methods	Booklet 2, *Running Standard Curve Experiments*.	4470050
Using the Relative Standard Curve quantification method	Part 1 of this booklet	4470050
Selecting an endogenous control	Application Note *Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies*	127AP05-03
Reference samples (also known as calibrators) and endogenous controls	*User Bulletin #2: Relative quantification of Gene Expression*	4303859
Using alternative setup	Chapter 2 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

9 Prepare the Reactions

This chapter explains how to prepare the PCR reactions for the Comparative C_T ($\Delta\Delta C_T$) example experiment.

This chapter covers:

- Assemble required materials 81
- Prepare the template 81
- Prepare the sample dilutions 82
- Prepare the reaction mix (“cocktail mix”). 82
- Prepare the reaction plate 83
- Tips for preparing reactions for your own experiments. 84
- For more information. 85

Assemble required materials

- Items listed in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.
- 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 one of the Invitrogen VILO™ kits to carry out the 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 using one of the Invitrogen VILO™ kits, SuperScript® VILO™ cDNA Synthesis Kit (PN 4453650).

Prepare the template

Use the Invitrogen VILO™ kits to reverse-transcribe cDNA from the total RNA samples. Follow the procedures in the *Invitrogen VILO Kits Protocol* (PN 100002284) to:

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 (µL)
1	Liver	19
2	Heart	19
3	Brain	19
4	Lung	19

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

Tube	Sample name	Volume (µL)
1	Liver	1.0
2	Heart	1.0
3	Brain	1.0
4	Lung	1.0

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 ("cocktail 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 (µL) for 1 reaction	Volume (µL) for 16 reactions (plus 10% excess)
TaqMan® Fast Universal PCR Master Mix (2X)	10.0	176.0
ACTB Assay Mix (20X)	1.0	17.6
Water	8	140.8
Total Reaction Mix Volume	19.0	158.4

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 steps 2 through 5 for the TGFB1, GH1, LPIN1, LIPC, and CCKAR assays.

   Note: Do not add the sample at this time.

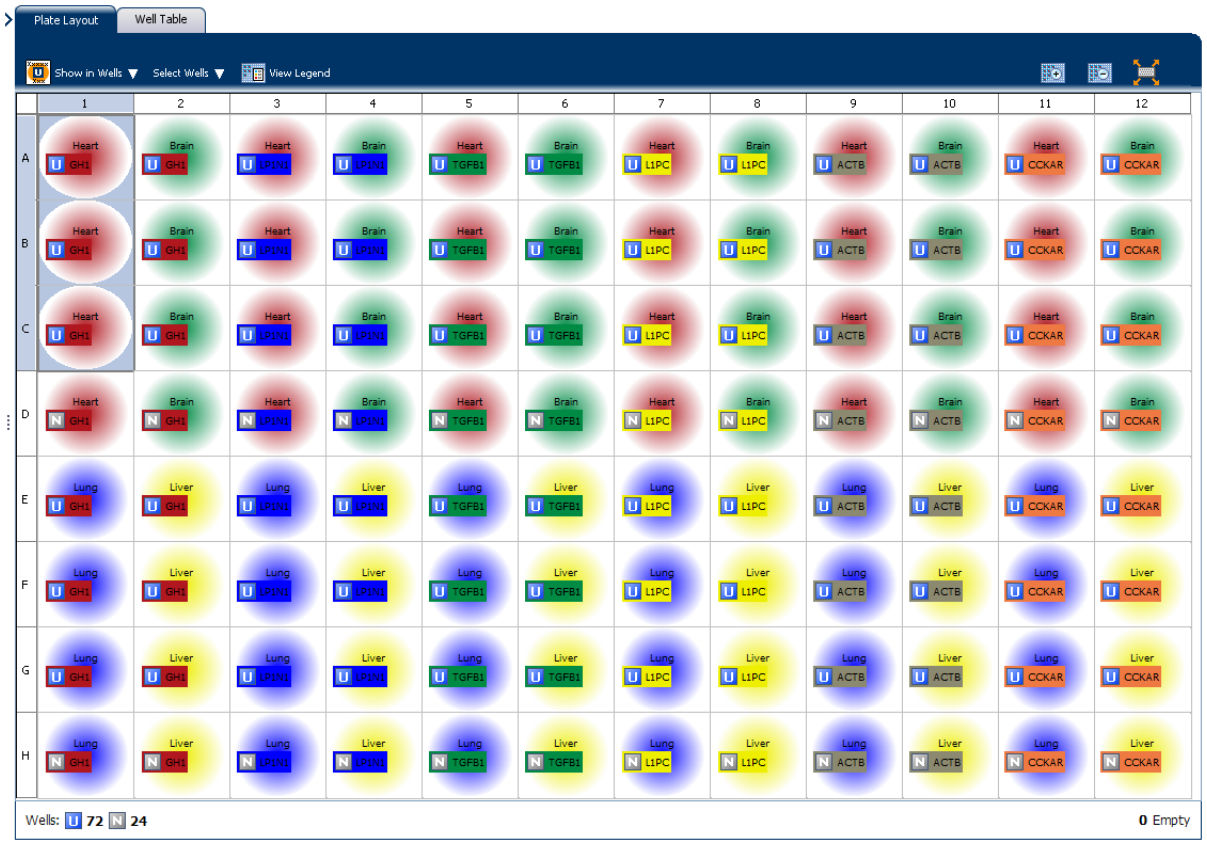
Prepare the reaction plate

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

The plate layout experiment looks like this:



To 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 cocktail 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, Life Technologies recommends 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

For more information on...	Refer to...	Part number
Assigning the reaction plate components	*Chapter 1 in Booklet 1, Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

10 Run the Experiment

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

This chapter covers:

- Start the run 87
- Monitor the run 87

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 QuantStudio™ 12K Flex Instrument is in operation.

Start the run

1. Open the Comparative C_T example file that you created using instructions in Chapter 8.
2. Load the reaction plate into the instrument.
3. Start the run.

Monitor the run

Monitor the example experiment run:

- From the QuantStudio™ 12K Flex Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio™ 12K Flex Software (to monitor an experiment started from another computer or from the QuantStudio™ 12K Flex Instrument touchscreen).
- From the QuantStudio™ 12K Flex Instrument touchscreen.

From the Instrument Console of the QuantStudio™ 12K Flex Software

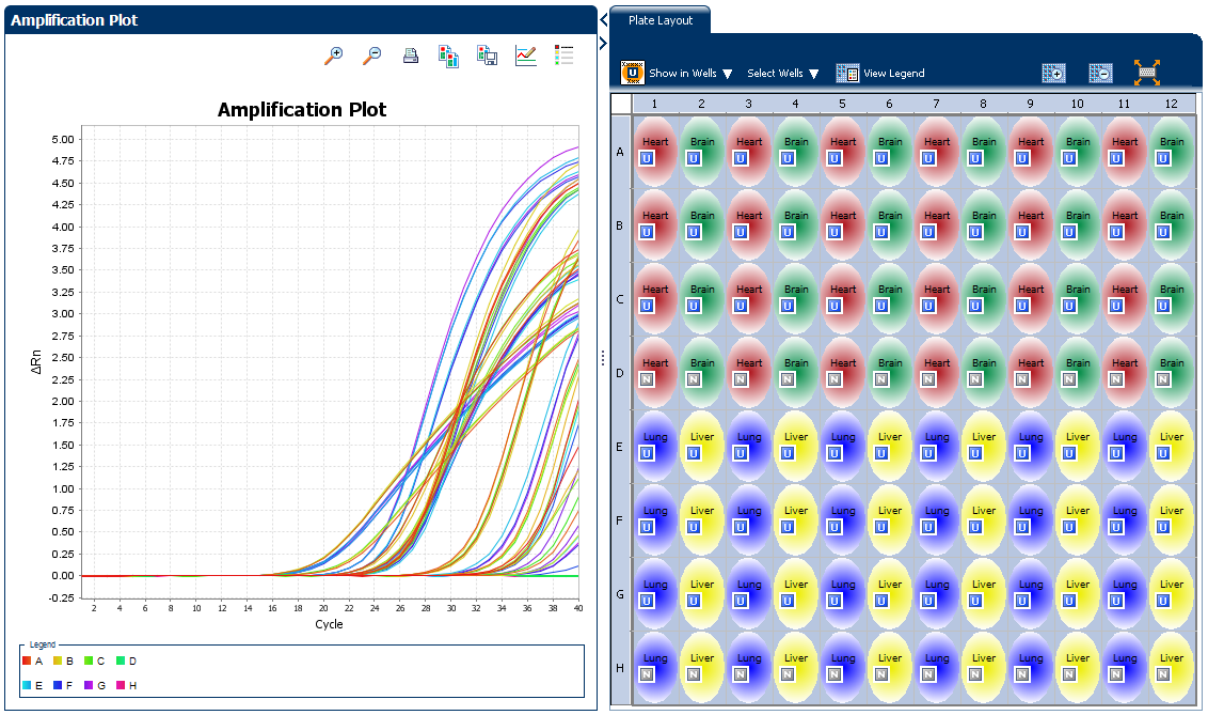
1. In the Instrument Console screen, select the instrument icon.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. In the Manage Instrument screen, click **Monitor Running Experiment** to access the Run screen.

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 t to view.

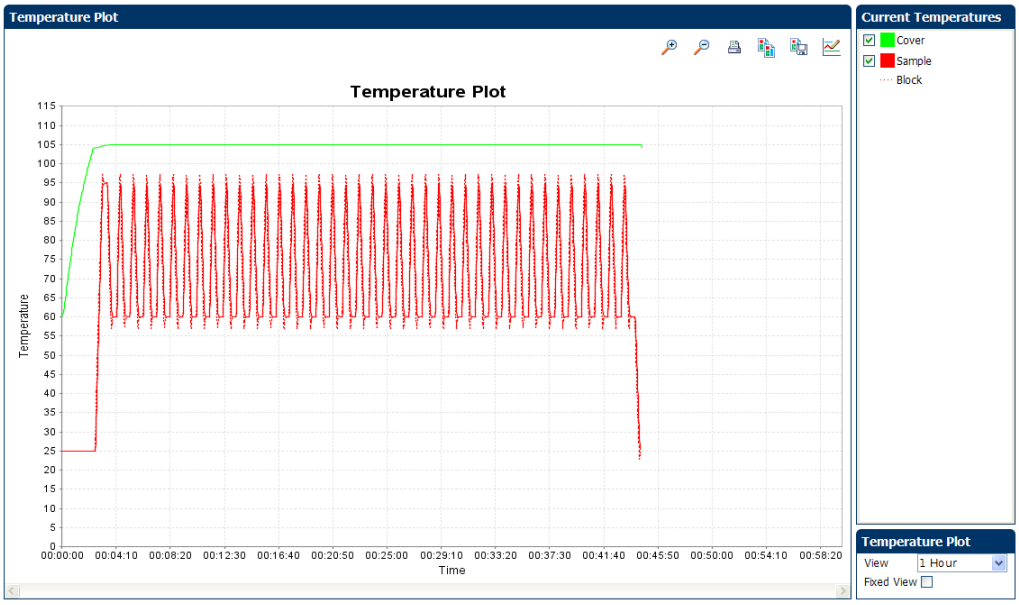
The figure below shows the Amplification Plot screen as it appears at the end of the example experiment.



View the Temperature Plot

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

The figure below shows the Temperature Plot screen as it appears during the example experiment.

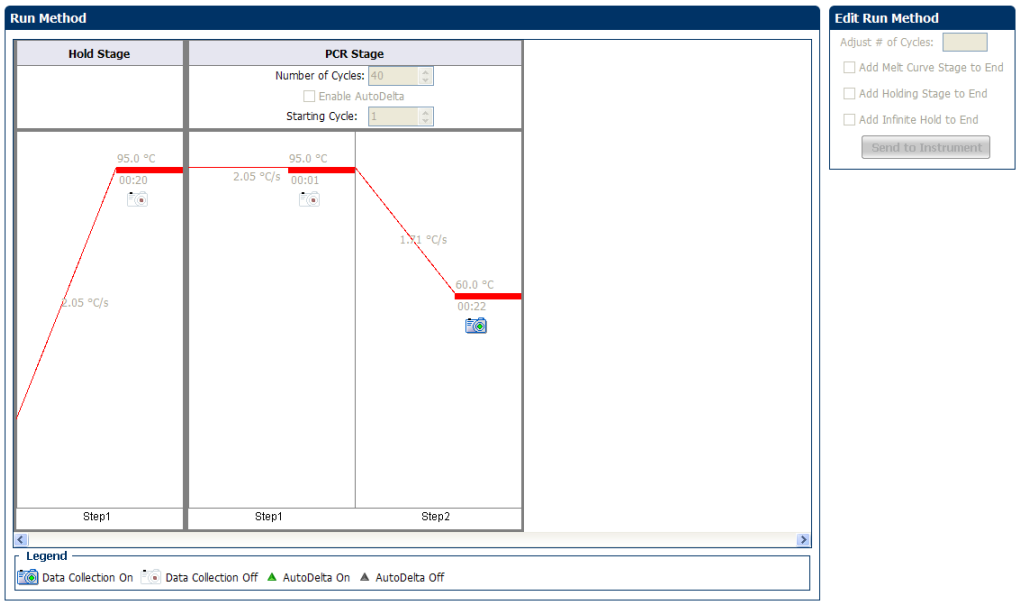


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

View the Run Method

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

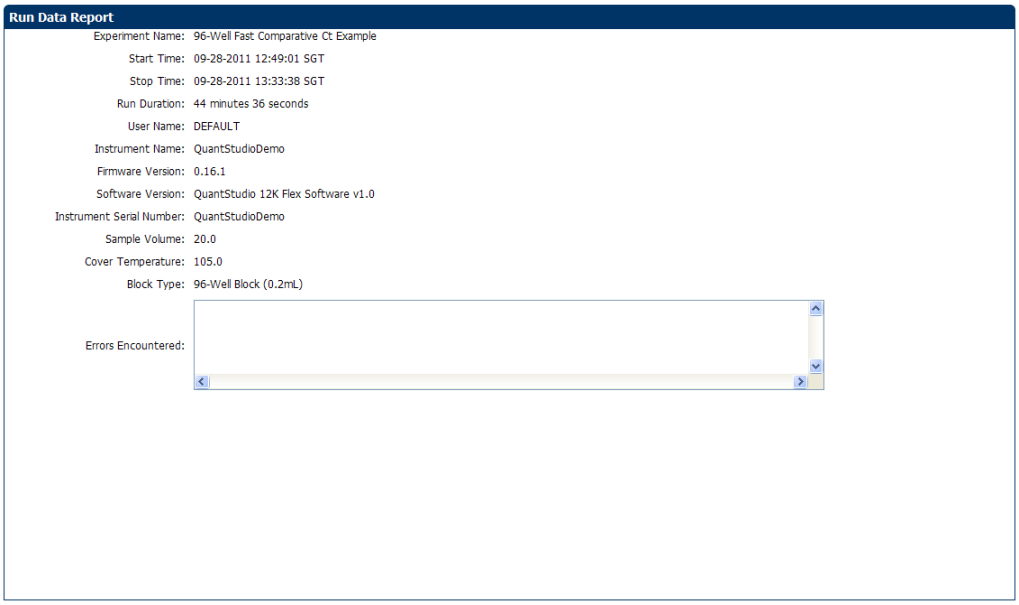
The figure below shows the Run Method screen as it appears in the example experiment.



View Run Data

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

Your View Run Data screen should like this:

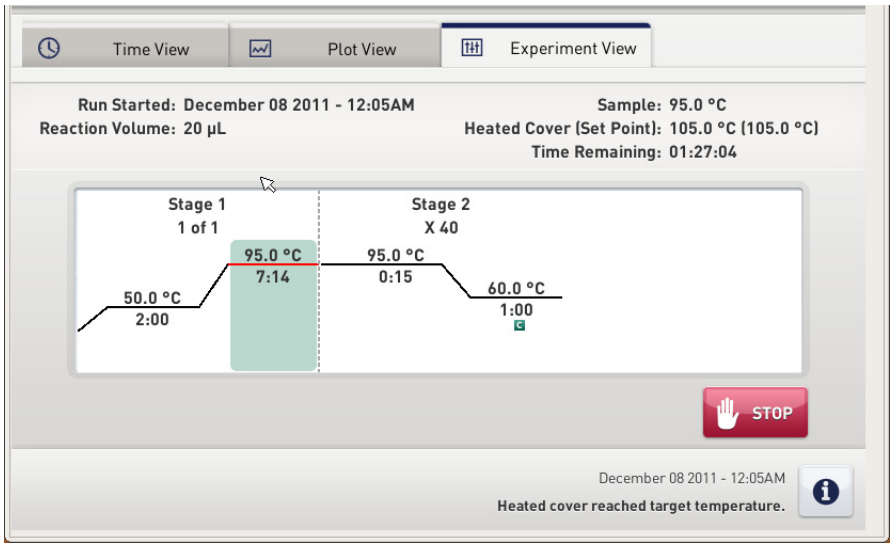


From the QuantStudio™ 12K Flex Instrument touchscreen

You can also view the progress of the run from the touchscreen of the QuantStudio™ 12K Flex Instrument.

The Run Method screen on the QuantStudio™ 12K Flex Instrument touchscreen looks like this:

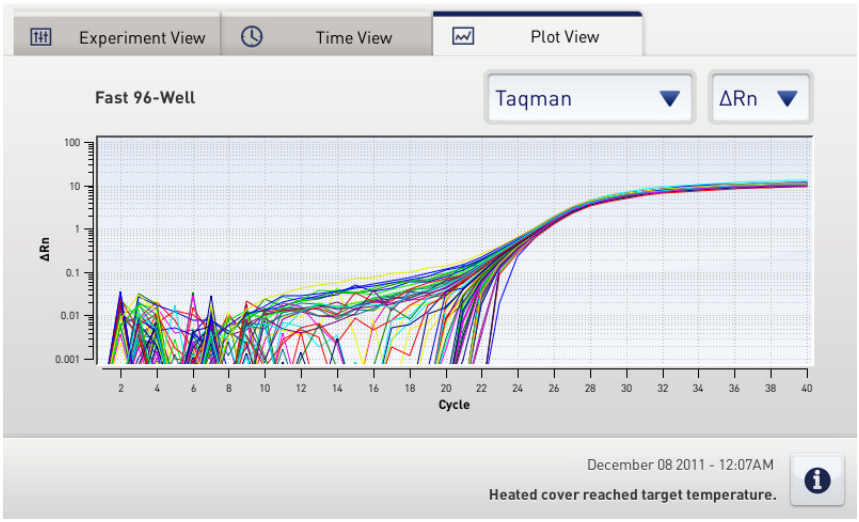
Experiment view



Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

Time View

Plot View



11 Review Results and Adjust Experiment Parameters

In Section 11.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 11.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

Section 11.1 Review Results 95

- Analyze the example experiment. 95
- Assess the gene expression profile using the Gene Expression Plot. 95
- Identify well problems using the Well Table 97
- Assess amplification results using the Amplification Plot. 99
- Confirm accurate dye signal using the Multicomponent Plot. 106
- Determine signal accuracy using the Raw Data Plot 108
- View the endogenous control profile using the QC Plot 110
- Review the flags in the QC Summary 111
- For more information. 113

Section 11.2 Adjust parameters for re-analysis of your own experiments 115

- Adjust analysis settings 115
- Improve C_T precision by omitting wells. 118

Section 11.1 Review Results

Analyze the example experiment

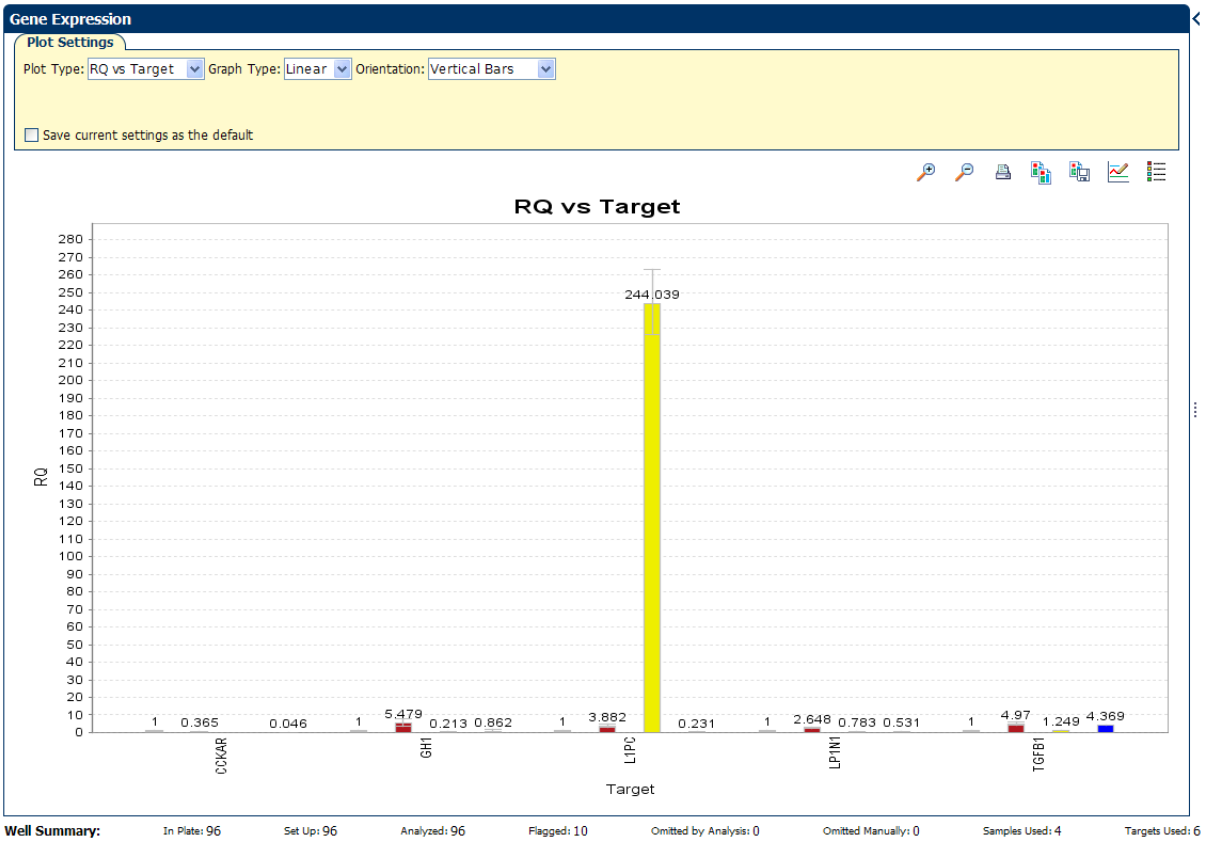
1. Open the example experiment file that you ran in Chapter 10.
2. Click **Analyze**. The software analyzes the data using the default analysis settings.

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

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. The Gene Expression plot when viewed as a linear graph type looks like this:



- **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.

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. From the Experiment Menu pane, select **Analysis** ▸ **Gene Expression**.

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

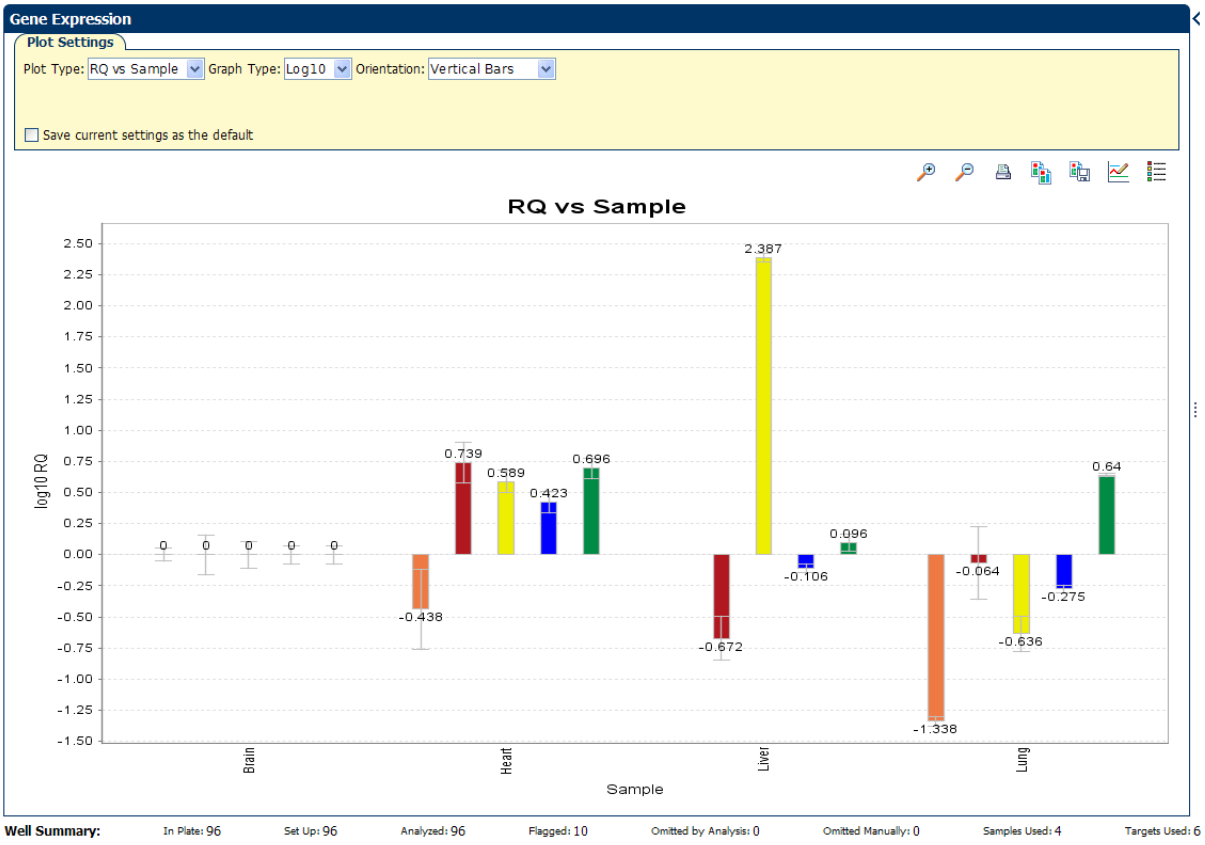
2. In the Gene Expression Plot screen, select:

Menu	Selection
Plot Type	RQ vs Sample (default)
Graph Type	Log10
Orientation	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. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**, then click the **Well Table** tab.
2. From the Group By drop-down menu, 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. You will omit these wells in the troubleshooting section ("Improve C_T precision by omitting wells" on page 118).

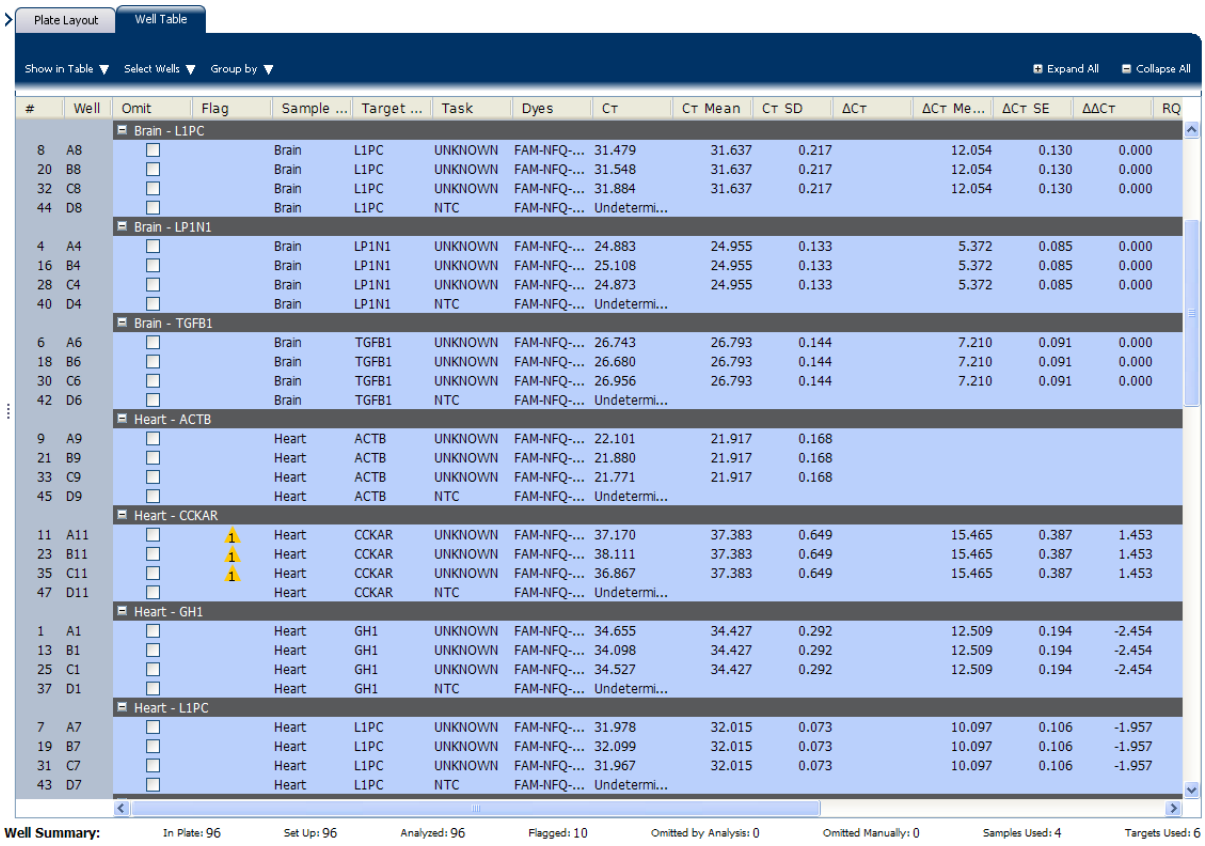


Plate Layout | Well Table

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

#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	CT	CT Mean	CT SD	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...							

Well Summary: In Plate: 96 Set Up: 96 Analyzed: 96 Flagged: 10 Omitted by Analysis: 0 Omitted Manually: 0 Samples Used: 4 Targets Used: 6

Note: To show/hide columns in the Well Table, select/deselect the column name from the Show in Table drop-down menu.

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 C_T precision by omitting wells" on page 118).

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. You can 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. 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.

Purpose

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

- Correct baseline and threshold values
- Outliers

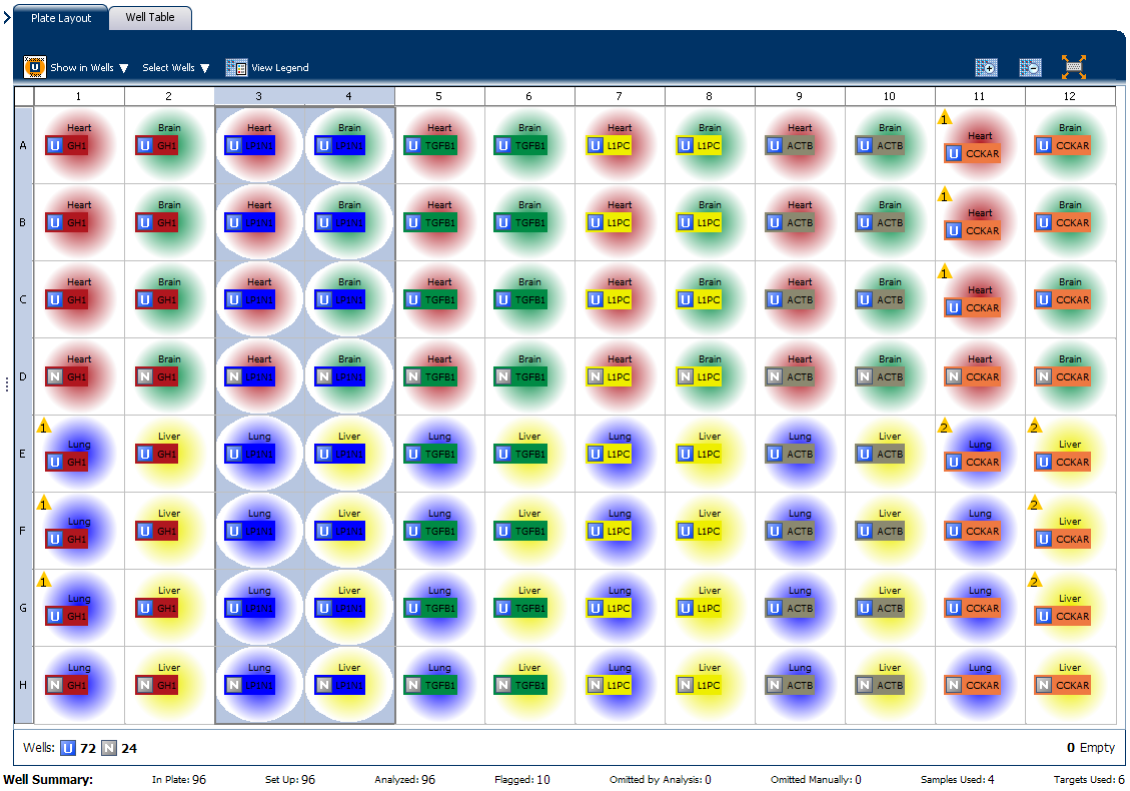
View the Amplification Plot

1. From the Experiment Menu pane, select **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. From the Select Wells drop-down menus, select **Target**, then **LP1N1**.

The Plate Layout screen should look like this:

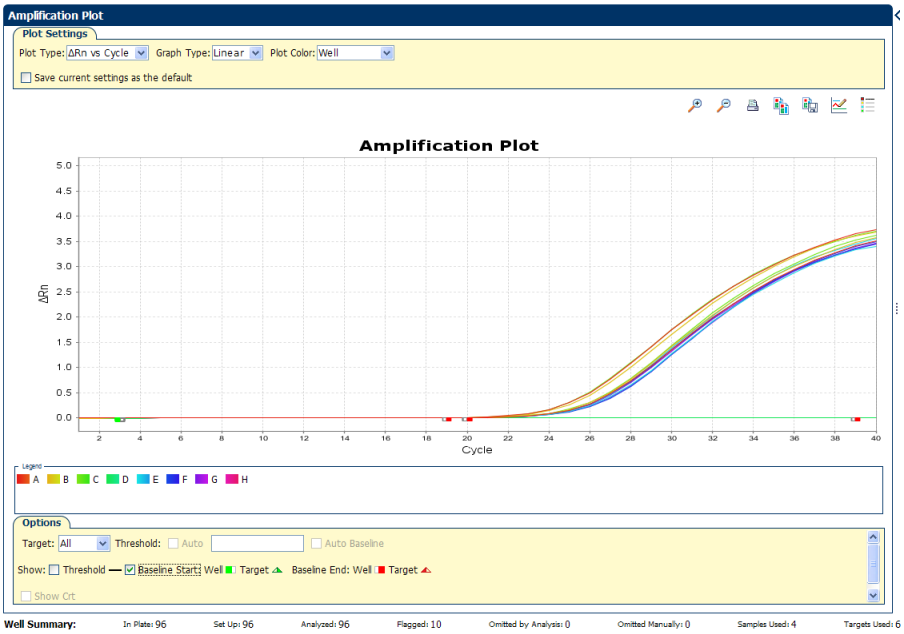


3. In the Amplification Plot screen, enter:

Menu	Selection
Plot Type	ΔRn vs Cycle (default)
Plot Color	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 drop-down menu, select **Linear**.
   b. Select the **Baseline** check box to show the start cycle and end cycle.

c. Verify 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.



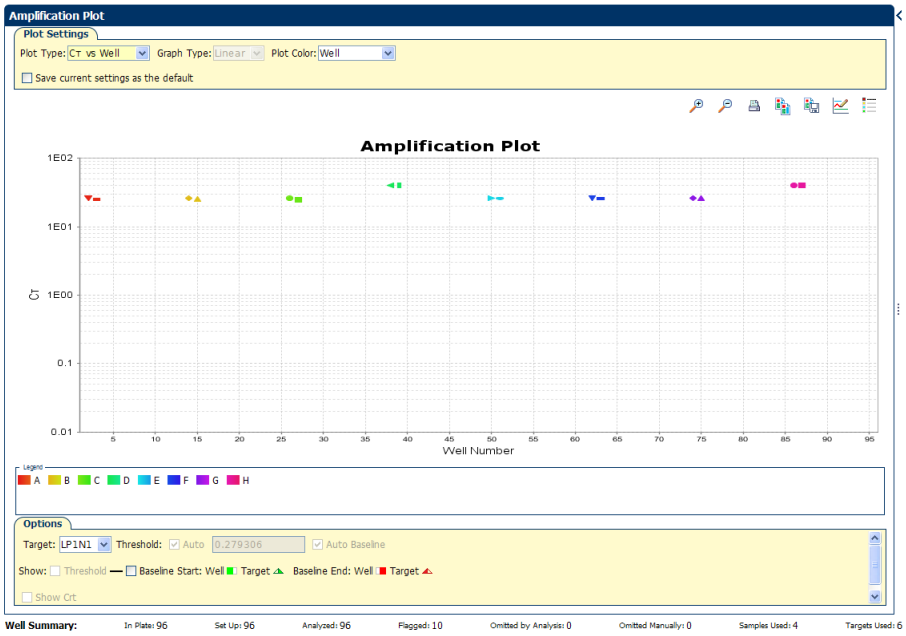
5. View the threshold values:

Menu	Select
Graph Type	Log
Target	LP1N1

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

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

6. Locate any outliers:
   a. From the Plot Type drop-down menu, 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 steps 2 through 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. You will omit these wells in the troubleshooting section ("Improve C_T precision by omitting wells" on page 118).

Tips for analyzing your own experiments

When you analyze your own Comparative C_T experiment, look for:

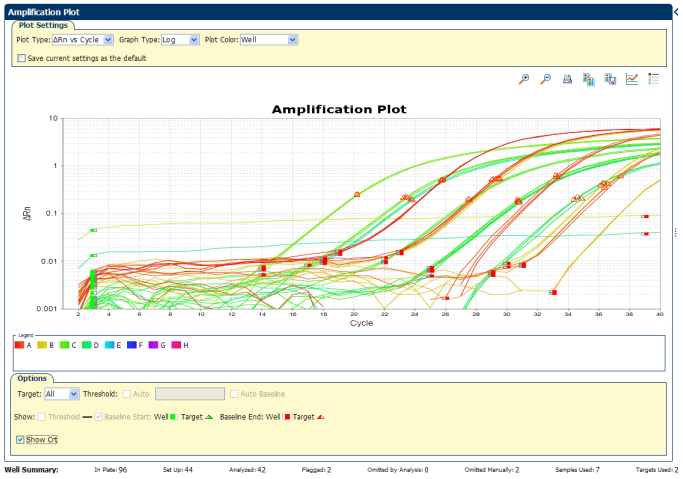
- **Outliers**
- **A typical amplification plot** – The QuantStudio™ 12K Flex Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear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

– Exponential (geometric phase)
– Baseline

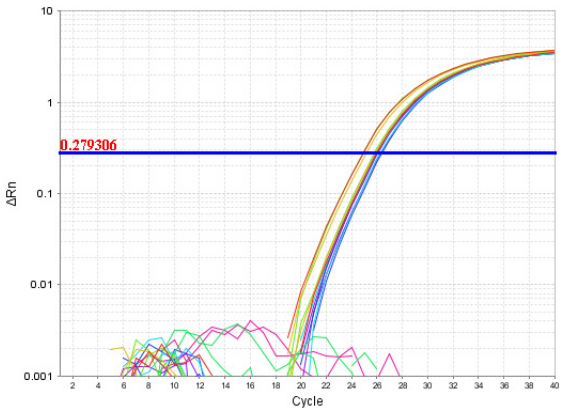
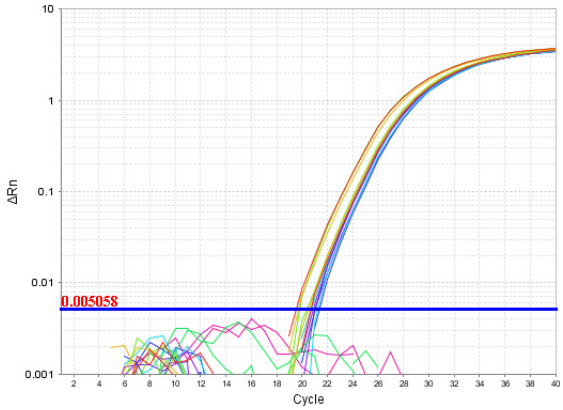
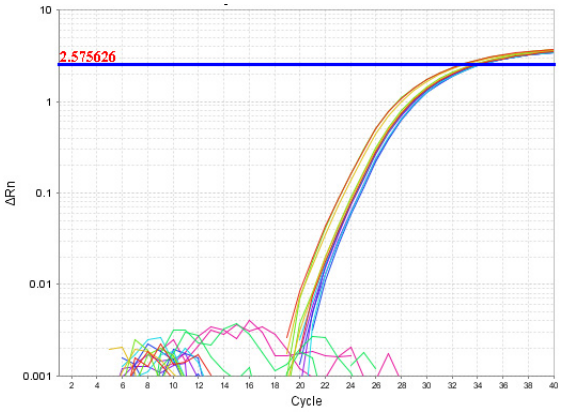
A typical amplification plot should look like this:

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. Therefore, Life Technologies recommends that you examine the Amplification Plot screen and review the assigned baseline and threshold values for each well after analysis completes.

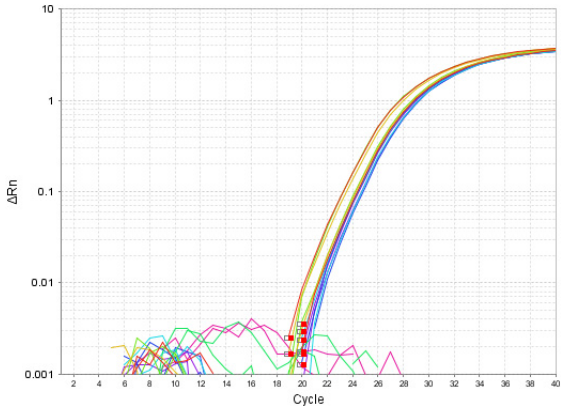
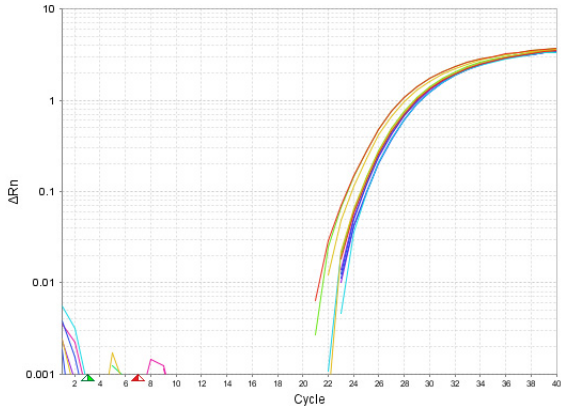
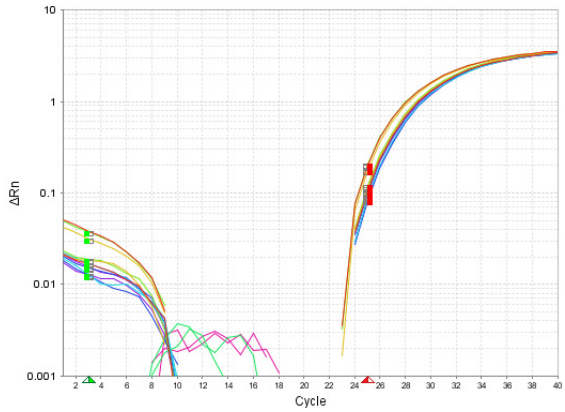
Note: If you use the Relative Threshold algorithm to analyze an experiment that includes amplification, select to view the analysis results using the ΔRn vs Cycle, Rn vs Cycle, or C_{RT} vs Well plot type and Linear or Log graph type. Also select the **Show Crt** check box to view the derived fractional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



- **Correct threshold values**

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**

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 115.

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

- Omit wells (see "Improve C_T precision by omitting wells" on page 118).

  Or

- Manually adjust the baseline and/or threshold (see "Adjust analysis settings" on page 115).

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:

- 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 shown in the Multicomponent Plot screen.

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

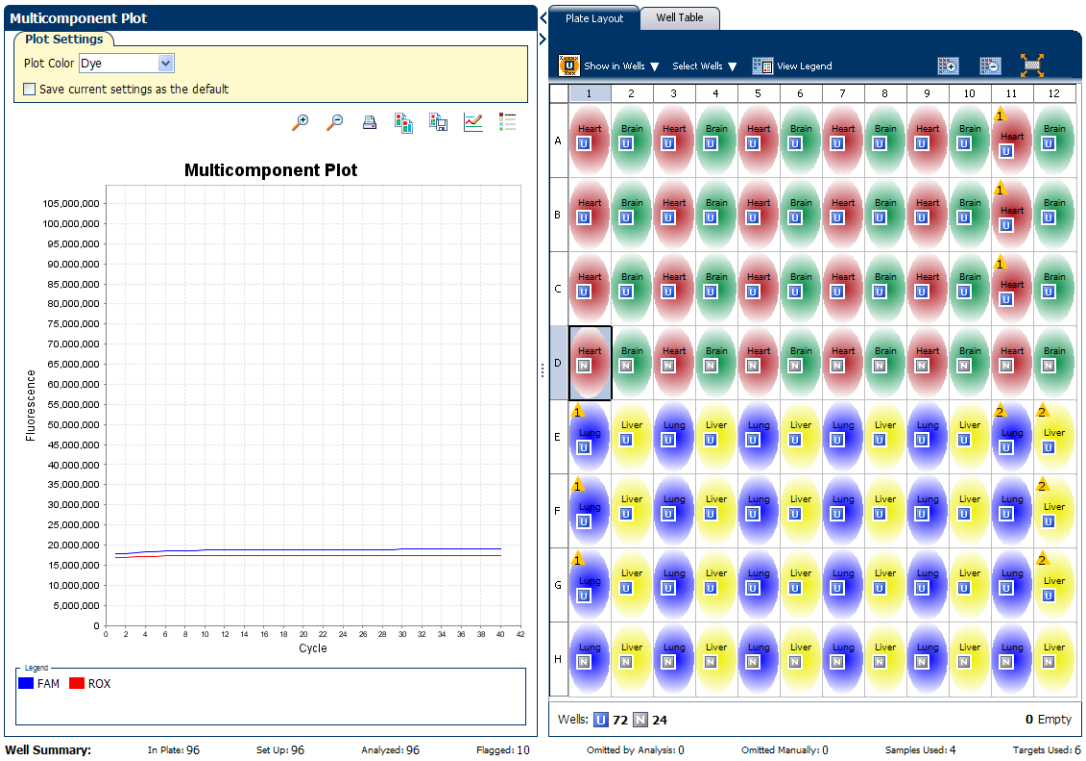
3. From the Plot Color drop-down menu, select **Dye**.

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:

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

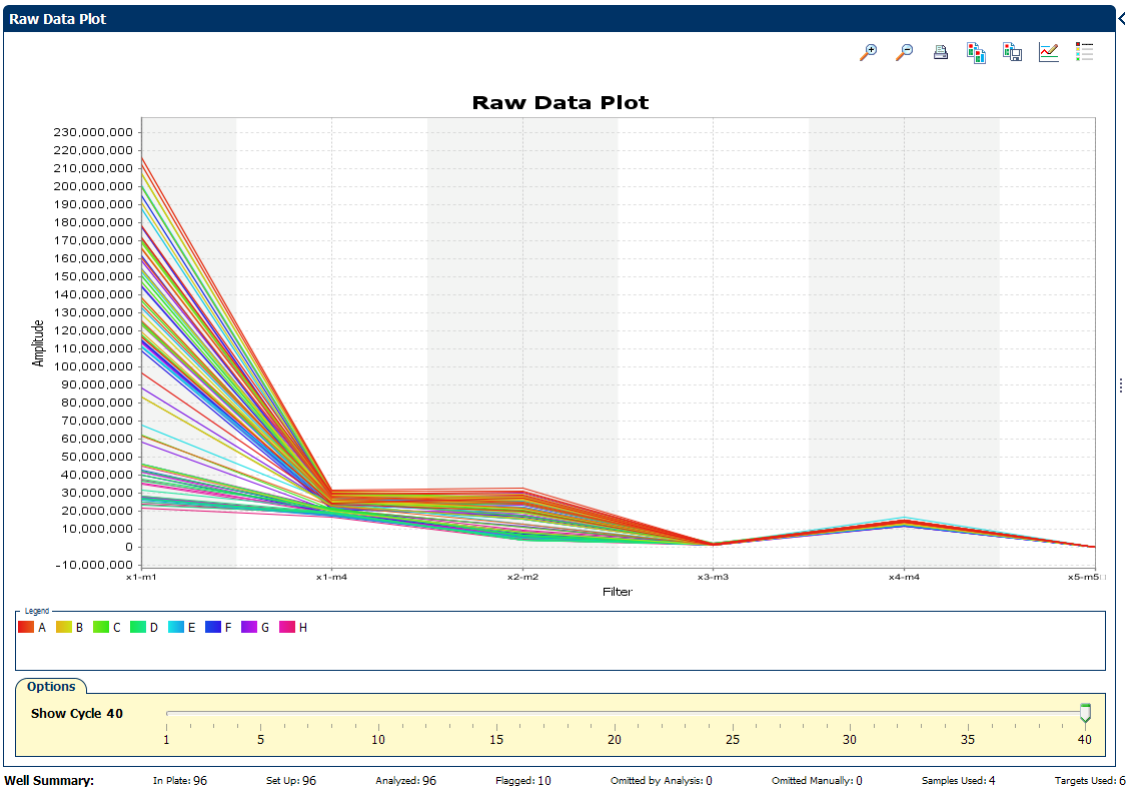
   Note: If no data are displayed, click **Analyze**.

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).

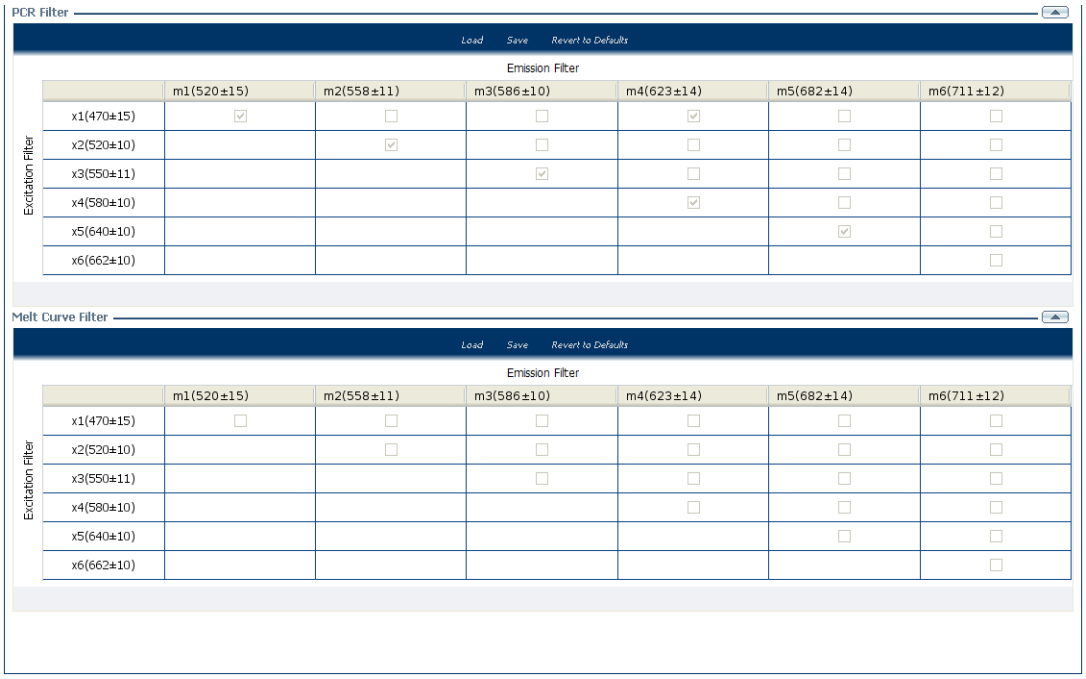
   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

   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.



The filters used for the example experiment are:



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

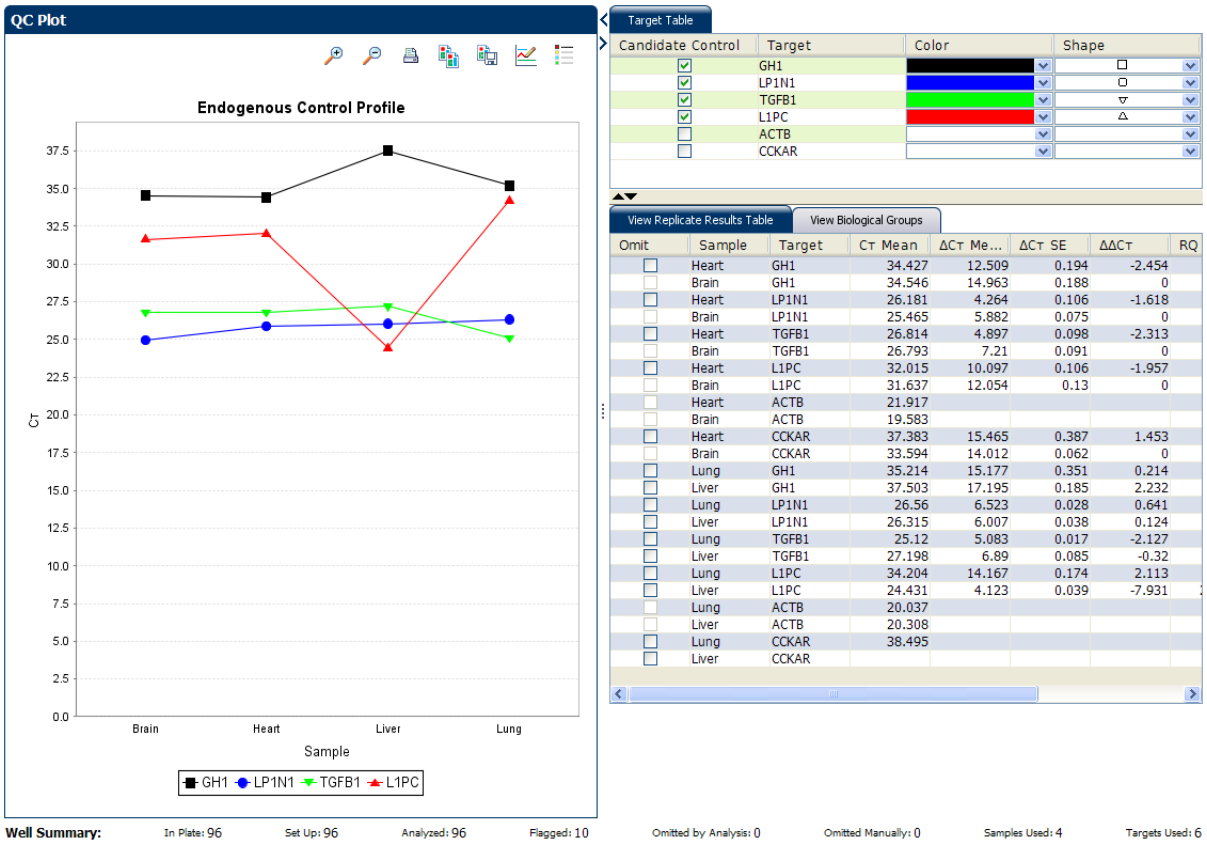
1. From the Experiment Menu pane, select **Analysis ▸ QC Plot**.

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

2. In the QC Plot screen, click **Target Table**.
   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 for each target, from the Color drop-down menu.
   c. Select a shape for each target, from the Shape drop-down menu.
3. Click the **View Replicate Results Table**.
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.

The QC Plot in the Comparative C_T example experiment looks like this:



Note: 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, and wells E11, E12, F12, and G12 have data that triggered the EXPFAIL flag.

View the QC Summary

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 > 0, the flag appears somewhere in the experiment; the well position is listed in the Wells column.

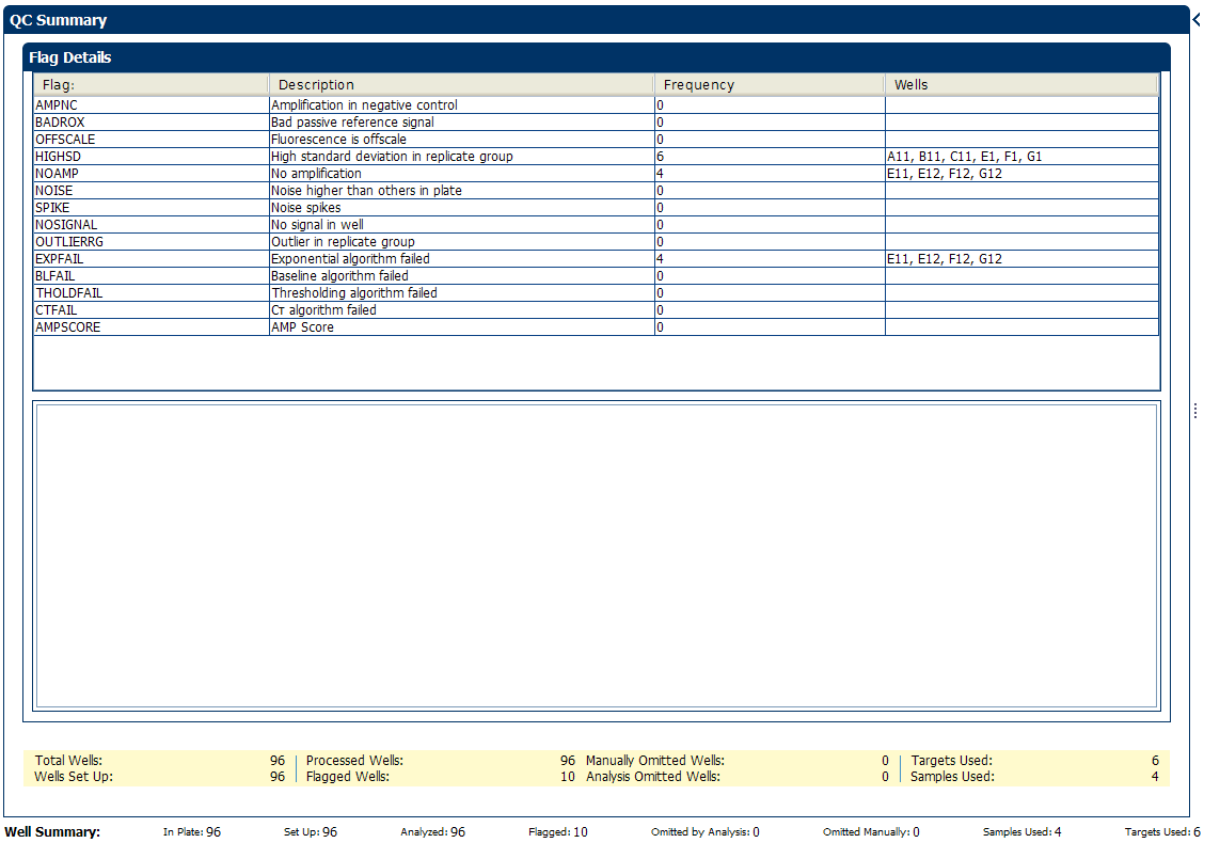
   In the example experiment, there are ten flagged wells.

3. In the Flag Details table, click each flag with a frequency >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 >0, click the troubleshooting link to view information on correcting the flag.

   The QC Summary for the example experiment looks like this:



QC Summary

Flag Details

Flag:	Description	Frequency	Wells
AMPNC	Amplification in negative control	0	
BADROX	Bad passive reference signal	0	
OFFSCALE	Fluorescence is offscale	0	
HIGHSD	High standard deviation in replicate group	6	A11, B11, C11, E1, F1, G1
NOAMP	No amplification	4	E11, E12, F11, G12
NOISE	Noise higher than others in plate	0	
SPIKE	Noise spikes	0	
NOSIGNAL	No signal in well	0	
OUTLIERRG	Outlier in replicate group	0	
EXPFAIL	Exponential algorithm failed	4	E11, E12, F12, G12
BLFAIL	Baseline algorithm failed	0	
THOLDFAIL	Thresholding algorithm failed	0	
CTFAIL	Ct algorithm failed	0	
AMPSCORE	AMP Score	0	

Total Wells:	96	Processed Wells:	96	Manually Omitted Wells:	0	Targets Used:	6
Wells Set Up:	96	Flagged Wells:	10	Analysis Omitted Wells:	0	Samples Used:	4

Well Summary: In Plate: 96 Set Up: 96 Analyzed: 96 Flagged: 10 Omitted by Analysis: 0 Omitted Manually: 0 Samples Used: 4 Targets Used: 6

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

Flag	Description
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	
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

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

Section 11.2 Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

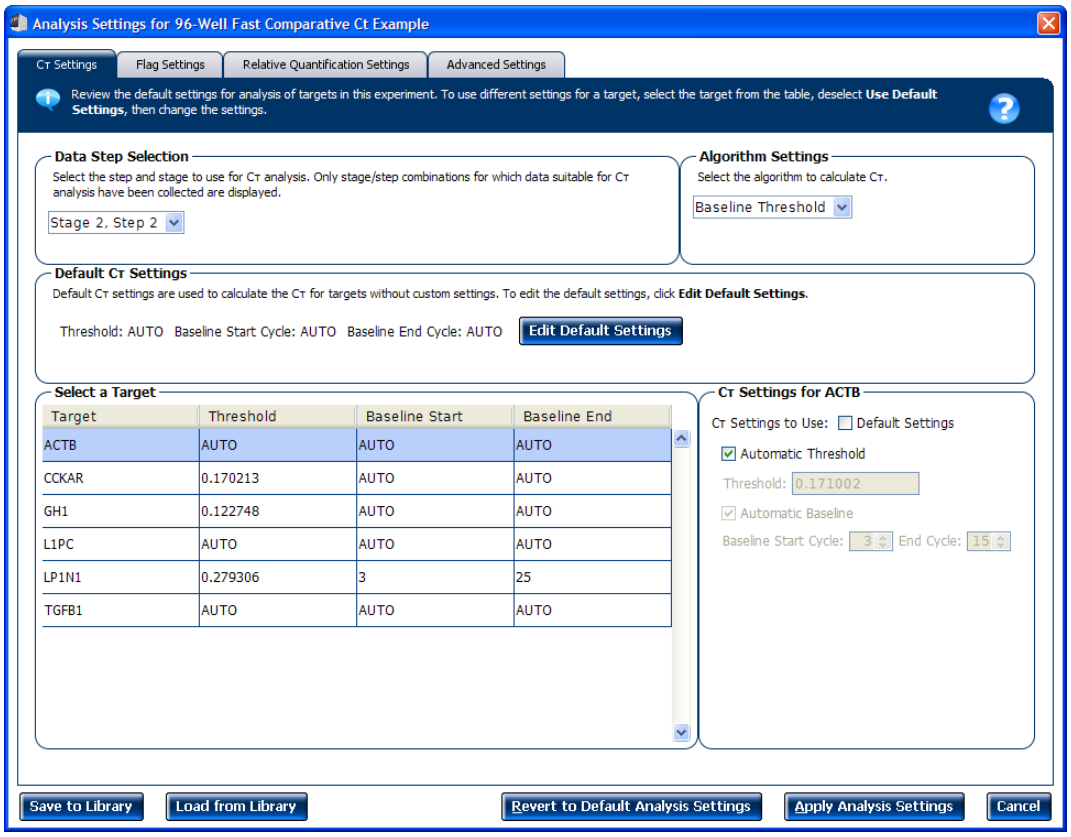
The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, and advanced options.

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
   - Relative Quantification Settings
   - Advanced Settings

   The Analysis Settings dialog box for a Comparative C_T experiment looks like this:



3. View and, if necessary, change the analysis settings (see "Adjust analysis settings" below).

   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 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.

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, Life Technologies recommends:

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: This setting is applicable only to the Baseline Threshold algorithm.

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:

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

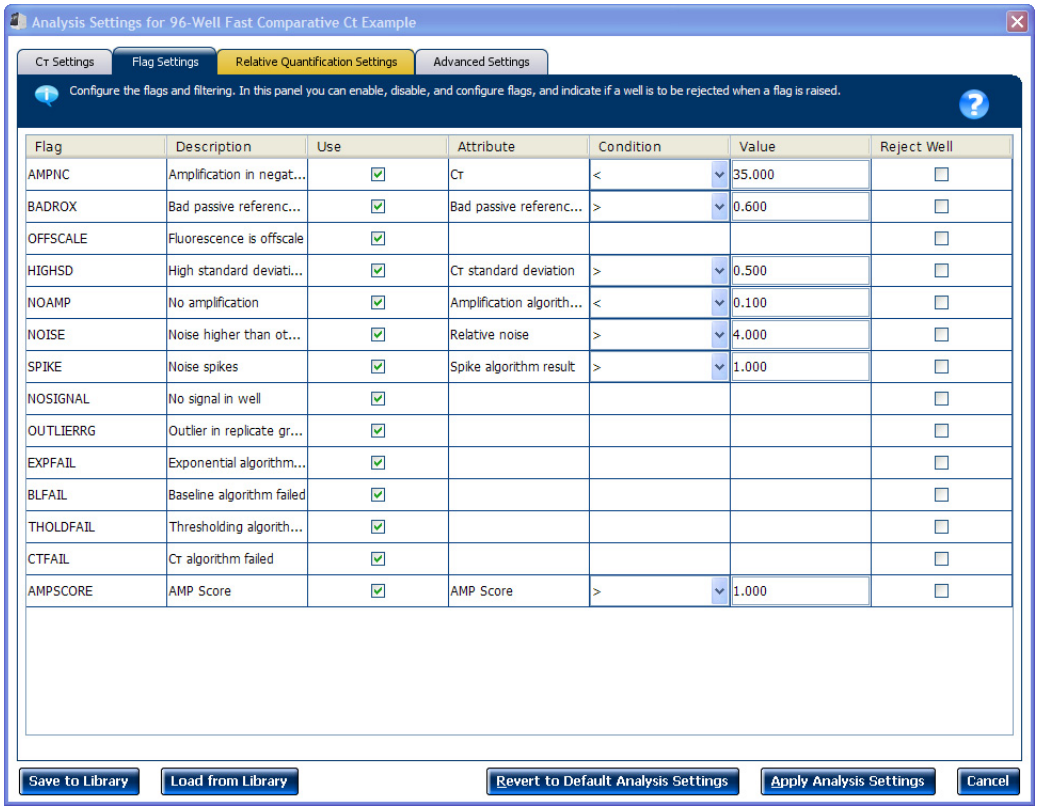
To adjust the flag settings

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.

   The Flag Settings tab looks like this:



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_Tvalues 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.

To use 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** check box.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, then enter the baseline start cycle and baseline end cycle.

Improve C_T precision by omitting wells

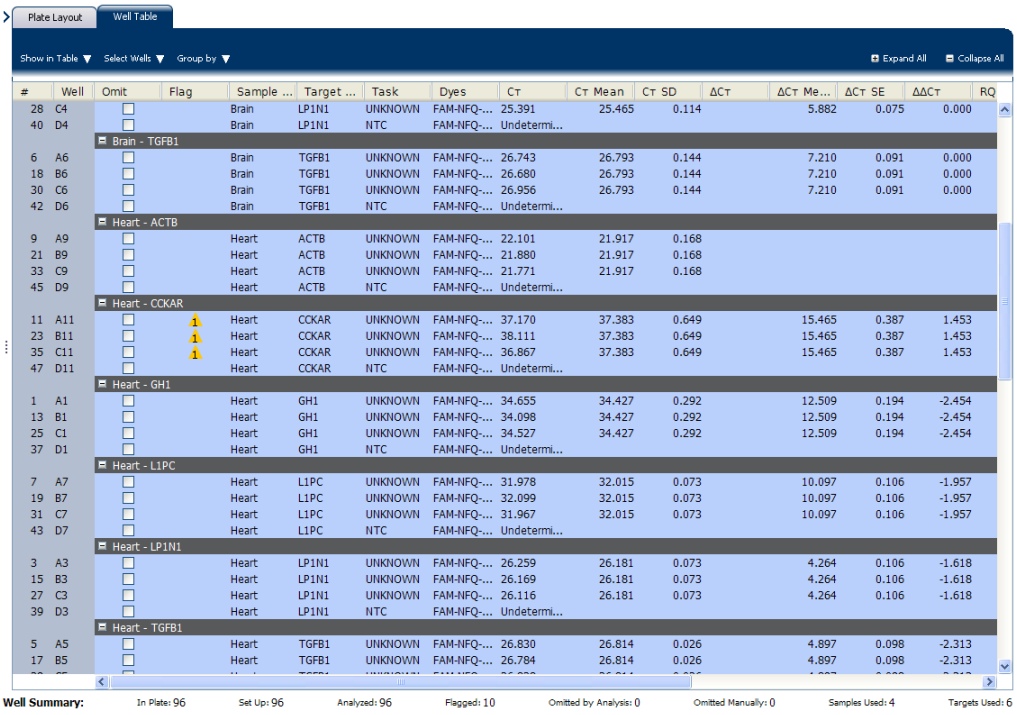
Experimental error may cause some wells to be amplified insufficiently or not at all. These wells typically produce C_T values that differ significantly from the average for the associated replicate wells. If included in the calculations, these outliers can result in erroneous measurements; to ensure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

In the Comparative C_T example experiment, there are seven outliers. To remove these wells from analysis.

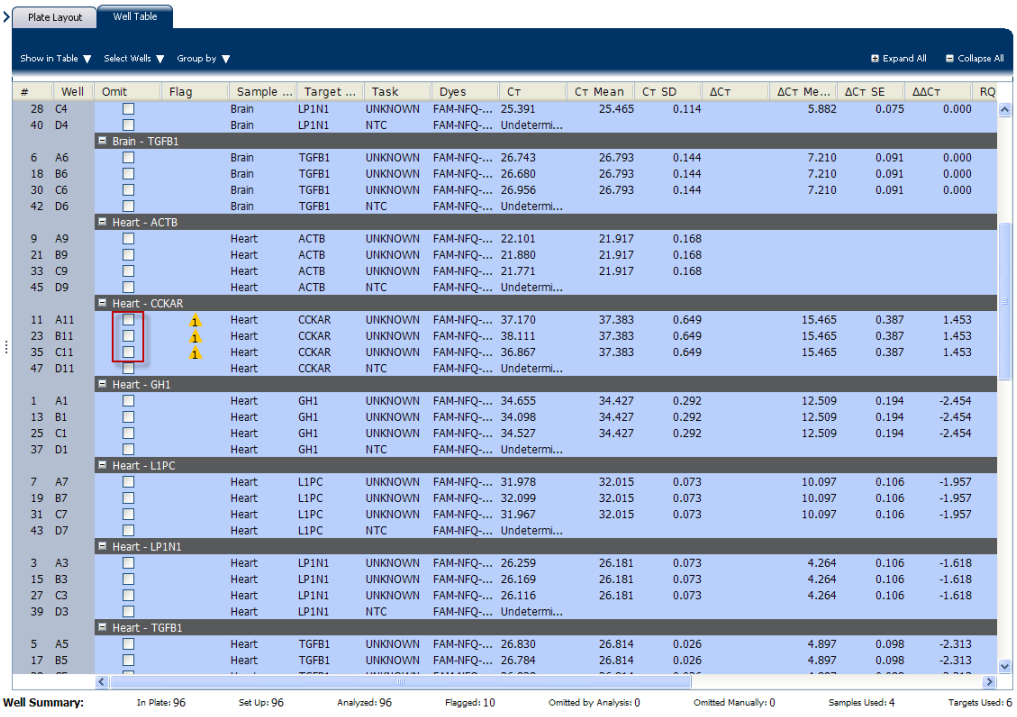
1. From the Experiment Menu pane, select **Analysis ▸ Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.
2. In the Amplification Plot screen, select **C_T vs Well** from the Plot Type drop-down menu.
3. Select the **Well Table** tab.

4. In the Well Table, identify outliers:
   a. From the Group By drop-down menu, 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.



   c. Select the **Omit** check box next to outlying well(s).



5. Click **Analyze** to reanalyze the experiment data with the outlying well(s) 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 and select **Omit**.

12 Export Analysis Results

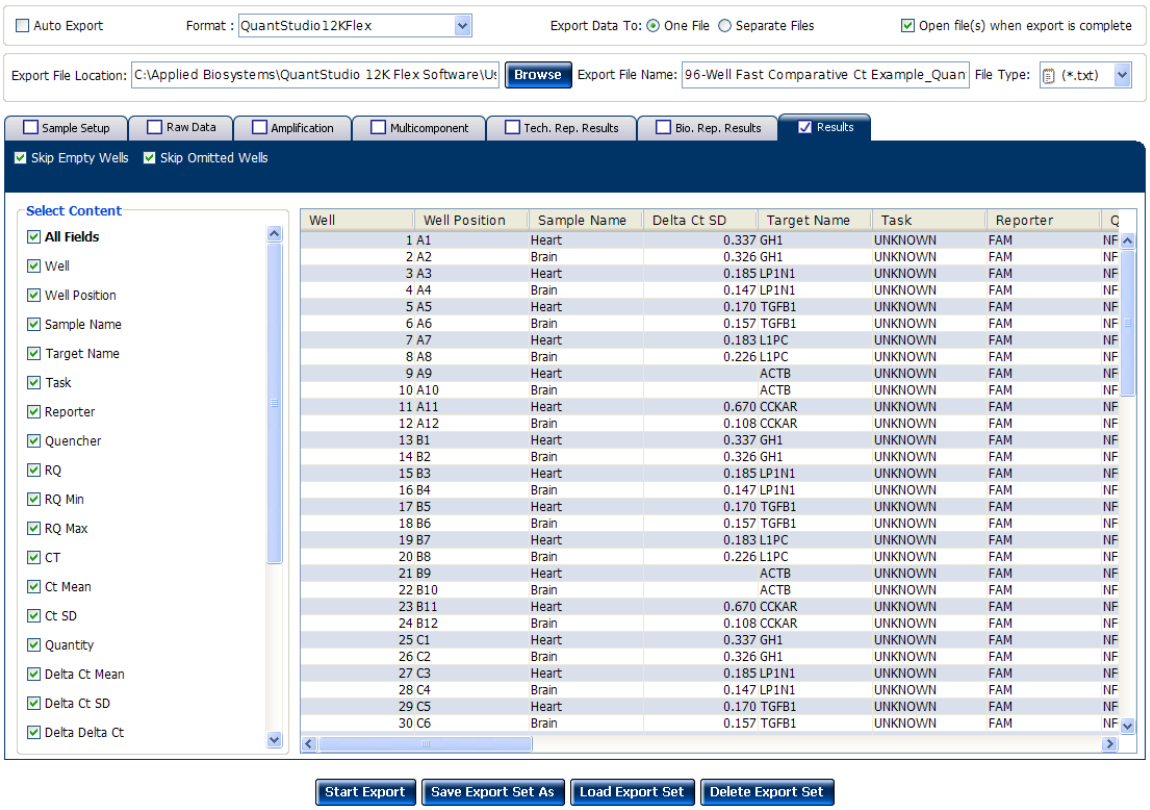
1. Open the Comparative C_T example experiment file that you analyzed in Chapter 11.
2. In the Experiment Menu, click  **Export.**

   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.
3. Select **QuantStudio™ 12K Flex format**.

   Note: Select **7900** Format if you want to export the Clipped Data.
4. Complete the Export dialog box as shown below:

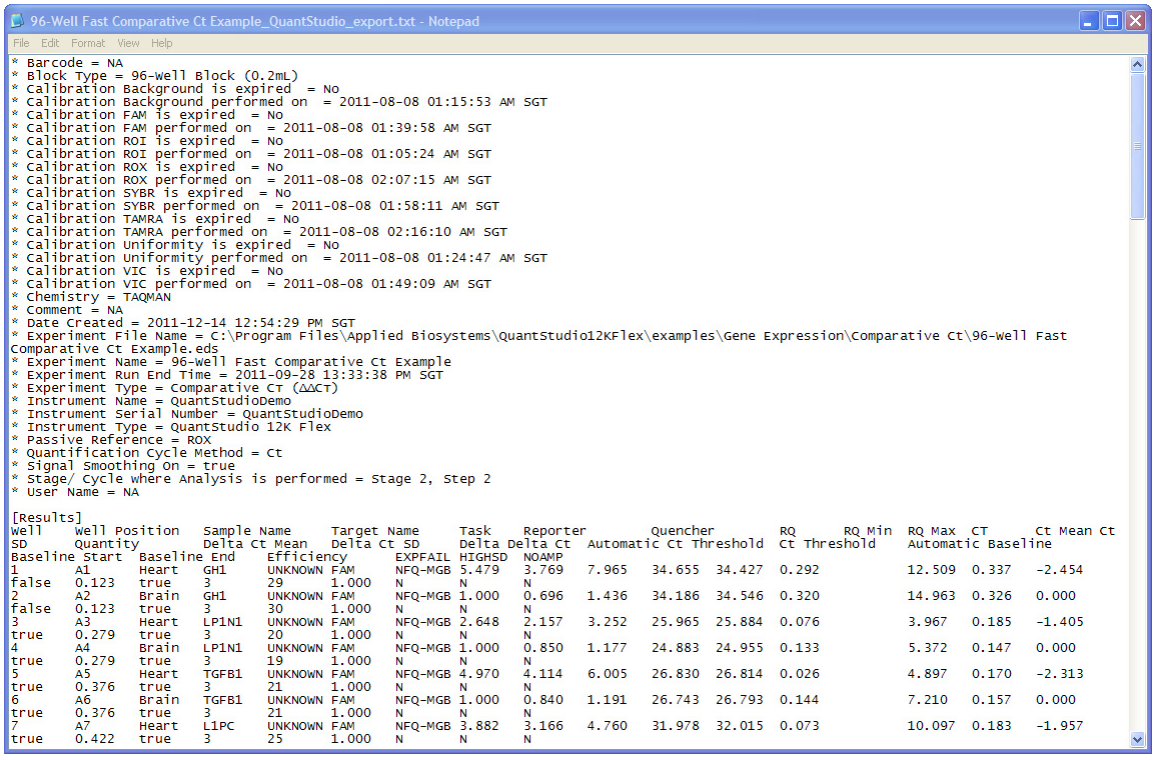
Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	96-Well Fast Comparative Ct Example_QuantStudio_export
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments

Your Export screen should look like this:



Well	Well Position	Sample Name	Delta Ct SD	Target Name	Task	Reporter	Q
1	A1	Heart	0.337	GH1	UNKNOWN	FAM	NF
2	A2	Brain	0.326	GH1	UNKNOWN	FAM	NF
3	A3	Heart	0.185	LP1N1	UNKNOWN	FAM	NF
4	A4	Brain	0.147	LP1N1	UNKNOWN	FAM	NF
5	A5	Heart	0.170	TGFB1	UNKNOWN	FAM	NF
6	A6	Brain	0.157	TGFB1	UNKNOWN	FAM	NF
7	A7	Heart	0.183	L1PC	UNKNOWN	FAM	NF
8	A8	Brain	0.226	L1PC	UNKNOWN	FAM	NF
9	A9	Heart		ACTB	UNKNOWN	FAM	NF
10	A10	Brain		ACTB	UNKNOWN	FAM	NF
11	A11	Heart	0.670	CCKAR	UNKNOWN	FAM	NF
12	A12	Brain	0.108	CCKAR	UNKNOWN	FAM	NF
13	B1	Heart	0.337	GH1	UNKNOWN	FAM	NF
14	B2	Brain	0.326	GH1	UNKNOWN	FAM	NF
15	B3	Heart	0.185	LP1N1	UNKNOWN	FAM	NF
16	B4	Brain	0.147	LP1N1	UNKNOWN	FAM	NF
17	B5	Heart	0.170	TGFB1	UNKNOWN	FAM	NF
18	B6	Brain	0.157	TGFB1	UNKNOWN	FAM	NF
19	B7	Heart	0.183	L1PC	UNKNOWN	FAM	NF
20	B8	Brain	0.226	L1PC	UNKNOWN	FAM	NF
21	B9	Heart		ACTB	UNKNOWN	FAM	NF
22	B10	Brain		ACTB	UNKNOWN	FAM	NF
23	B11	Heart	0.670	CCKAR	UNKNOWN	FAM	NF
24	B12	Brain	0.108	CCKAR	UNKNOWN	FAM	NF
25	C1	Heart	0.337	GH1	UNKNOWN	FAM	NF
26	C2	Brain	0.326	GH1	UNKNOWN	FAM	NF
27	C3	Heart	0.185	LP1N1	UNKNOWN	FAM	NF
28	C4	Brain	0.147	LP1N1	UNKNOWN	FAM	NF
29	C5	Heart	0.170	TGFB1	UNKNOWN	FAM	NF
30	C6	Brain	0.157	TGFB1	UNKNOWN	FAM	NF

Your exported file when opened in Notepad should look like this:




```
* 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
```

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GETTING STARTED GUIDE

applied biosystems®
by life technologies™

Booklet 4 - Running Genotyping Experiments

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1 About Genotyping Experiments

This chapter covers:

- About data collection 5
- About TaqMan® SNP Genotyping assays 6
- About TaqMan® MGB probes 6
- 5′ nuclease assay 6
- Minimizing non-specific fluorescence 7
- Reading and analyzing the plates 7
- About the example experiment 8

IMPORTANT! First-time users of the QuantStudio™ 12K Flex System please read Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* and Booklet 7, *QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes* of this binder thoroughly. The booklets provide information and general instructions that are applicable to all the experiments described in this binder

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

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.

About TaqMan® SNP Genotyping assays

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:

- 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

About 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.

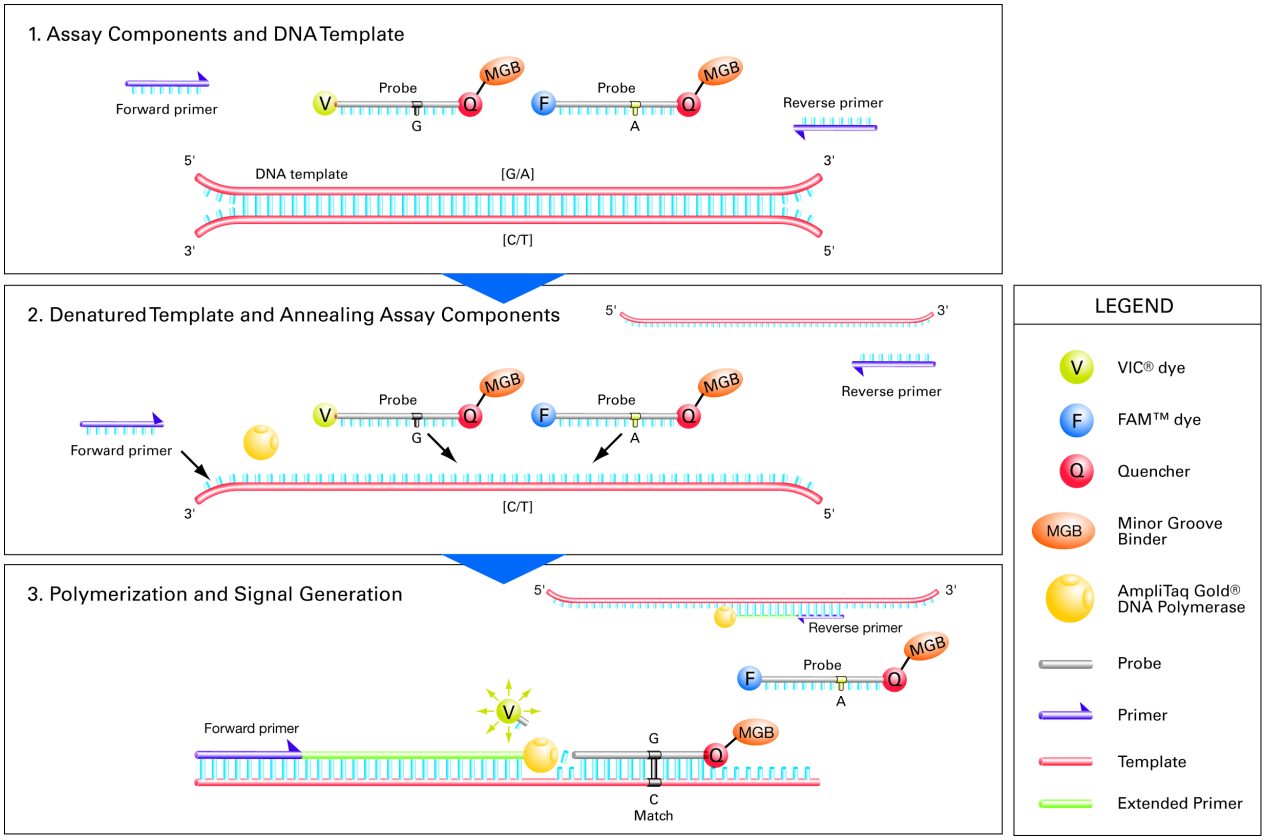
  The Allele 1 VIC® dye-labeled probe corresponds to the first nucleotide inside the square brackets of the context sequence in the assay information file (AIF) shipped with each order. The Allele 2 FAM™ dye-labeled probe corresponds to the second nucleotide inside the square brackets of the context sequence 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.

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

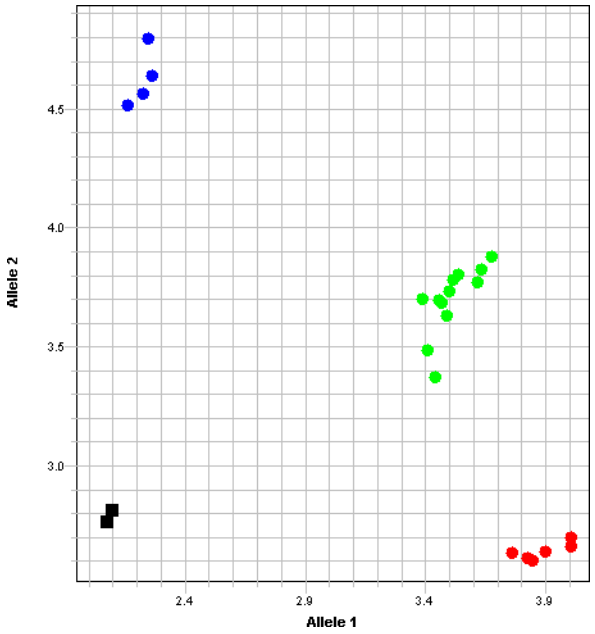
In TaqMan® assays, fluorescence from nonspecifically bound probes is reduced, because nucleotide mismatches between a probe and a sequence reduce the chances that the probe will be cleaved. The probe's short length means that a one-base-pair mismatch will a have a larger negative effect on the binding. 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.

A substantial increase in...	Indicates...
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 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 and run according to the protocol that is described in the *Performing a TaqMan® Drug Metabolism Genotyping Assay.*

2 Design the Experiment

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

This chapter covers:

- Define the experiment properties. 11
- Define SNPs and samples 12
- Assign markers and samples 14
- Set up the run method 16
- For more information. 17

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 on Auto Export, refer to Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.

Define the experiment properties

Click **Experiment Setup** ▸ **Experiment Properties** to create a new experiment in the QuantStudio™ Software. Enter:

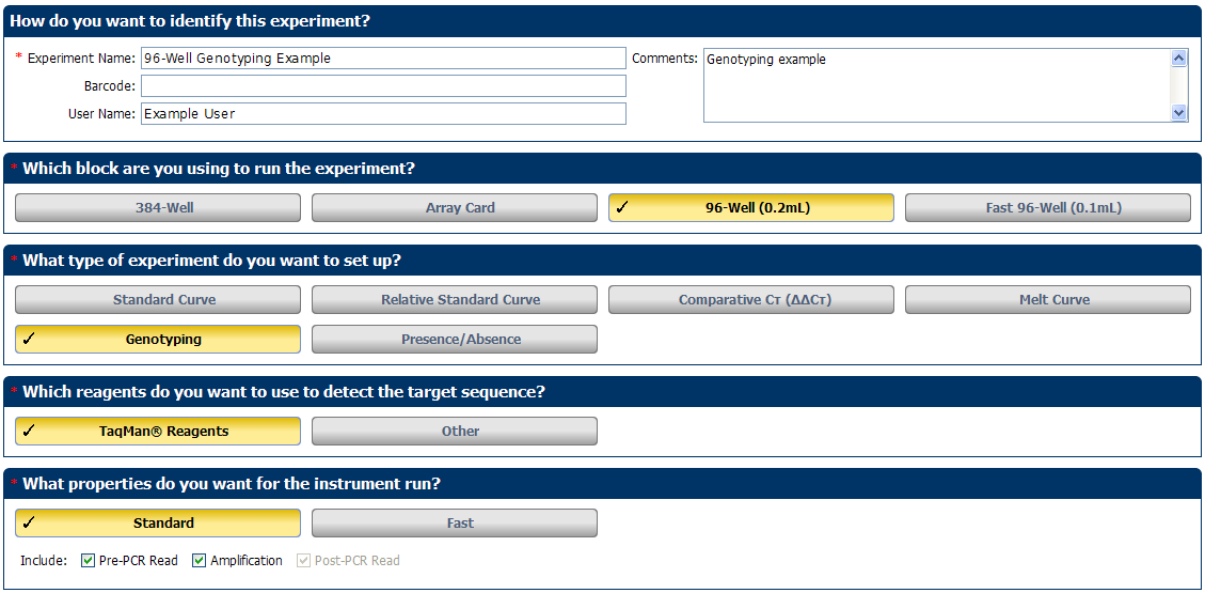
Field	Entry
Experiment Name	96-Well Genotyping Example
Barcode	Leave field empty
User Name	Example User
Comments	Genotyping example
Block	96-Well (0.2mL)
Experiment Type	Genotyping
Reagents	TaqMan® Reagents
Ramp speed	Standard

Select all three data-collection check boxes: Pre-PCR, Amplification, and Post-PCR collection methods:

Pre-PCR Read	Checked
Amplification	Checked
Post-PCR Read	Checked

Save the experiment.

Your Experiment Properties screen should look like this:



Define SNPs and samples

Click **Define** to access the Define screen. Enter:

1. SNP Assays

SNP assay name	NCBI SNP reference	Context sequence	Allele 1	Reporter	Quencher	Allele 2	Reporter	Quencher	Color
SNP Assay 1			Allele1	VIC	NFQ-MGB	Allele2	FAM	NFQ-MGB	
SNP Assay 2			Allele1	VIC	NFQ-MGB	Allele2	FAM	NFQ-MGB	

Note: The NCBI SNP reference and Context sequence fields are optional fields and are used for reference. They are not required to run an experiment.

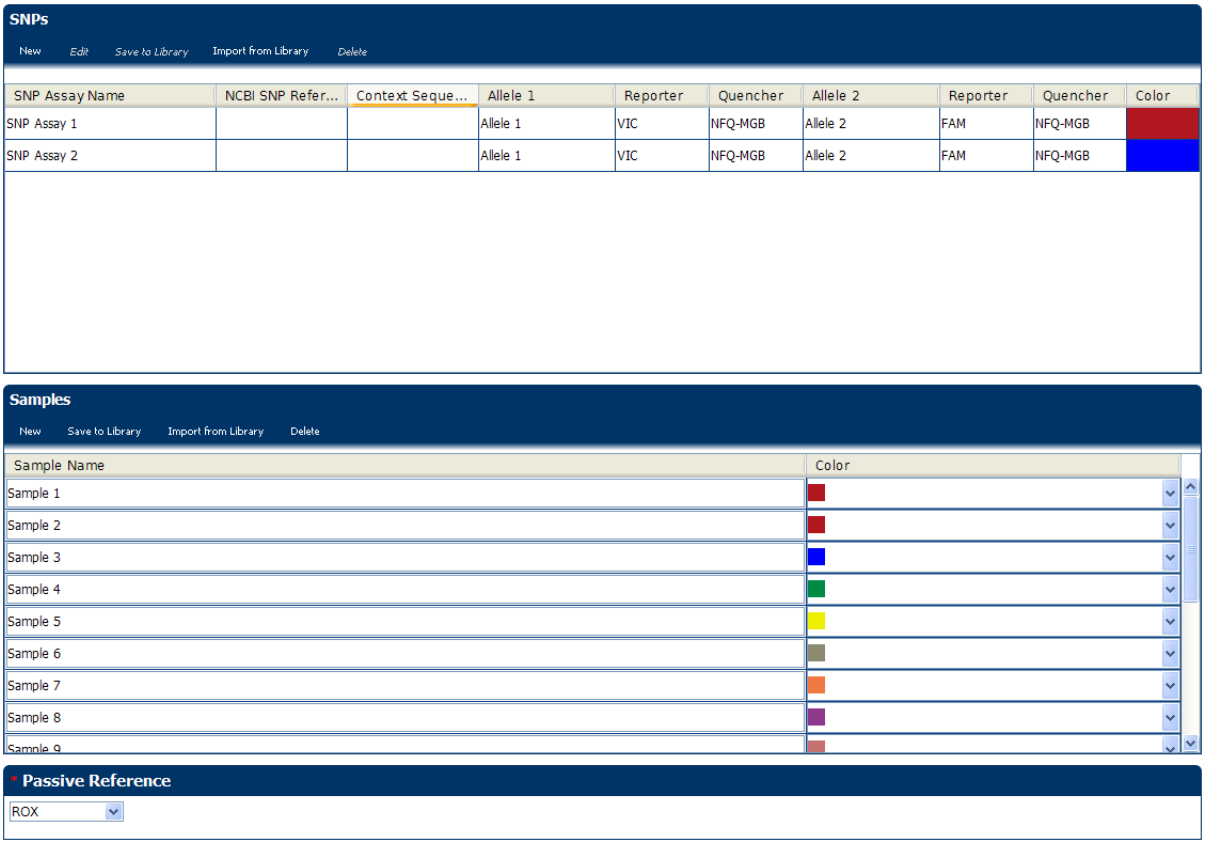
2. Samples

Sample name	Color	Sample name	Color
Sample 1		Sample 11	
Sample 2		Sample 12	
Sample 3		Sample 13	
Sample 4		Sample 14	

Sample name	Color	Sample name	Color
Sample 5		Sample 15	
Sample 6		Sample 16	
Sample 7		Sample 17	
Sample 8		Sample 18	
Sample 9		Sample 19	
Sample 10			

3. Dye to be used as a Passive Reference
   ROX

Your Define screen should look like this:



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

Assign markers and samples

Click **Assign** to access the Assign screen. Enter the SNP assays and samples:

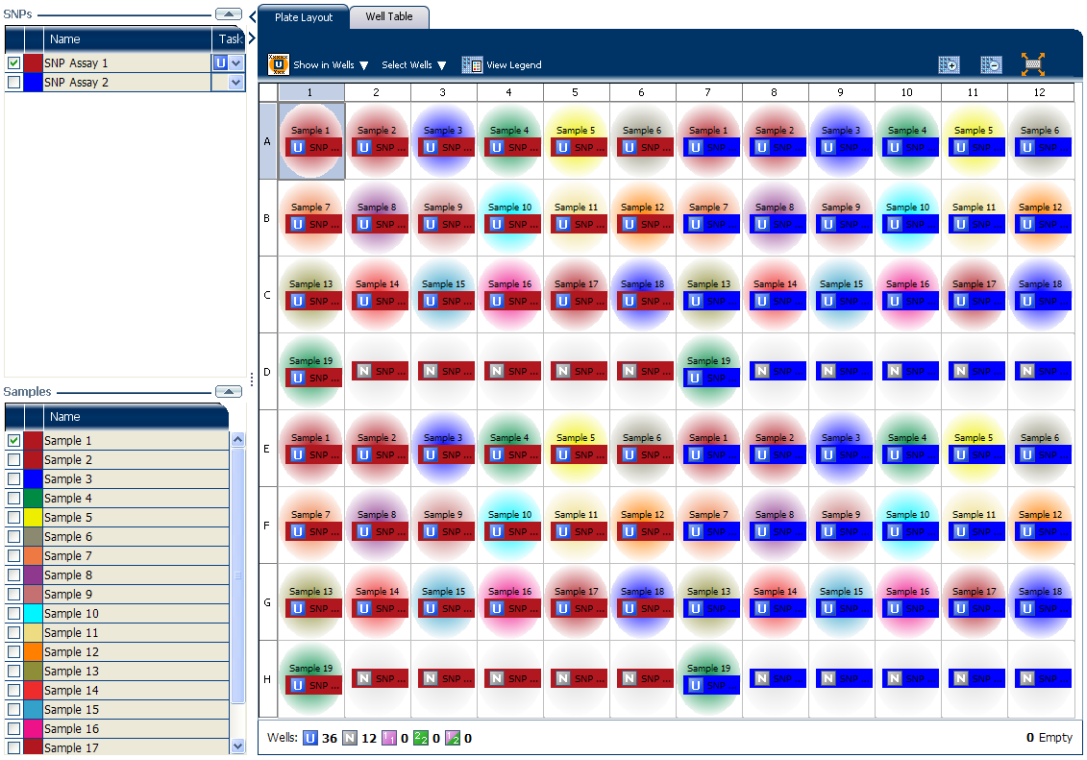
- SNP Assay 1

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
	A6, E6		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		

- 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
	B11, F11		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		

Your Assign screen should look like this:



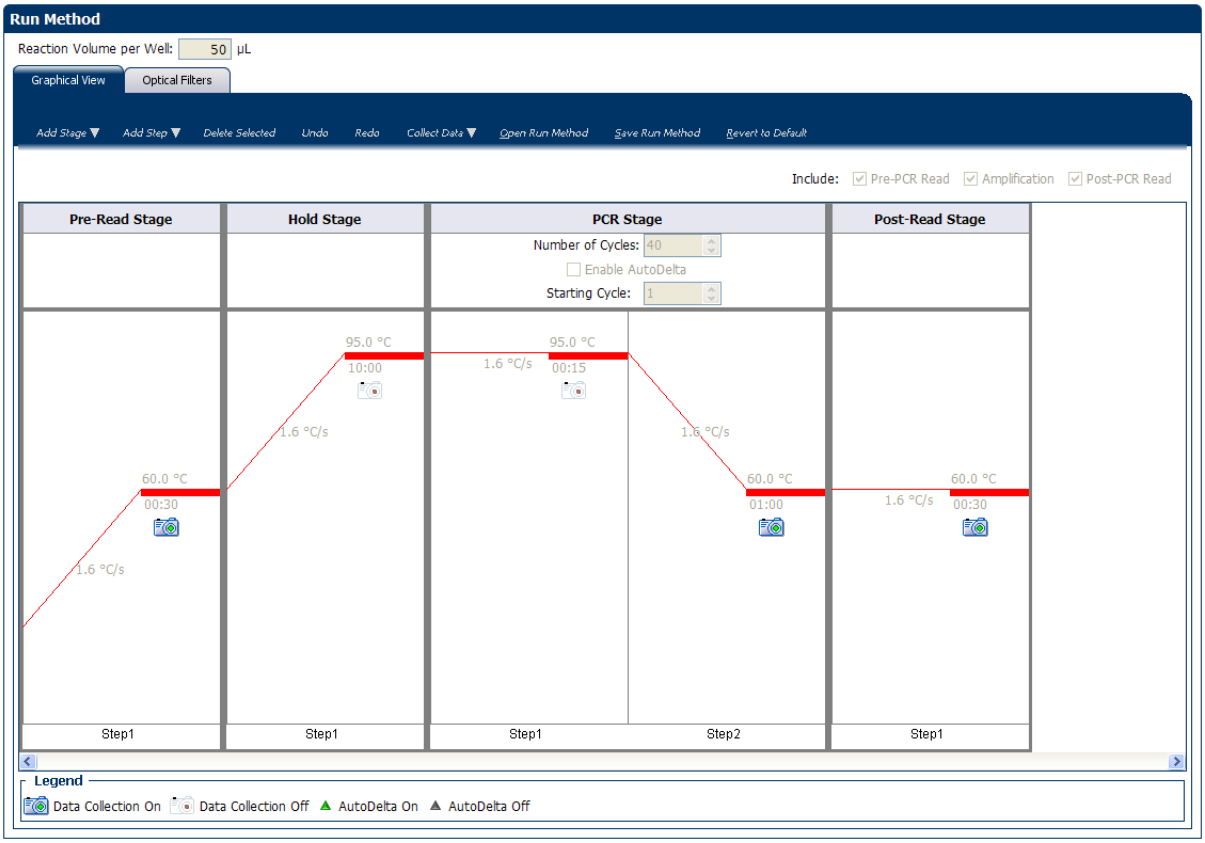
Set up the run method

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

- 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

Your Run Method screen should look like this:



For more information

For more information on...	Refer to...	Part number
Consumables	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* Appendix A in Booklet 7, *QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes*	4470050
Data collection	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*	127AP05-03
Using alternative setup	Chapter 2 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

3 Prepare the Reactions

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

This chapter covers:

- Assemble required materials 19
- Prepare the sample dilutions 19
- Prepare the reaction mix (“cocktail mix”). 20
- Prepare the reaction plate 20
- Tips for preparing reactions for your own experiments. 22
- For more information. 22

Assemble required materials

- Items listed in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*
- Samples - Sample 1 - Sample 19
- Example experiment reaction mix components:
  - **TaqMan® Genotyping Master Mix (2X)**
  - SNP 1 Assay Mix (20×)
  - SNP 2 Assay Mix (20×)

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.

To prepare the sample dilutions:

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 ("cocktail 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 cocktail by adding the required volumes of each component to the SNP 1 reaction tube, as detailed below.

Reaction component	Reaction volume			
	Per well (µL)		38 Reactions + 10% excess (µL)	
	Dry	Wet	Dry	Wet
TaqMan® Genotyping Master Mix (2X)	10.0	10.0	420.0	420.0
SNP Assay Mix (20X)	1.0	1.0	42.0	42.0
H_2O, DNase-free	39.0	37.0	1,638.0	1,554.0
Total Reaction Mix Volume	50.00	48.00	2,100.0	2,016.0

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 through 5 for the SNP 2 assay.

   Note: Do not add the sample at this time.

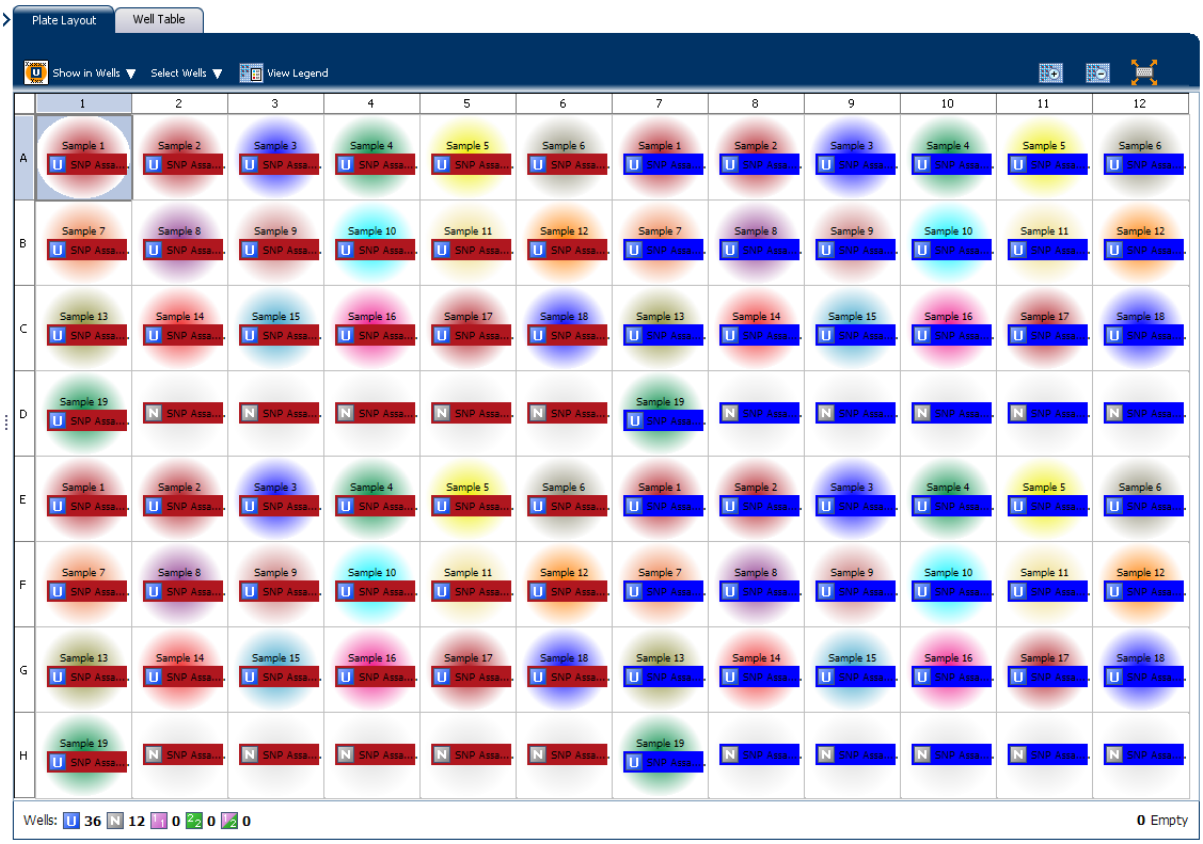
Prepare the reaction plate

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 U

The reaction plate for the example experiment looks like this:



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 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! Make 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 to 5 sec.

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 to 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:

- 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, make sure you prepare the reactions for each SNP separately.

Prior to use:

- 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

When you prepare the reaction plate for your own experiment:

- Make sure the reaction locations match the plate layout in the QuantStudio™ 12K Flex Software.
- Load 1 to 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

For more information on...	Refer to...	Part number
Assigning the reaction plate components	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

4 Run the Experiment

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

This chapter covers:

- Start the run 23
- Monitor the run 23

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 QuantStudio™ 12K Flex Instrument is in operation.

Start the run

1. Open the Genotyping example file that you created using instructions in Chapter 2.
2. Load the reaction plate into the instrument.
3. Start the run.

   Note: To collect real-time data during a run, click the  button on the Run Method screen in the Experiment Setup menu.

Monitor the run

Monitor the example experiment run:

- From the QuantStudio™ 12K Flex Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio™ 12K Flex Software (to monitor an experiment started from another computer or from the QuantStudio™ 12K Flex Instrument touchscreen).
- From the QuantStudio™ 12K Flex Instrument touchscreen.

From the Instrument Console of the QuantStudio™ 12K Flex Software

1. In the Instrument Console screen, select the instrument icon.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. On the Manage Instrument screen, click **Monitor Running Instrument** 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.

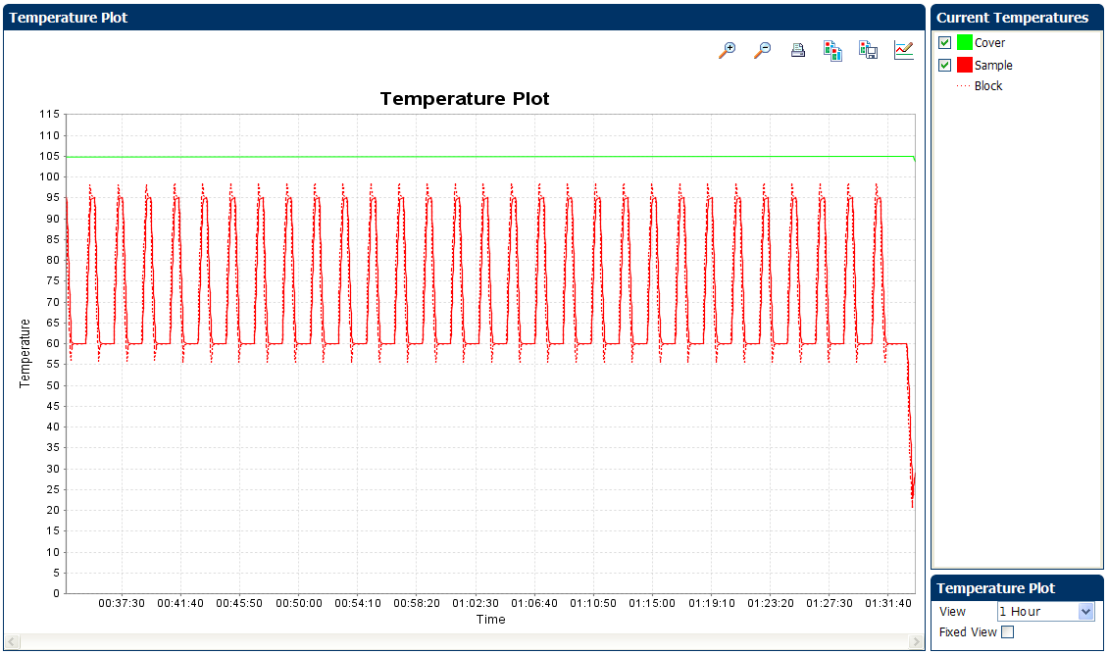
The figure below shows the Amplification Plot screen as it appears at the end of the example experiment.

Note: The Amplification Plot is not available for experiments that do not include the PCR process.

View the Temperature Plot

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

The figure below shows the Temperature Plot screen as it appears during the example experiment.

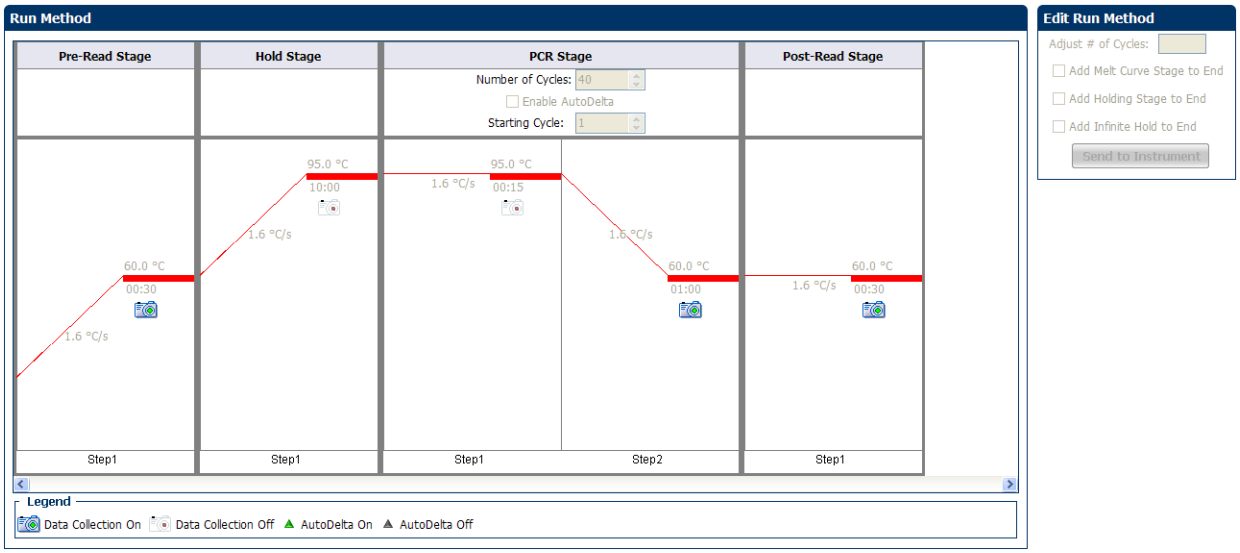


Note: The sample temperature displayed in the Current Temperatures group is a calculated value.

View the Run Method

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

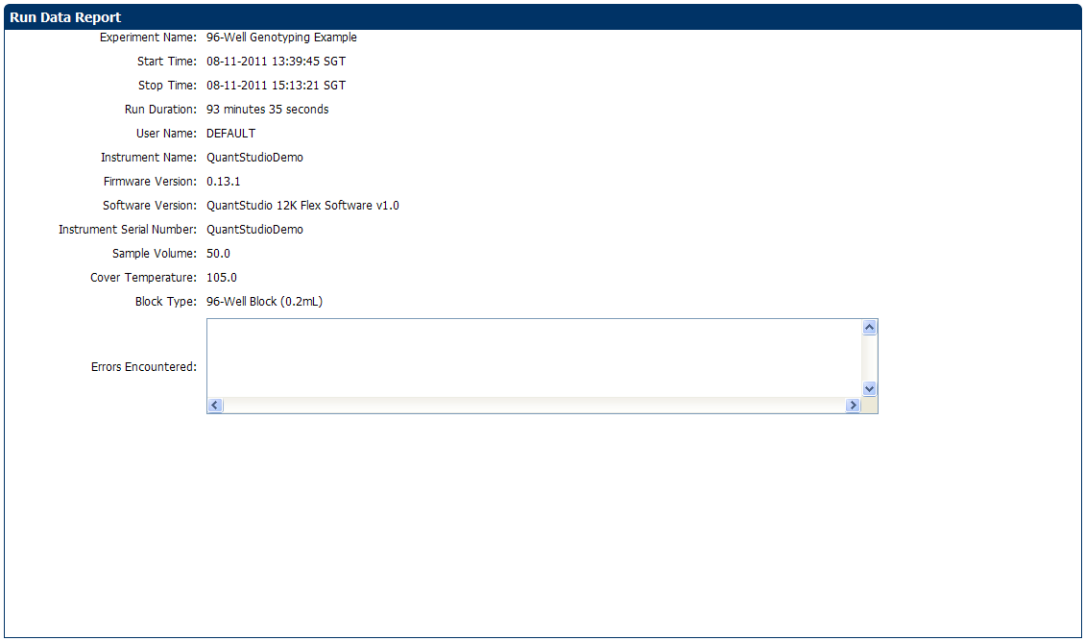
The figure below shows the Run Method screen as it appears in the example experiment.



View run data

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

The figure below shows the View Run Data screen as it appears in the example experiment.

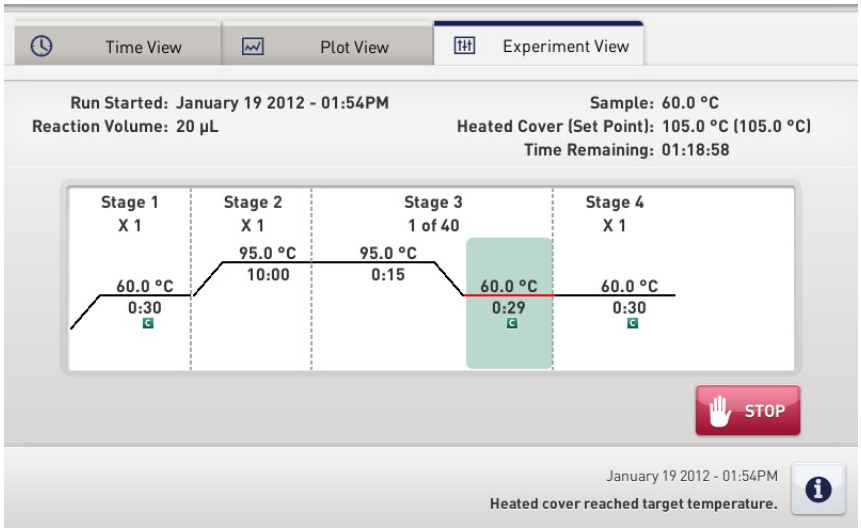


From the QuantStudio™ 12K Flex Instrument touchscreen

You can also view the progress of the run from the touchscreen of the QuantStudio™ 12K Flex Instrument.

The Run Method screen on the **QuantStudio™ 12K Flex Instrument** touchscreen looks like this:

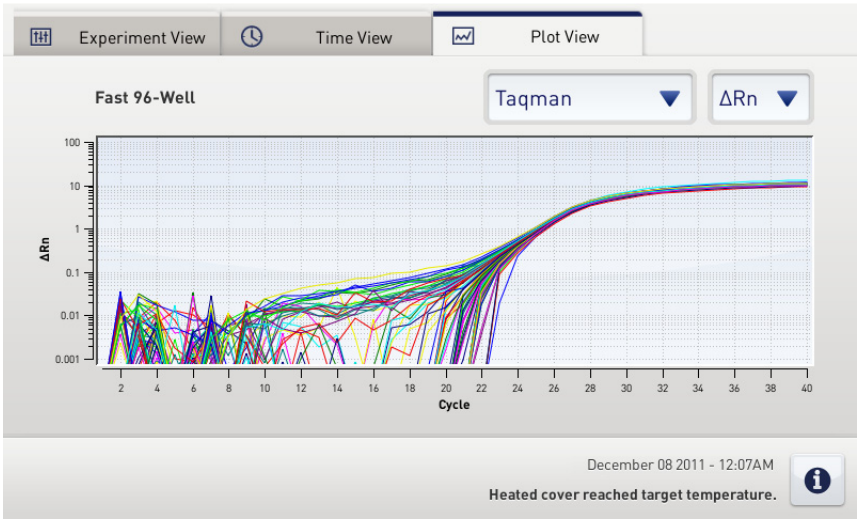
Experiment View



Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

Time View

Plot View



Note: You will see the Plot View only if your experiment includes the PCR process.

5 Review Results and Adjust Experiment Parameters

In Section 5.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 5.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

Section 5.1 Review Results 31

- Analyze the example experiment. 31
- View clusters in the Allelic Discrimination Plot 31
- Confirm setup accuracy using Plate Layout. 34
- Assess amplification results using the Amplification Plot. 37
- Identify well problems using the Well Table 40
- Confirm accurate dye signal using the Multicomponent Plot. 43
- Determine signal accuracy using the Raw Data Plot 46
- Review the flags in the QC Summary 47
- For more information. 49

Section 5.2 Adjust parameters for re-analysis of your own experiments. 51

- Adjust analysis settings 51
- For more information. 55

Section 5.1 Review Results

Analyze the example experiment

1. Open the example experiment file that you ran in Chapter 4.
2. Click **Analyze**. The software analyzes the data using the default analysis settings.

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

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:

- Clusters for the three possible genotypes (Allele 1 homozygous, Allele 2 homozygous, and Allele 1/2 heterozygous).
- A cluster for the no template controls.

To 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 menu, then enable Autocaller.

   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

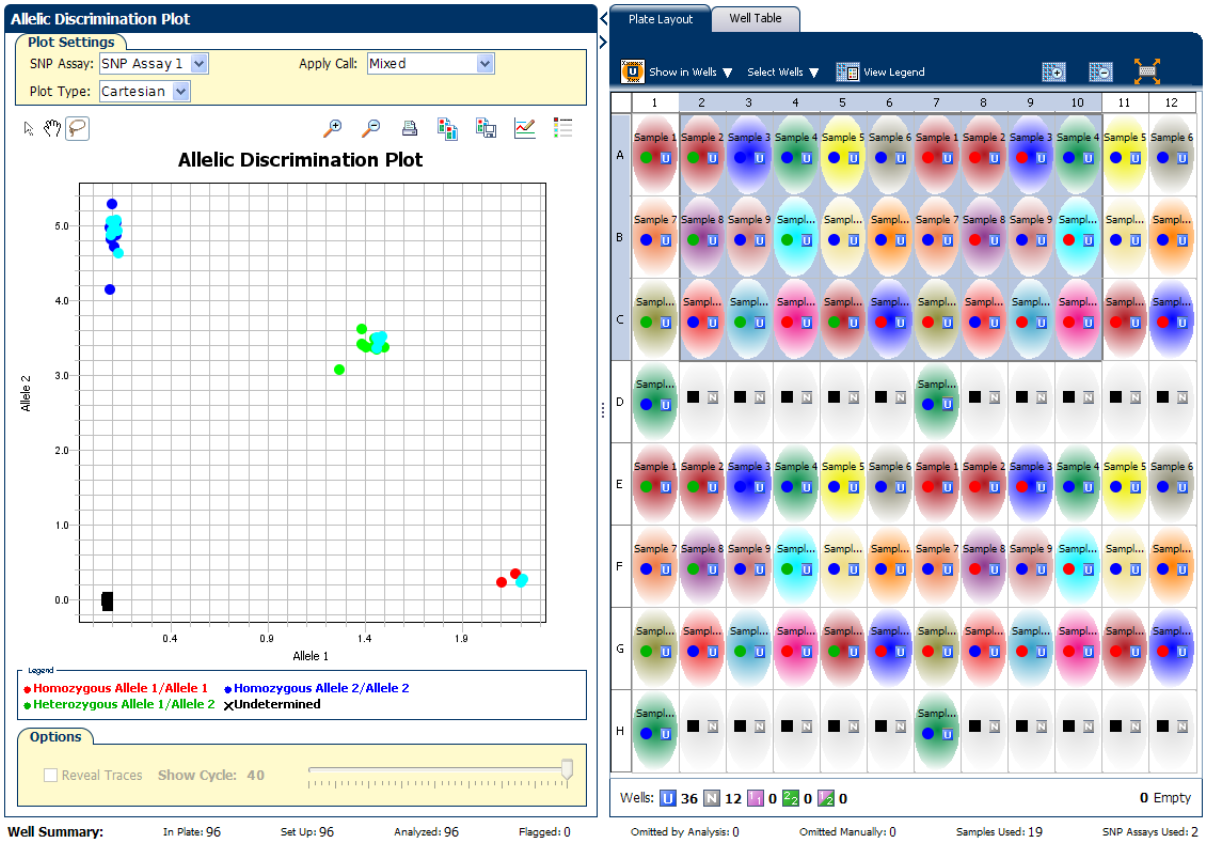
Note: If the Autocaller is not enabled, the Allelic Discrimination Plot displays a crossmark (X – Undetermined) for each sample.

4. 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 steps a and b for all other clusters in the plot.
   d. The table below describes the elements of the Allelic Discrimination Plot.

Element	Description
SNP Assay drop-down menu	Determines the SNP assay data that the QuantStudio™ 12K Flex Software displays in the plot.
Plot Type drop-down menu	Determines the type of plot (Cartesian or Polar) that the QuantStudio™ 12K Flex Software uses to display the data.
Apply Call drop-down menu	When a datapoint is selected, this menu allows you to assign an allele call to the datapoint within the scatterplot.
Toolbar	Contains tools for manipulating 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 52.

The Allelic Discrimination plot for the example experiment looks like this:



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 and 2 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. 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 QuantStudio™ 12K Flex Software called:

- 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.

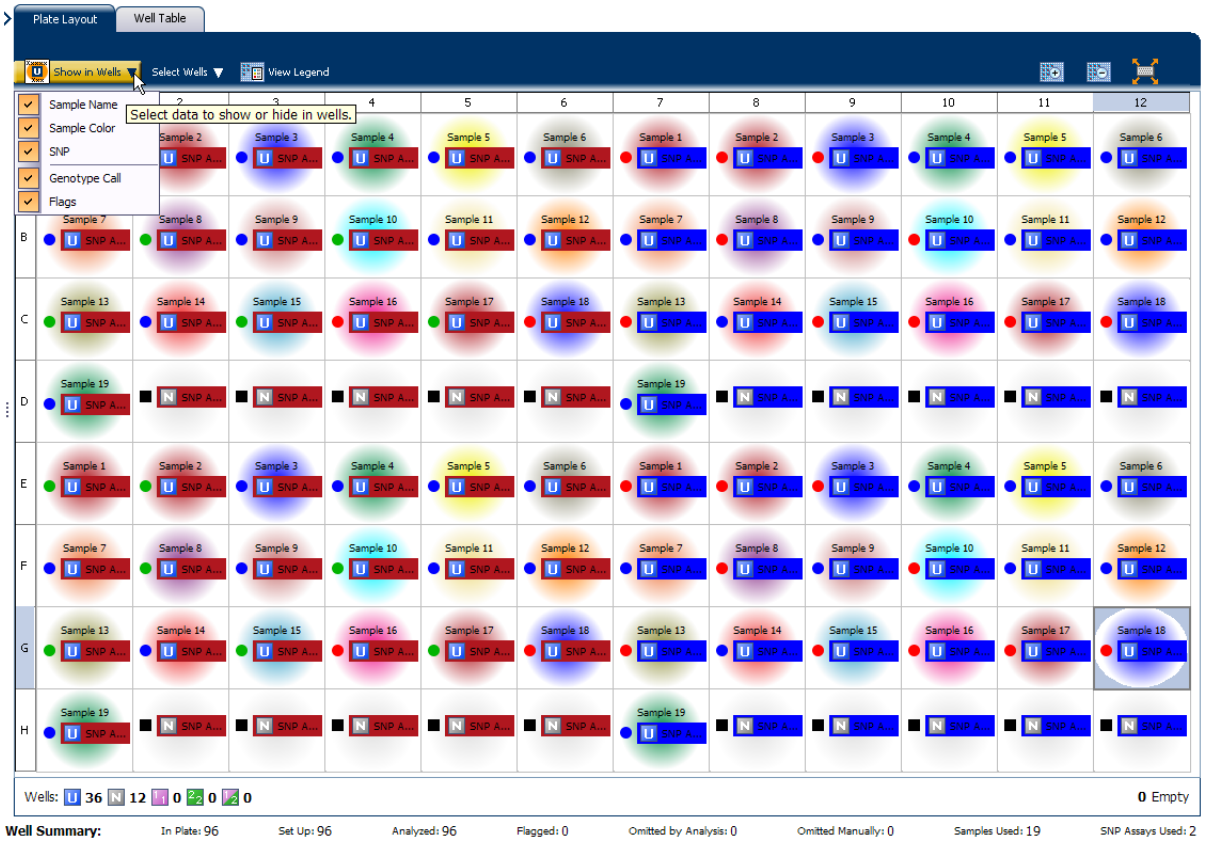
View the layout

1. Click the ‹ icon 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.

Parameter	Description
Task	The task assigned to the well:  – Unknown  – No Template Control  – Positive Control - Allele 1  – Positive Control - Allele 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.

The following figure shows the plate layout of the example Genotyping experiment.



Tips for troubleshooting plate setup in your own experiment

You can 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 menu in the Plate Layout tab to select wells of a specific type using the well-selection criteria.
- Use the (Zoom In), (Zoom Out), and (Fit Plate) buttons to magnify or compress the view of the wells shown.
- Use the ‹ arrow tabs 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 ΔR_n 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.

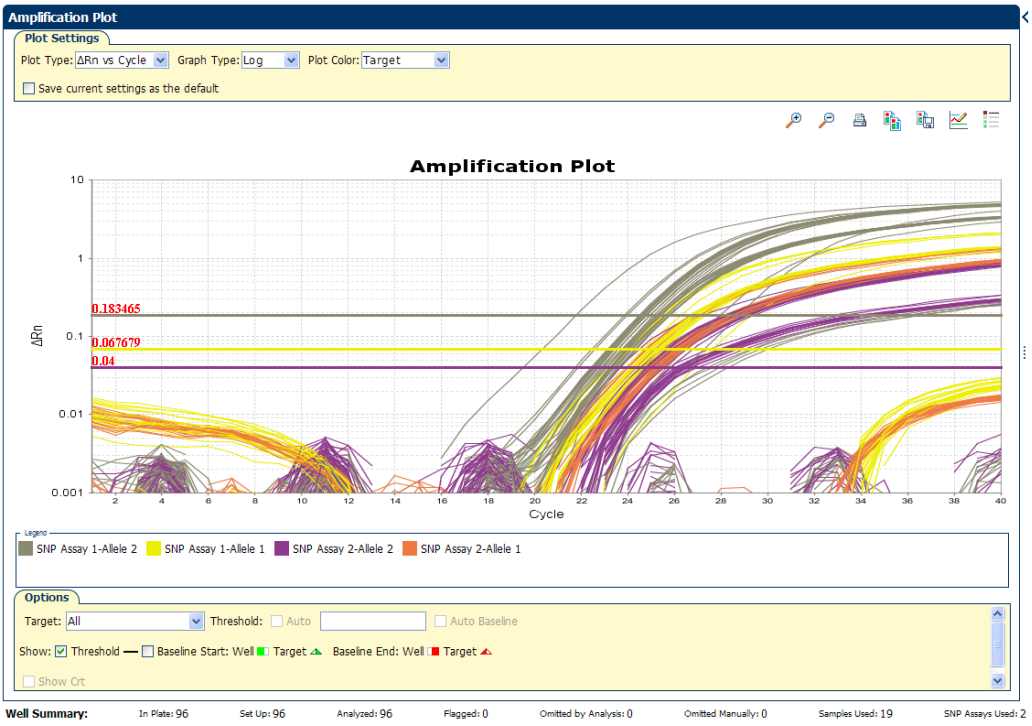
View the ΔRn vs. Cycle plot

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

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

2. Select the plot type and format:

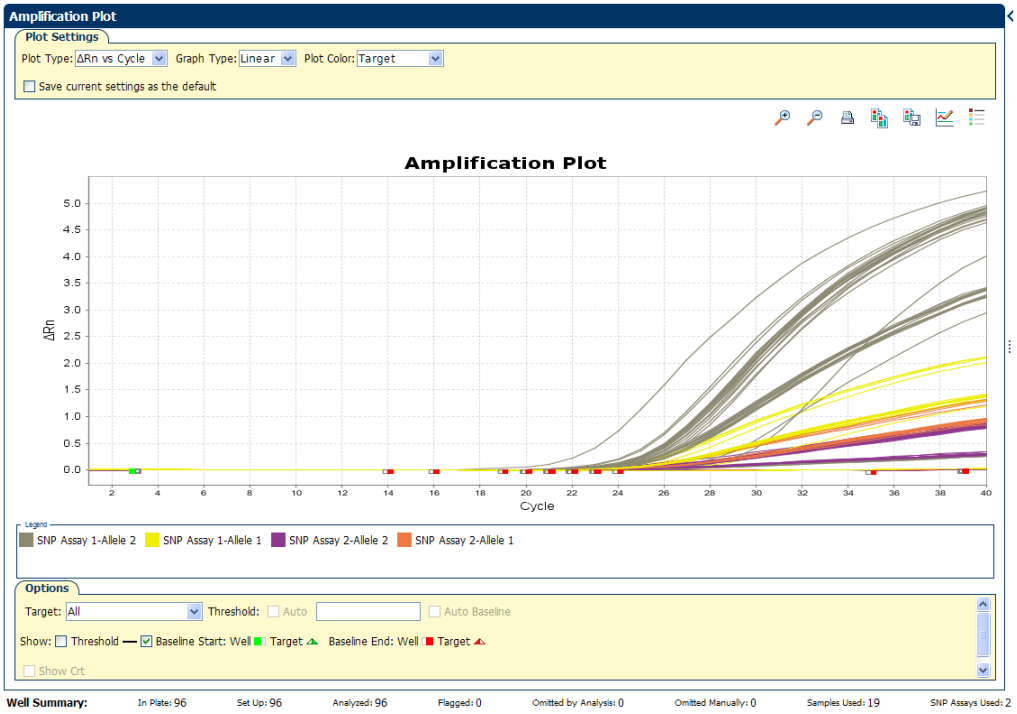
Menu	Selection
Plot Type	ΔRn vs. Cycle
Plot Color	Target
 (This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.)	Check (default)



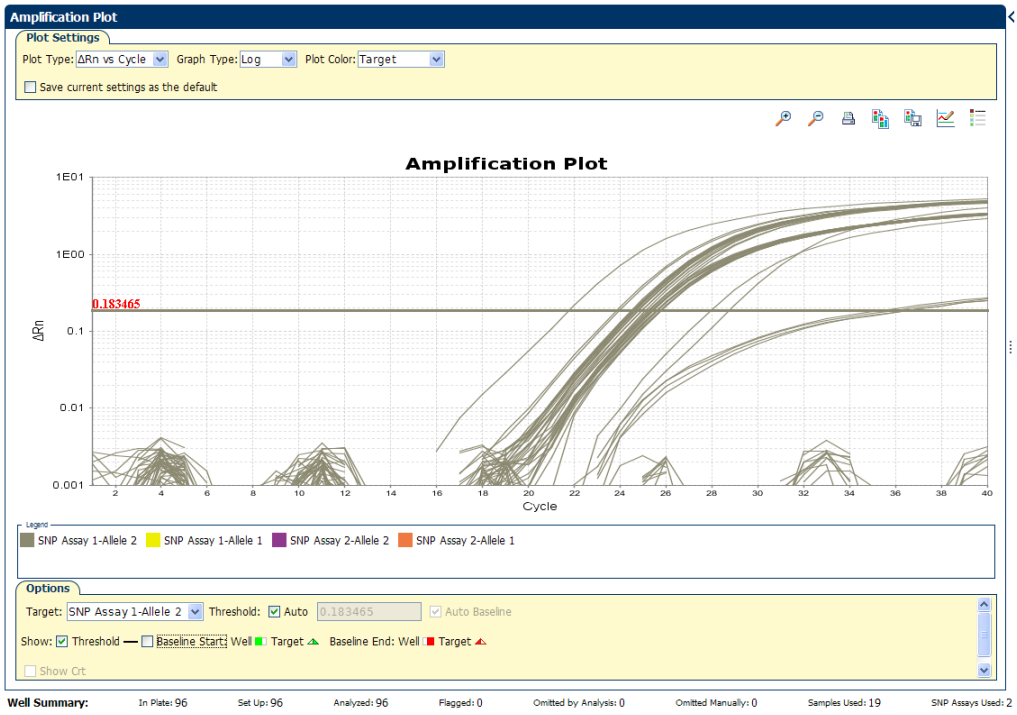
3. View the baseline values:

   a. From the Graph Type drop-down menu, select **Linear**.

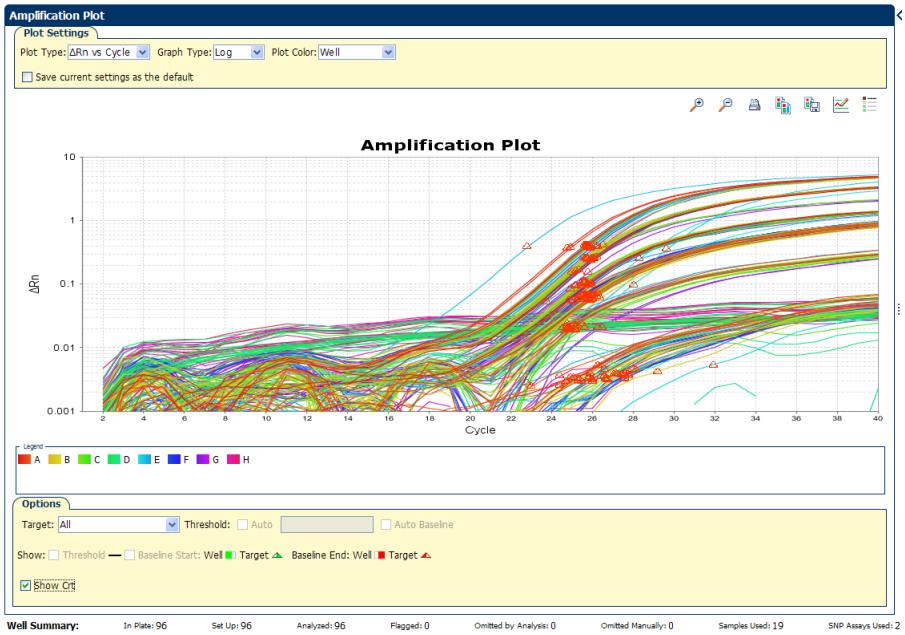
b. Select Baseline to show the start cycle and end cycle.



4. View the threshold values:
   a. From the Graph Type drop-down menu, select **Log**.
   b. From the Target drop-down menu, select **SNP Assay 1-Allele 2**.
   c. Select the Threshold check box 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** check box 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.

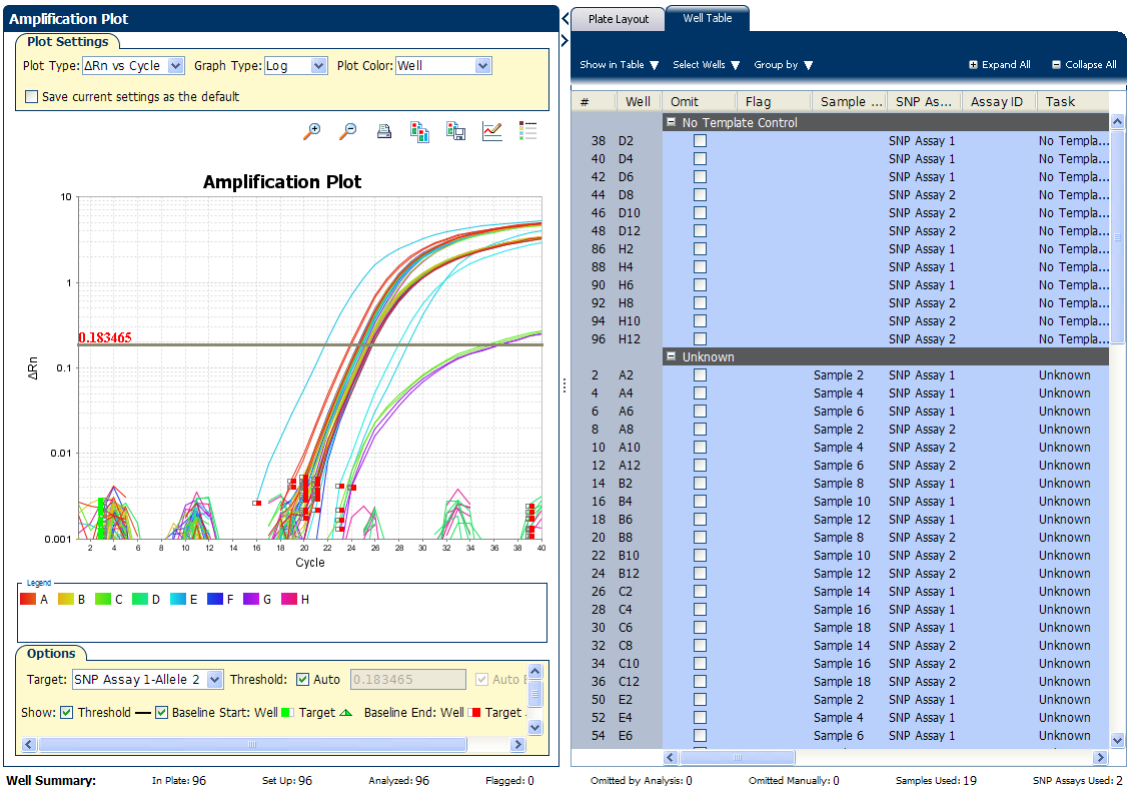
Example experiment values and flags

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. From 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 (▲).
   c. Click the ⊟ icon to collapse the negative and positive controls.

The figure below shows 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	The position of the well on the reaction plate.
Omit	A check mark indicates that the well has been removed from the analysis.
Flag	A (▲) indicates that the well triggered the number of flags listed inside the symbol.
Sample Name	The name of the sample.
SNP Assay Name	The name of the SNP assay evaluated by the well.
Assay ID	The Assay ID number of the SNP evaluated by the well.
Task	The task assigned to the well (Unknown, No Template Control, or Positive Control).
Allele 1 / 2	The name of the associated allele for the SNP evaluated by the well.
Allele 1 / 2 Dyes	The name of the reporter and quencher dyes of the associated allele for the SNP evaluated by the well.
Allele 1 / 2 R_n	Normalized signal (R_n) of the reporter dye of the associated allele for the SNP evaluated by the well.

Column	Description
Pass Ref	The signal of the passive reference dye for the well.
Call	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 • ■ No Template Control • ✕ Undetermined
Quality (%)	The quality value calculated for the genotype call.
Method	The method used to assign the call to the sample (Auto if assigned by the QuantStudio™ 12K Flex Software, or Manual if applied by a user).
Comments	Comments entered for the associated sample well.
Allele 1 / 2 C_T	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 QuantStudio™ 12K Flex Software does not display a corresponding column for the flag.

A (▲) 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 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.
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**.

Flag	Description
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. From the Group By menu, 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 (▲) in the Flag column, note the data and the flag(s) triggered by the associated well.
- Select areas of the table or wells of a specified type by:
  - 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 menu 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 menu. You can then collapse or expand the lists either by clicking the **+/-** icon next to individual lists, or by clicking **Collapse All** or **Expand All**.
- Omit a well from the analysis by selecting the **Omit** check box for that well. To include the well in the analysis, deselect the **Omit** check box.

  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. From 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.

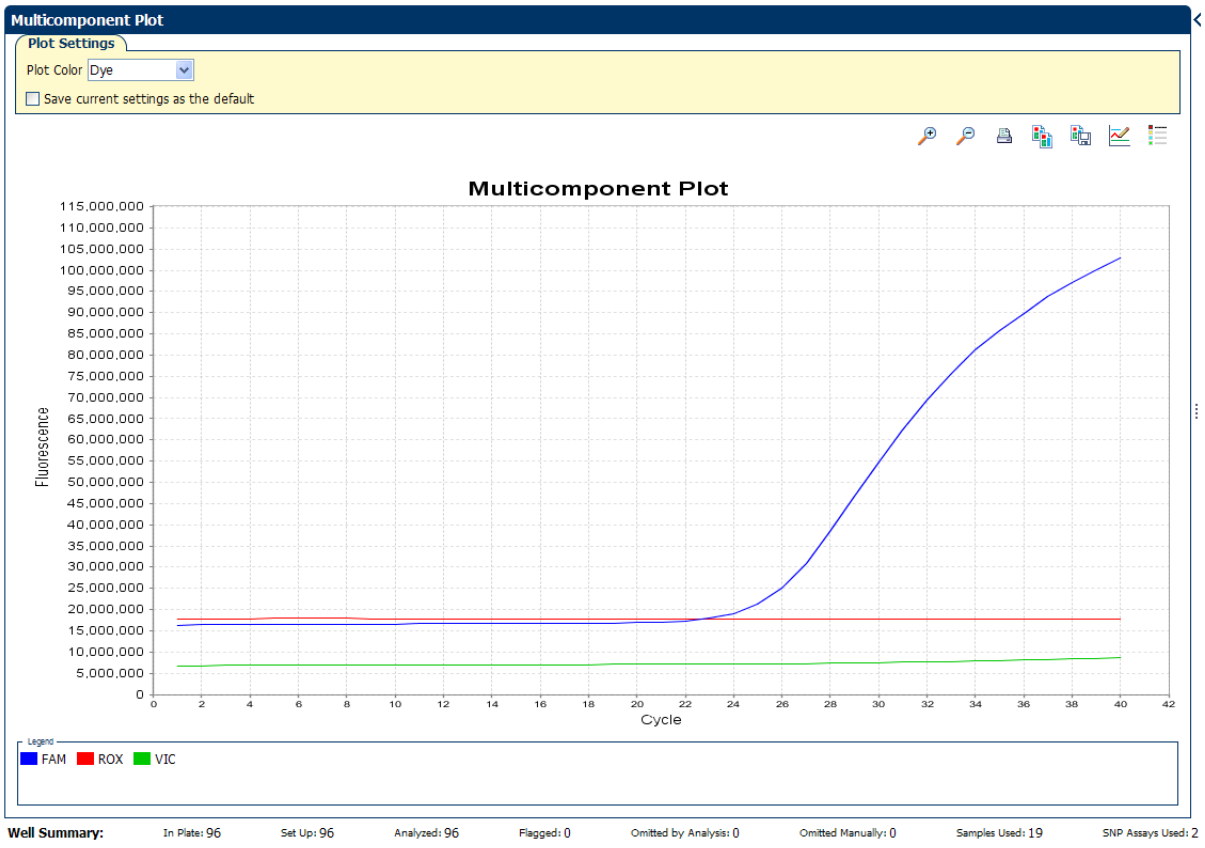
3. From the Plot Color drop-down menu, select **Dye**.

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; 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:

- **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.
- **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

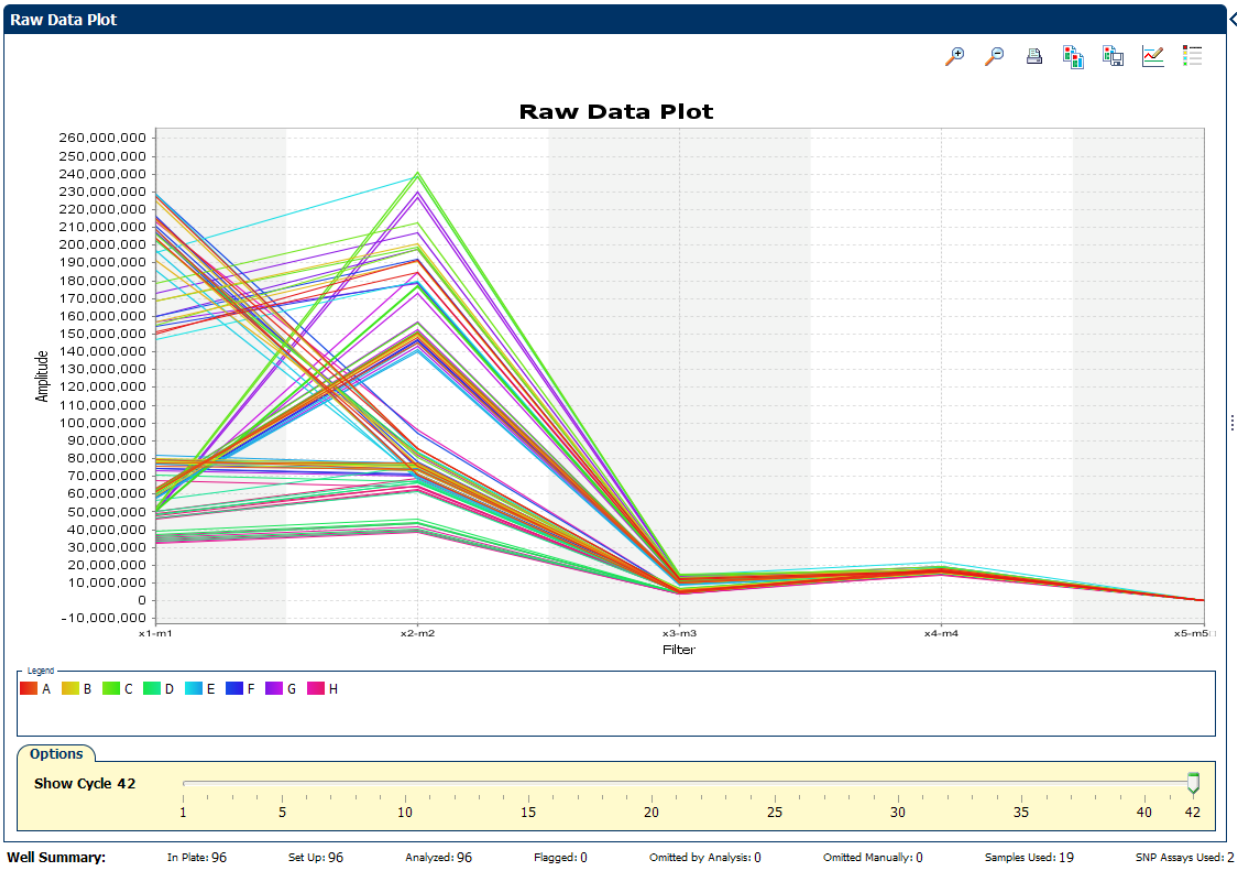
In the Genotyping example experiment, you review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the Raw Data Plot

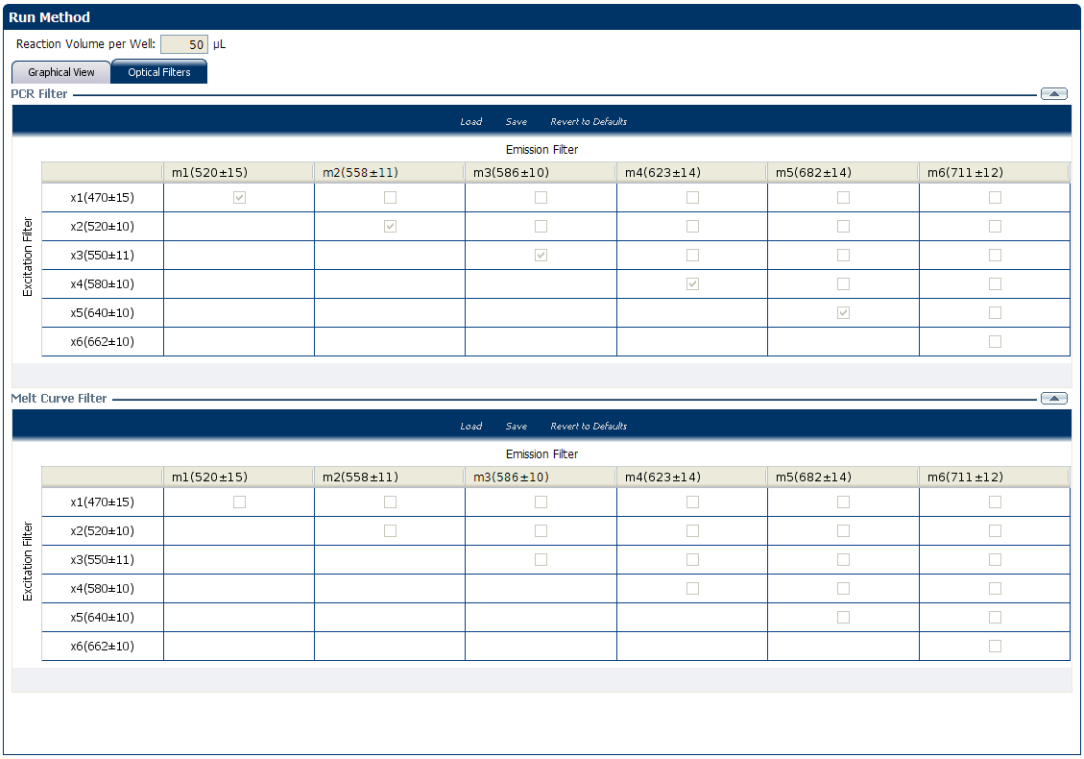
1. From the Experiment Menu pane, select **Analysis** ▸ **Raw Data Plot**.

   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 the legend in the Raw Data Plot shown below).
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.



The filters are:



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

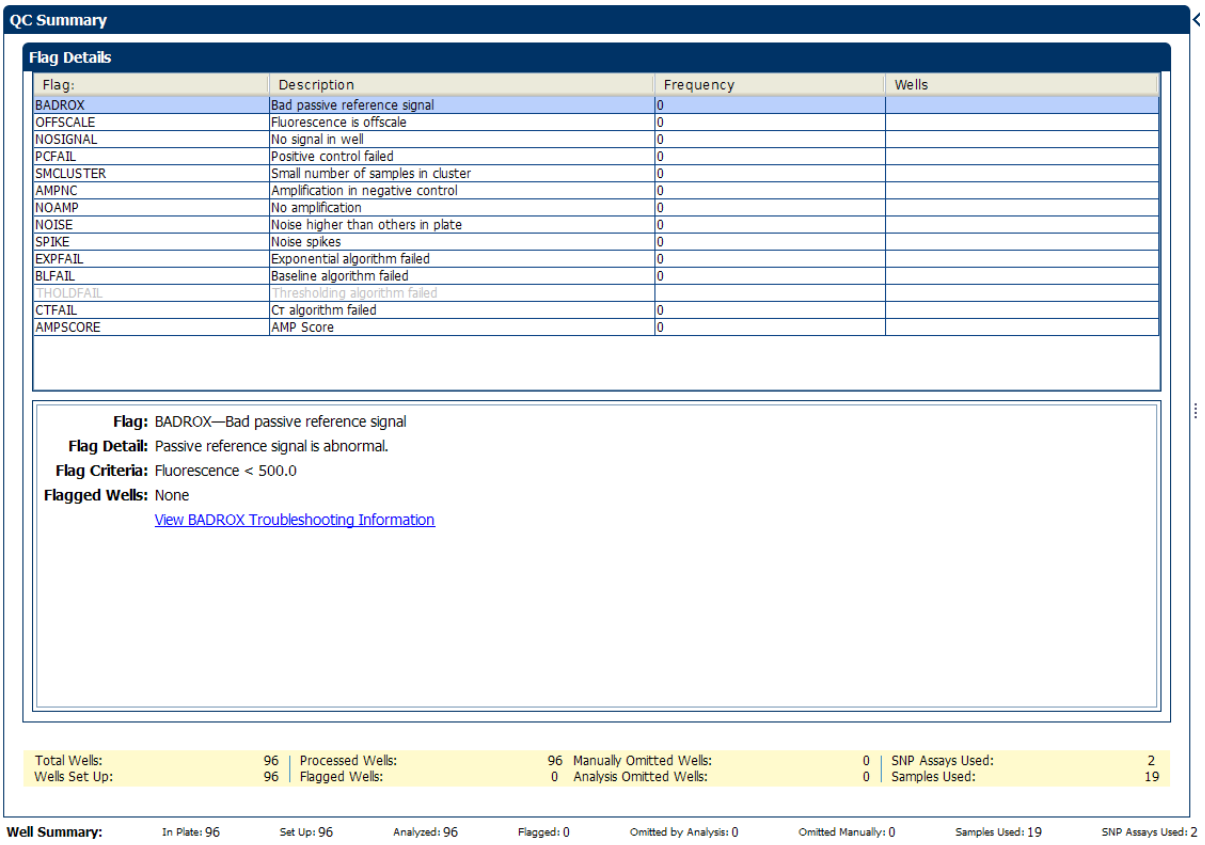
   Note: If no data are displayed, click **Analyze**.

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 >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 >0 to display detailed information about the flag.

4. *(Optional)* For those flags with frequency >0, click each flag with a frequency >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
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

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

Section 5.2 Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

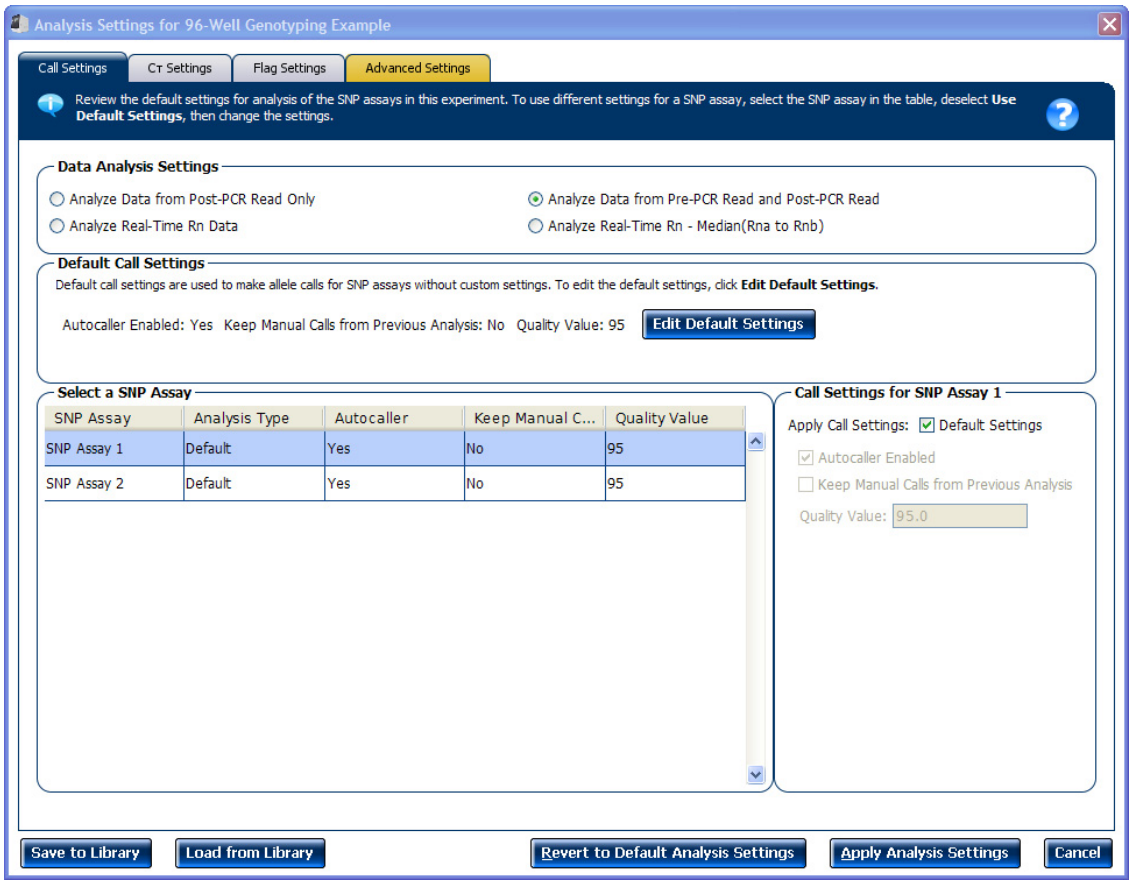
The Analysis Settings dialog box displays the analysis settings for the call, threshold cycle (C_T), flags, and advanced options.

You can change the settings in the Analysis Settings dialog box, then reanalyze your experiment.

View the analysis settings

1. 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:
   - Call Settings
   - C_T Settings
   - Flag Settings
   - Advanced Settings

   The Analysis Settings dialog box for a Genotyping experiment looks like this:



3. View and, if necessary, change the analysis (see "Adjust analysis settings" below).

   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 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Experiments*.

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:
  - Analyze data from Post-PCR Read only - 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 - 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 - 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 data from Rn - Avg (Rna to Rnb) - 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 scree, select either **Analyze Real-Time Rn Data** or **Analyze data from Rn - Avg (Rna - Rnb)**.
- Edit the default call settings. Click **Edit Default Settings**, then specify the default settings:
  - **Autocaller Enabled** - Select for the software to make genotype calls using the autocaller algorithm.
  - **Keep Manual Calls from Previous Analysis** - If the autocaller is enabled, select to maintain manual calls after reanalysis
  - **Quality Value** - Enter a value to use to make genotype calls. If the confidence value is less than the call setting, the call is undetermined.
- Use custom call settings for a SNP assay.
  - Select one or more SNP assays in the table, then deselect the **Default Settings** checkbox.
  - **Define the custom call 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 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, Life Technologies recommends:

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: This setting is applicable only to the Baseline Threshold algorithm.

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:

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

  To adjust the flag settings:

To adjust the flag settings:

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.

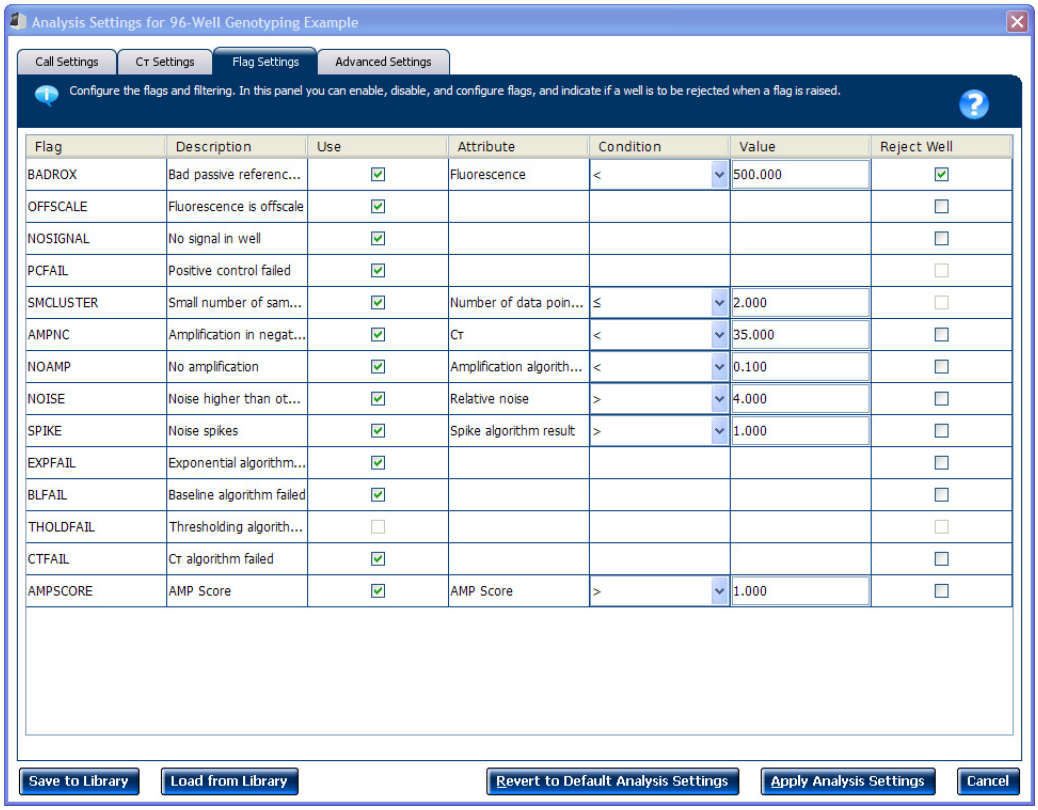
   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

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.

The Flag Settings tab looks like this:



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.

To use 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** check box.

3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, then enter the baseline start cycle and baseline end cycle.

For more information

For more information on...	Refer to...	Part number
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*	127AP05-03

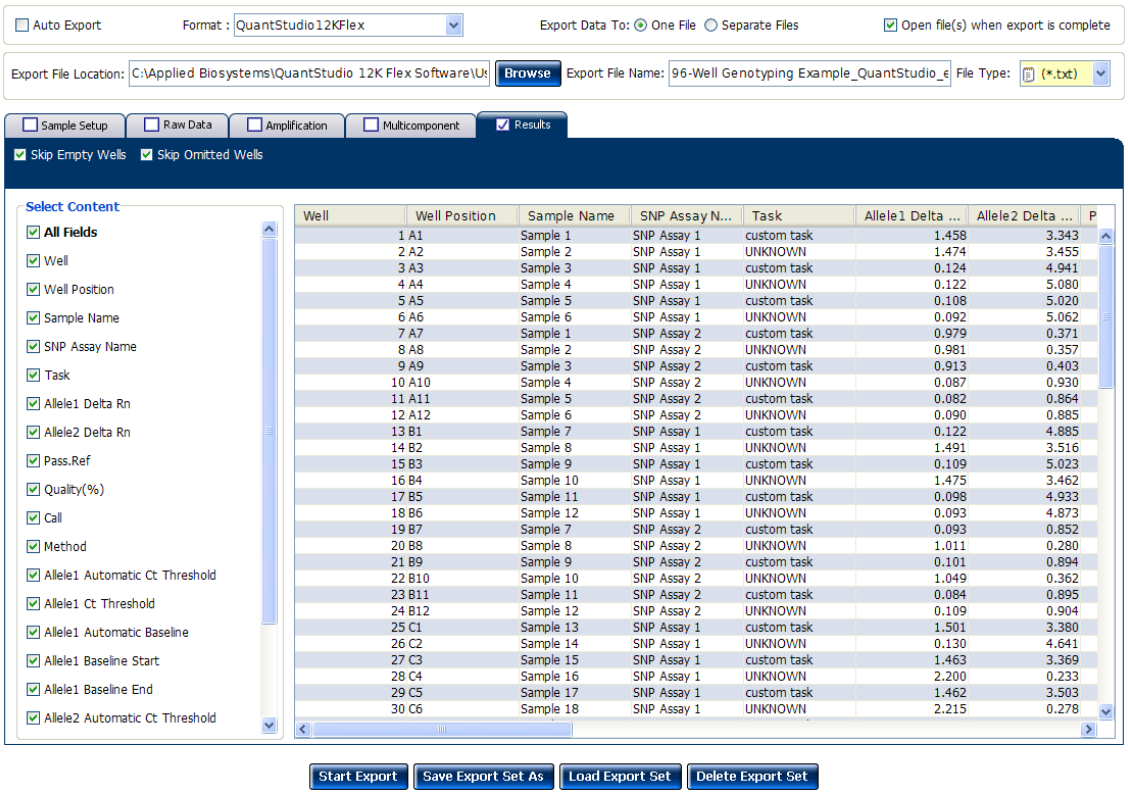
6 Export Analysis Results

1. Open the Genotyping example experiment file that you analyzed in Chapter 5.
2. In the Experiment Menu, click  **Export.**

   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.
3. Select **QuantStudio™ 12K Flex format**.
4. Complete the Export dialog box as shown below:

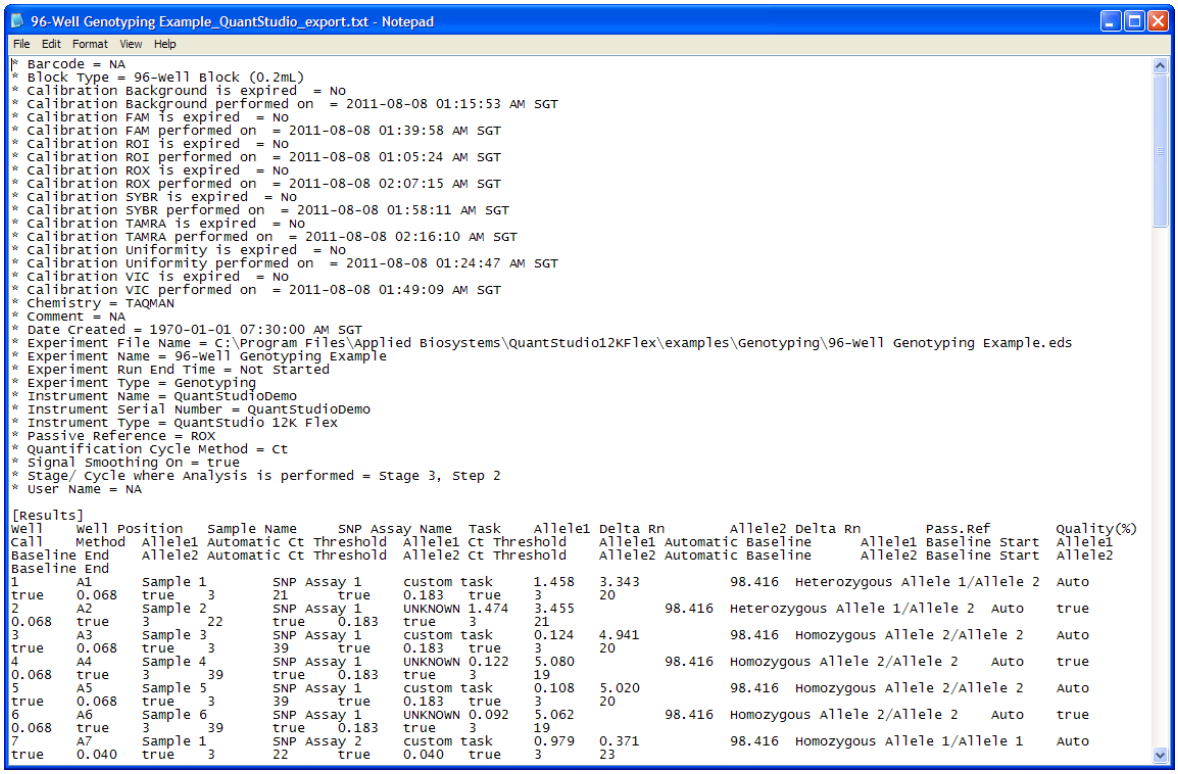
Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	96-Well Genotyping Example_QuantStudio_export
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments

Your Export screen should look like this:



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	

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
```

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GETTING STARTED GUIDE

applied biosystems® by life technologies™

Booklet 5 - Running Presence/Absence Experiments

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1 About Presence/Absence Experiments

This chapter covers:

- About data collection 5
- Setting up PCR reactions 6
- About the instrument run 6
- About the analysis 7
- About the example experiment 7

IMPORTANT! First-time users of the QuantStudio™ 12K Flex System, please read Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* and Booklet 7, *QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes* of this binder thoroughly. The booklet provides information and general instructions that are applicable to all the experiments described in this binder.

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 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 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 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. PCR reactions contain two primer/probe sets: One to detect the unknown target (unknown target primer set and TaqMan® probe to detect the unknown target) and one to detect the IPC (IPC primer set and a VIC dye-labeled TaqMan® probe to detect the IPC template). With this setup, there are three well types:

- **Unknown-IPC wells** contain sample template and IPC template; the presence of the target is not known.
- **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. Also called **IPC+**.
- **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. 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 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.

In the Presence/Absence example experiment:--

- DNA is extracted from samples using the PrepMan® Ultra Sample Preparation Reagent (PN 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.

2 Design the Experiment

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

This chapter covers:

- Define the experiment properties. 9
- Define targets and samples 10
- Assign targets and samples. 11
- Set up the run method 12
- For more information. 13

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 on Auto Export, refer to Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.

Define the experiment properties

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

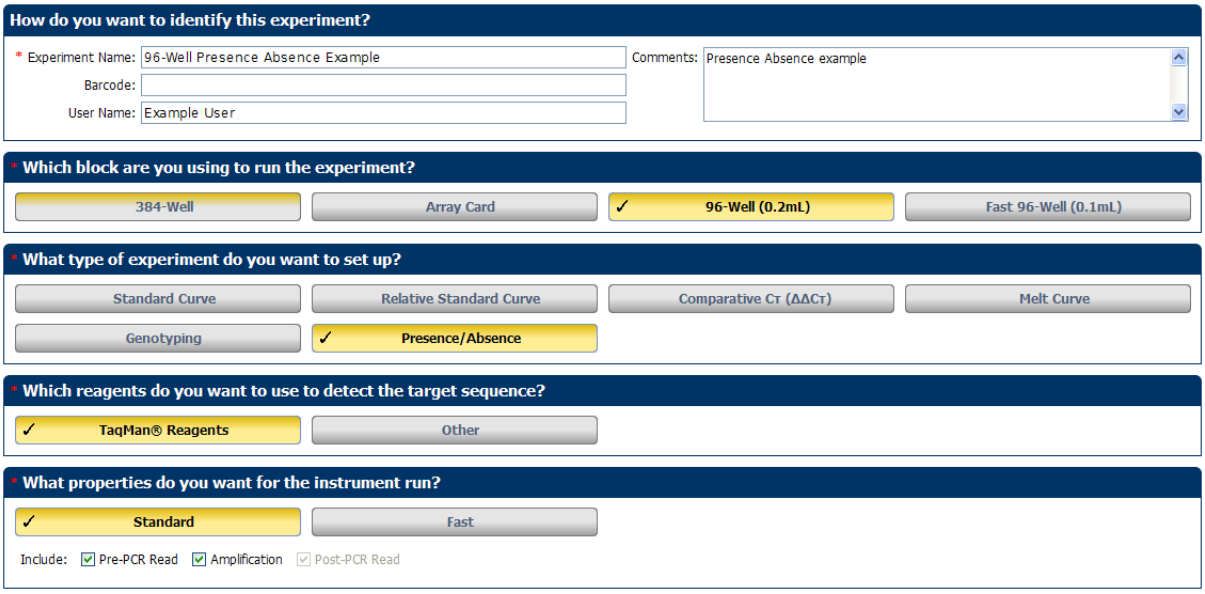
Field or Selection	Entry
Experiment Name	96-Well Presence Absence Example
Barcode	Leave field empty
User Name	Example User
Comments	Presence/Absence example
Block	96-Well (0.2mL)
Experiment Type	Presence/ Absence
Reagents	TaqMan® Reagents
Ramp speed	Standard

Select all three data-collection check boxes: Pre-PCR, Amplification, and Post-PCR collection methods:

Pre-PCR Read	Checked
Amplification	Checked
Post-PCR Read	Checked

Save the experiment.

Your Experiment Properties screen should look like this:



Define targets and samples

Click **Define** to access the Define screen. Enter:

1. Targets

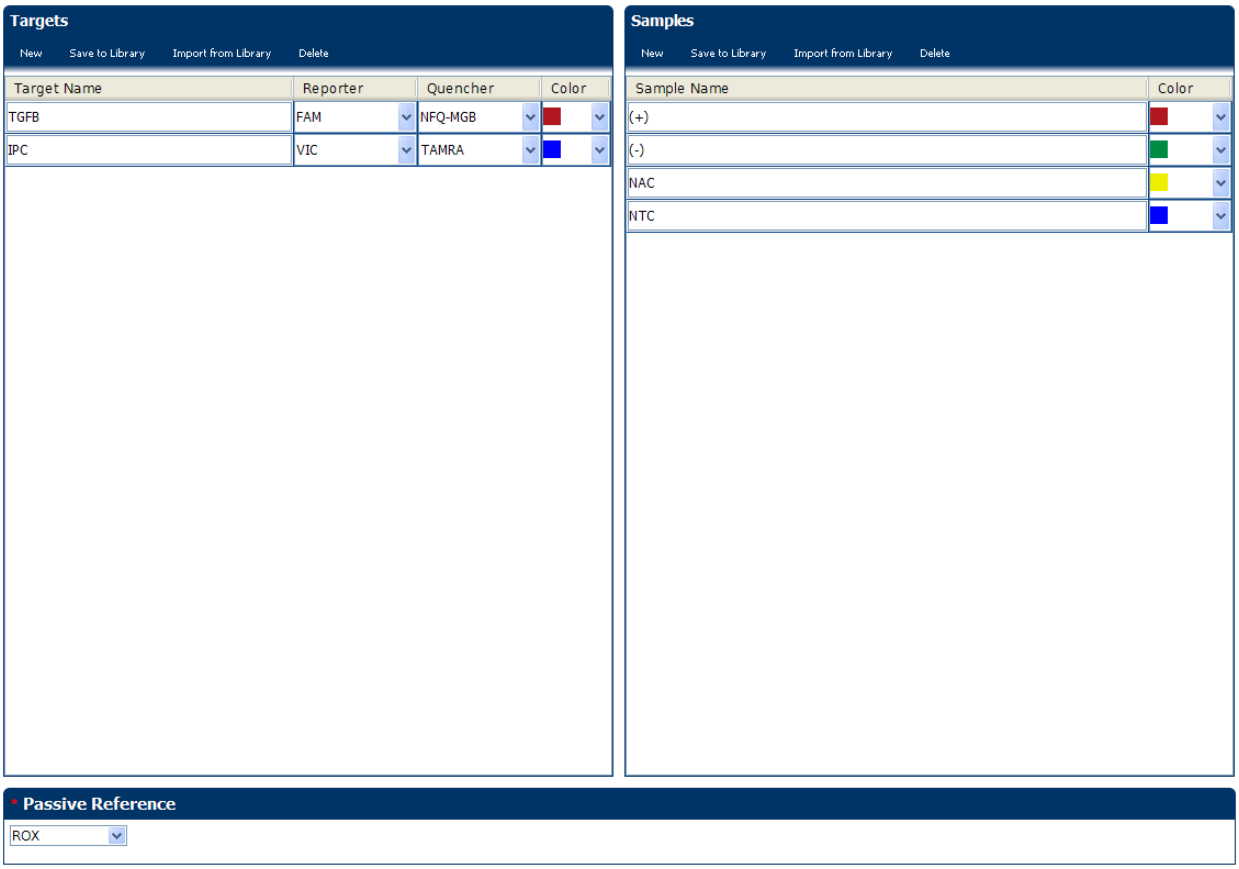
Target Name	Reporter	Quencher	Color
TGFB	FAM	NFQ-MGB	
IPC	VIC	TAMRA	

2. Samples

Sample Name	Color
(+)	
(-)	
NAC	
NTC	

3. Dye to be used as a Passive Reference
ROX

Your Define screen should look like this:

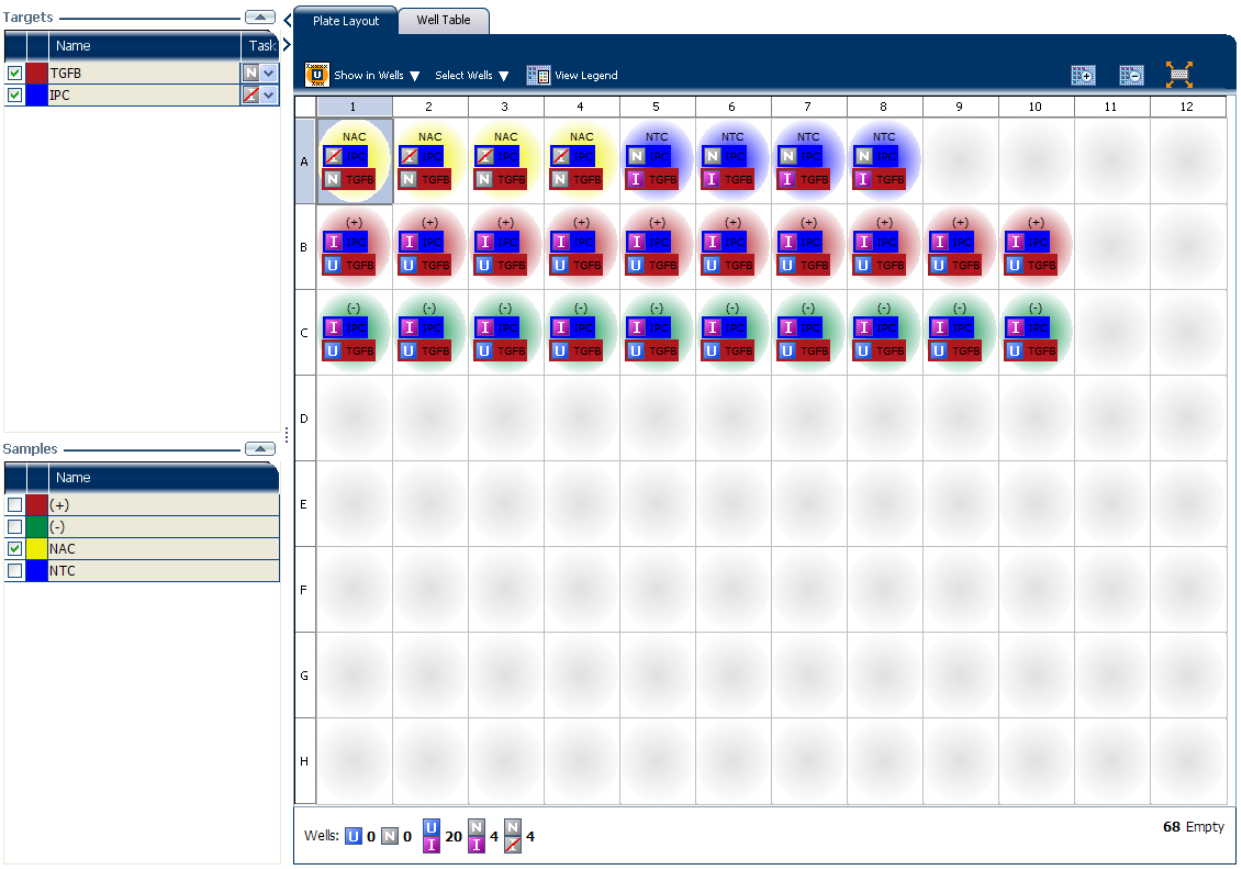


Assign targets and samples

Click **Assign** to access the Assign screen. Enter the 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	(-)

Your Assign screen should look like this:



Set up the run method

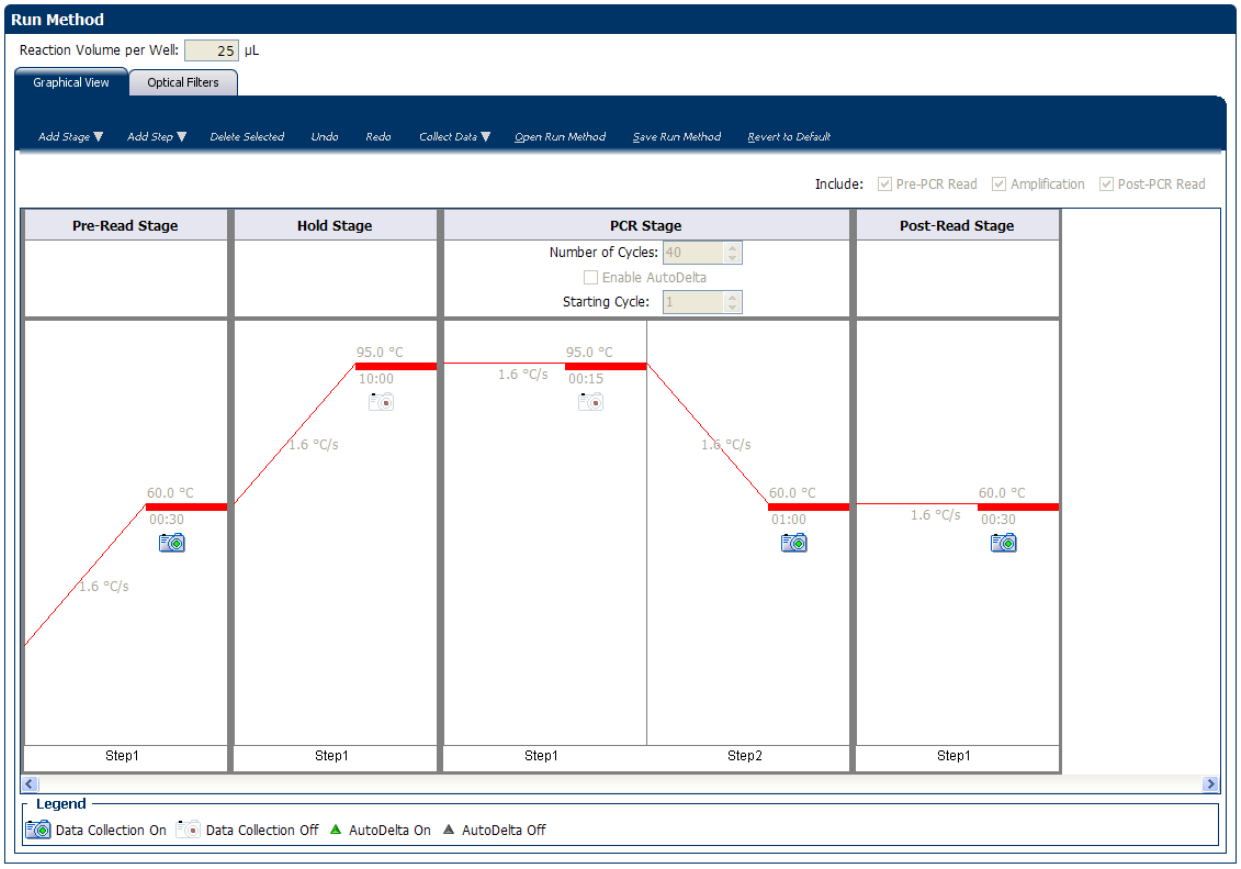
Click **Run Method** to access the Run Method screen. Set the thermal profile under the Graphical View tab. Enter:

- 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

Stage	Step	Ramp rate	Temperature	Time
Post-Read Stage	Step 1	1.6°C/s	60°C	30 seconds

Your Run Method screen should look like this:



For more information

For more information on...	Refer to	Part number
Consumables	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* Appendix A in Booklet 7, *QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes*	4470050
Data collection	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*	127AP05-03
Using alternative setup	Chapter 2 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

3 Prepare the Reactions

This chapter explains how to prepare the PCR reactions for the Presence/Absence example experiment.

This chapter covers:

- Assemble required materials 15
- Prepare the reaction mix (“cocktail mix”). 15
- Prepare the reaction plate 17
- For more information. 18

Assemble required materials

- Items listed in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*
- 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 (“cocktail mix”)

For the Presence/Absence example experiment, four cocktail mixes are used; one each for:

- (+)
- (-)
- 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 cocktail mixes.

Cocktail Mix	Reaction component	Volume for 1 reaction (µL)	Volume for 11 reactions (10 wells + 10% excess) (µL)
Cocktail Mix 1 for Sample (+)	TaqMan® Universal PCR Master Mix (2.0X)	12.50	137.50
	10X IPC Mix	2.50	27.5
	50X IPC DNA	0.50	5.5
	20X Primer/ Probe Mix	1.25	13.75
	Water/ Buffer	5.75	63.25
	Diluted unknown 1	2.5	27.5
	Total reaction mix volume	25.0	275
Cocktail Mix 2 for Sample (-)	TaqMan® Universal PCR Master Mix (2.0X)	12.50	137.50
	10X IPC Mix	2.50	27.5
	50X IPC DNA	0.50	5.5
	20X Primer/ Probe Mix	1.25	13.75
	Water/ Buffer	5.75	63.25
	Diluted unknown 2	2.5	27.5

Cocktail Mix	Reaction component	Volume for 1 reaction (µL)	Volume for 5 reactions (4 wells + 10% excess) (µL)
Cocktail Mix 3 for NTC/ IPC+	TaqMan® Universal PCR Master Mix (2.0X)	12.50	62.5
	10X IPC Mix	2.50	12.5
	50X IPC DNA	0.50	2.5
	20X Primer/ Probe Mix	1.25	6.25
	Water/ Buffer	8.25	41.25
	Total reaction mix volume	25.0	125.0
Cocktail Mix 4 for NAC/ IPC-	TaqMan® Universal PCR Master Mix (2.0X)	12.50	62.5
	10X IPC Mix	2.50	12.5
	50X IPC DNA	0.50	2.5
	20X Primer/ Probe Mix	1.25	6.25
	IPC Block	2.5	12.5
	Water/ Buffer	5.75	28.75
	Total reaction mix volume	25.0	125.0

To prepare the reaction mix for each of the four types:

1. Label four appropriately sized tubes for the reaction mixes: **Sample (+), Sample (-), NTC, NAC.**
2. Add the required volumes of each cocktail 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 cocktail mix on ice until you prepare the reaction plate.

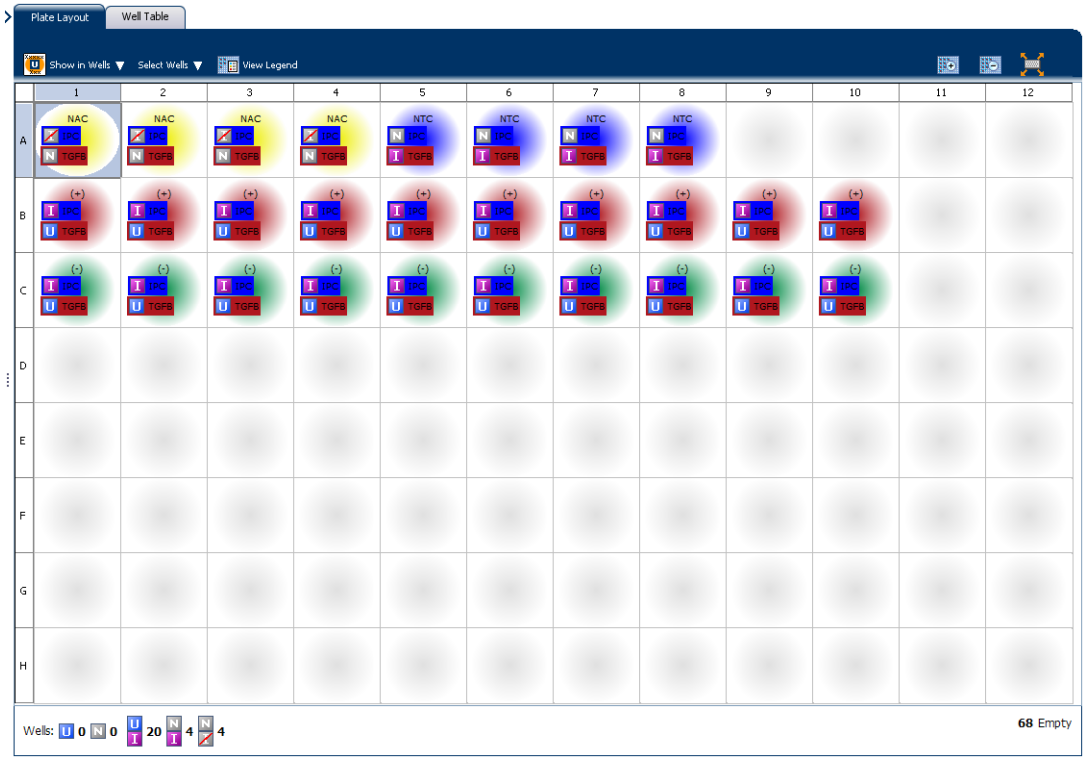
   Note: You can separately add the sample to the reaction plate, as opposed to preparing individual reaction mixes for each sample.

Prepare the reaction plate

The reaction plate for the Presence/Absence example experiment contains:

- 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

The plate layout looks like this:



To prepare the reaction plate:

1. Add 25 µL of Cocktail mix 1 to wells B1 - B10.
2. Add 25 µL of Cocktail mix 2 to wells C1 - C10.
3. Add 25 µL of Cocktail mix 3 to wells A5 - A8.
4. Add 25 µL of Cocktail 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

For more information on...	Refer to...	Part number
Assigning the reaction plate components	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

4 Run the Experiment

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

This chapter covers:

- Start the run 19
- Monitor the run. 19

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 QuantStudio™ 12K Flex Instrument is in operation.

Start the run

1. Open the Presence/Absence example file that you created using instructions in Chapter 2.
2. Load the reaction plate into the instrument.
3. Start the run.

Monitor the run

Monitor the example experiment run:

- From the QuantStudio™ 12K Flex Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio™ 12K Flex Software (to monitor an experiment started from another computer or from the QuantStudio™ 12K Flex Instrument).
- From the QuantStudio™ 12K Flex Instrument touchscreen.

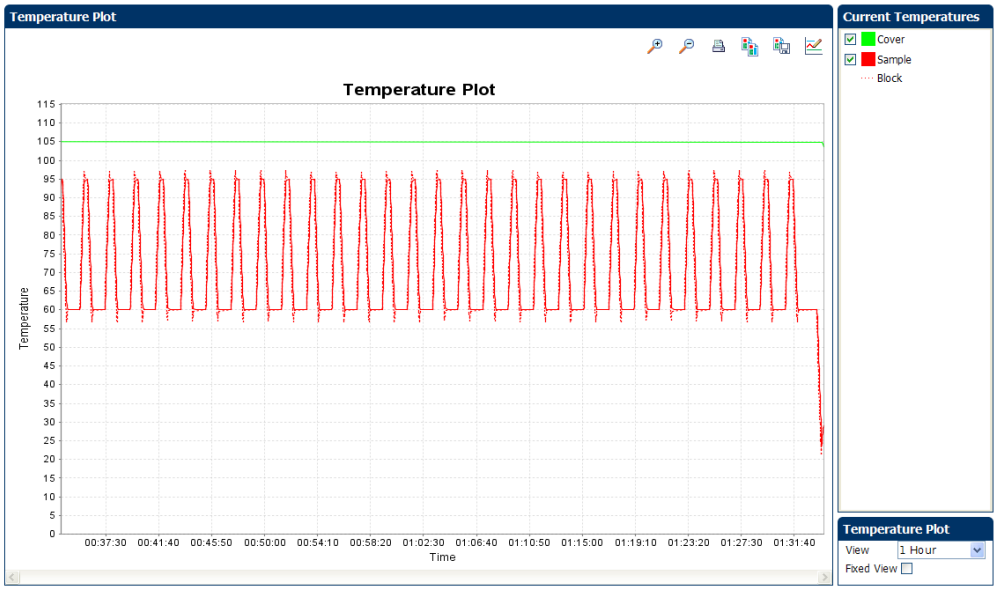
From the Instrument Console of the QuantStudio™ 12K Flex Software

1. In the Instrument Console screen, select the instrument icon.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. On the Manage Instrument screen, click **Monitor Running Instrument** to access the Run screen.

View the Temperature Plot

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

The figure below shows the Temperature Plot screen as it appears during the example experiment.

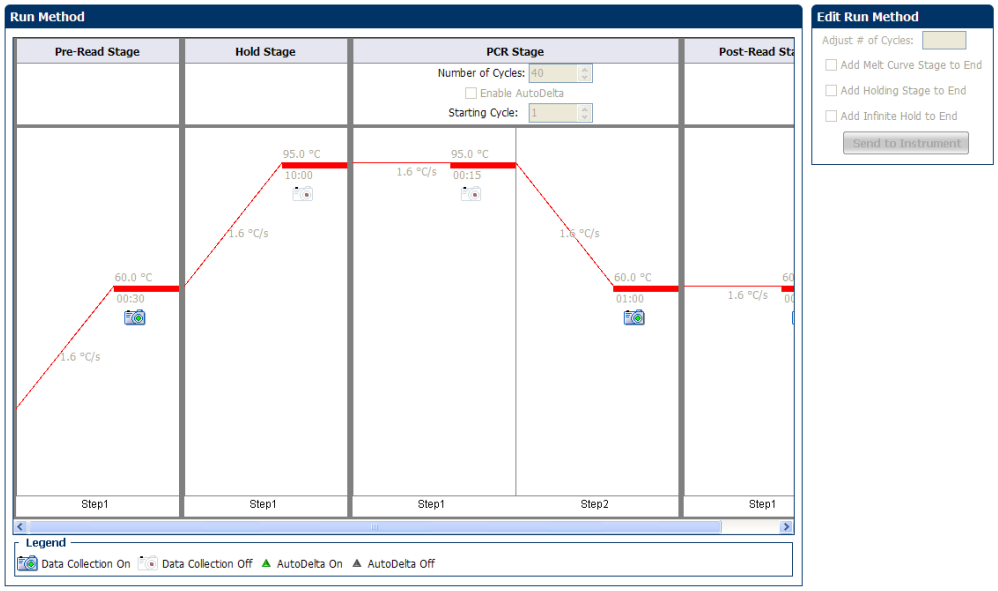


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

View the Run Method

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

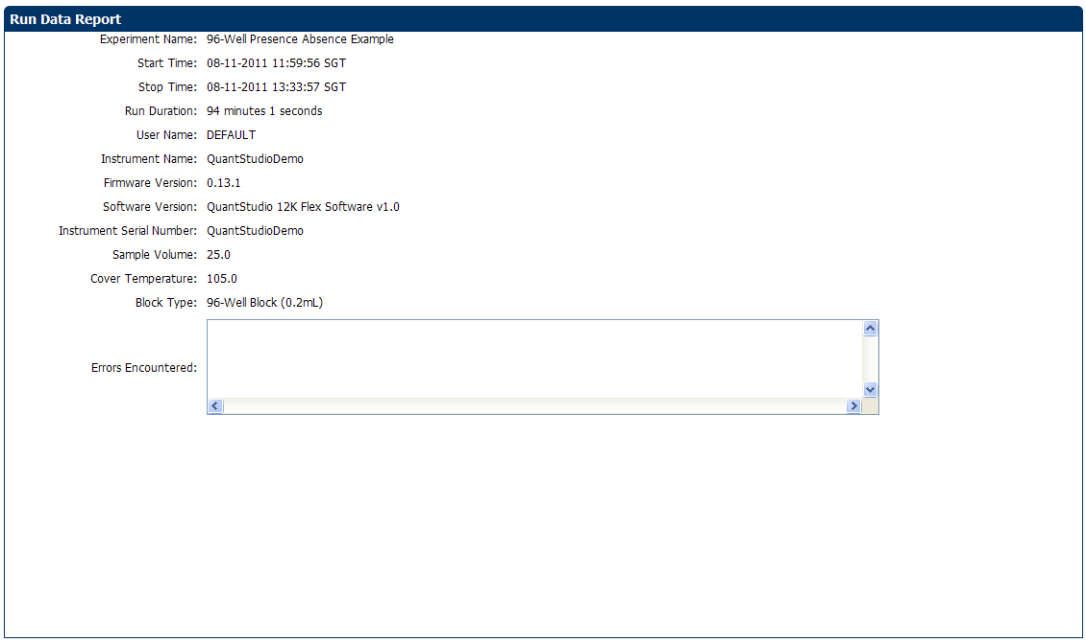
The figure below shows the Run Method screen as it appears in the example experiment.



View run data

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

The figure below shows the View Run Data screen as it appears in the example experiment.

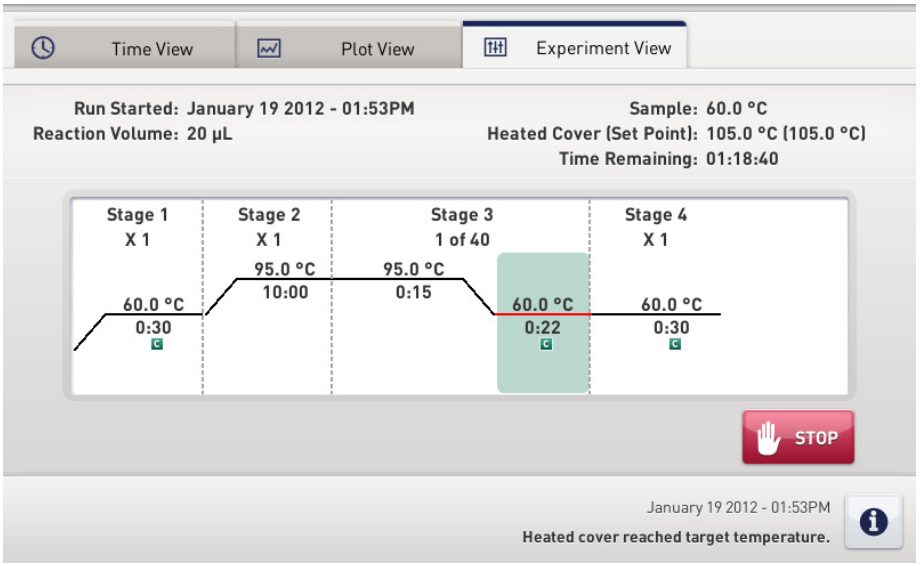


From the QuantStudio™ 12K Flex Instrument touchscreen

You can also view the progress of the run from the touchscreen of the QuantStudio™ 12K Flex Instrument.

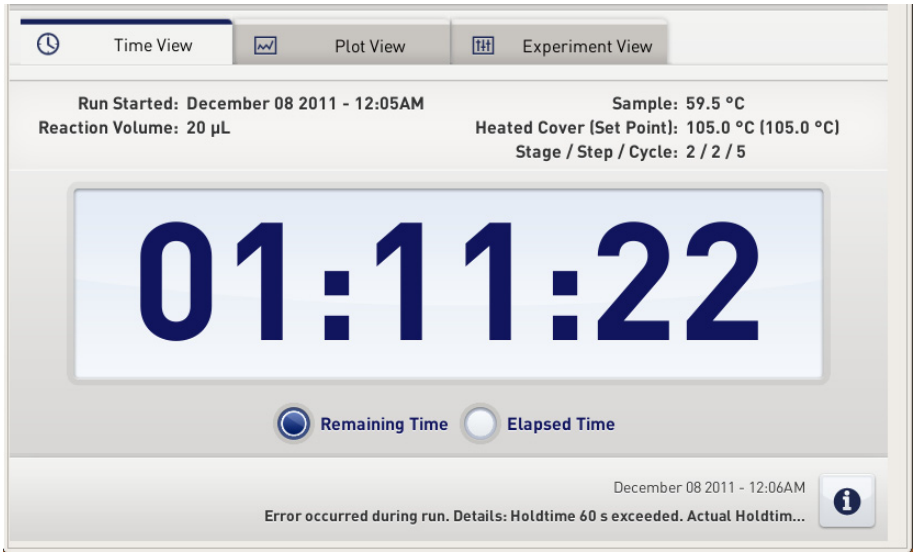
The Run Method screen on the **QuantStudio™ 12K Flex Instrument** touchscreen looks like this:

Experiment View

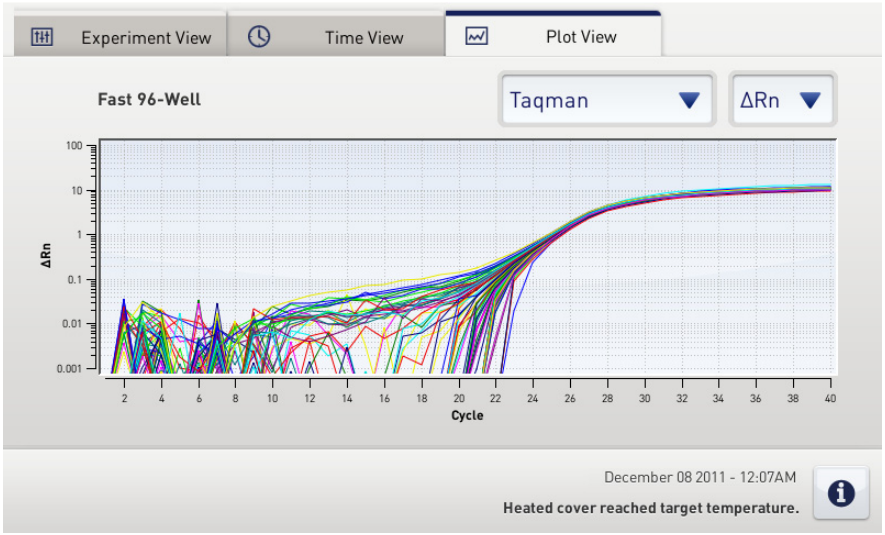


Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

Time View



Plot View



Note: You will see the Plot View only if your experiment includes the PCR process.

5 Review Results and Adjust Experiment Parameters

In Section 5.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 5.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

Section 5.1 Review Results 25

- Analyze the example experiment 25
- View the Presence/Absence Plot 25
- Assess amplification results using the Amplification Plot 27
- View the Well Table 32
- Confirm accurate dye signal using the Multicomponent Plot 35
- Determine signal accuracy using the Raw Data Plot 38
- Review the flags in the QC Summary 40
- For more information 42

Section 5.2 Adjust parameters for re-analysis of your own experiments 43

- Adjust analysis settings 43
- For more information 47

Section 5.1 Review Results

Analyze the example experiment

1. Open the Presence/Absence example experiment file that you ran in Chapter 4.
2. Click **Analyze**. The software analyzes the data using the default analysis settings.

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

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:

- Show IPC
- Show Controls

Purpose

The purpose of viewing the Presence/Absence Plot for the example experiment is to confirm that:

- 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

From the Experiment menu pane, select **Analysis ▸ Presence/Absence Plot.**

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

1. 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.
2. Enter the Plot Settings:

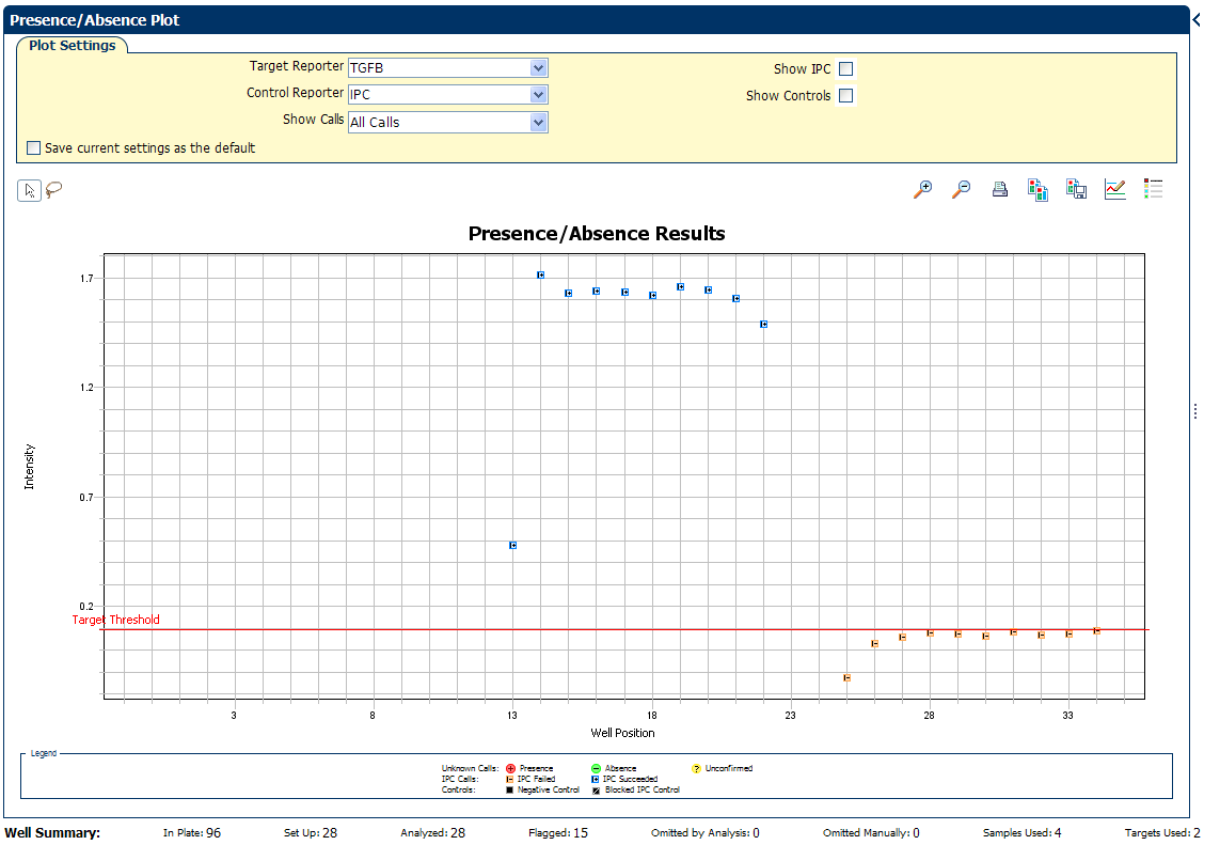
Menu	Selection
Target Reporter	TGFB
Control Reporter	IPC
Show Calls	All Calls

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

3. Click the **Show IPC** check box to view the fluorescence intensity of the IPC target in the Unknown-IPC wells.
4. Click the **Show Controls** check box to view the fluorescence intensity of the IPC target in the negative control-IPC wells and the negative control-Blocked IPC wells.
5. To view the fluorescence intensity of:
   - Presence calls– select **Presence** from the Show Calls drop-down menu.
   - Absence calls– select **Absence** from the Show Calls drop-down menu.
   - Unconfirmed calls– select **Unconfirmed** from the Show Calls drop-down menu.

     Note: The Presence/Absence example experiment does not contain any unconfirmed calls.

The Presence/Absence Plot for the example experiment looks like this:



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 target's intensity 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.
- T**arget 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 displays amplification of all samples in the selected wells. There are three amplification plot views available:

- **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.
- **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.

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

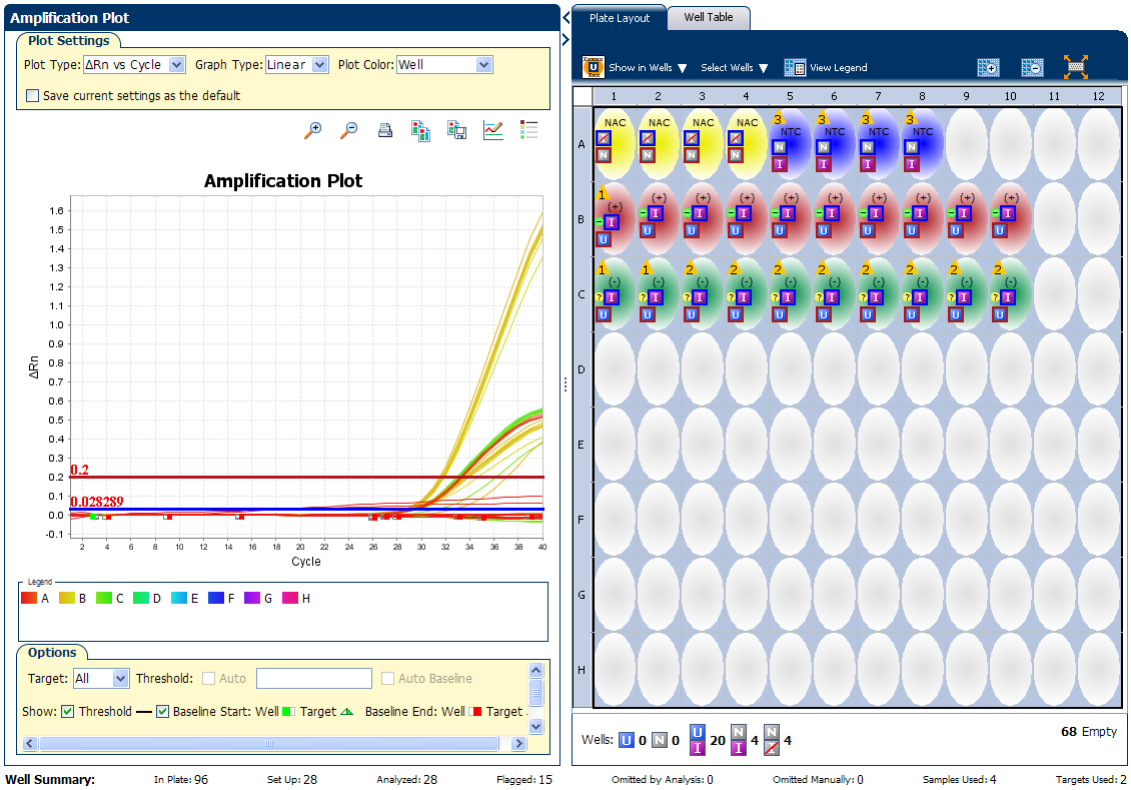
   Note: If no data are displayed, click **Analyze**.

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:

Menu	Selection
Plot Type	ΔRn vs Cycle
Plot Color	Well
 (This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.)	Check (default)

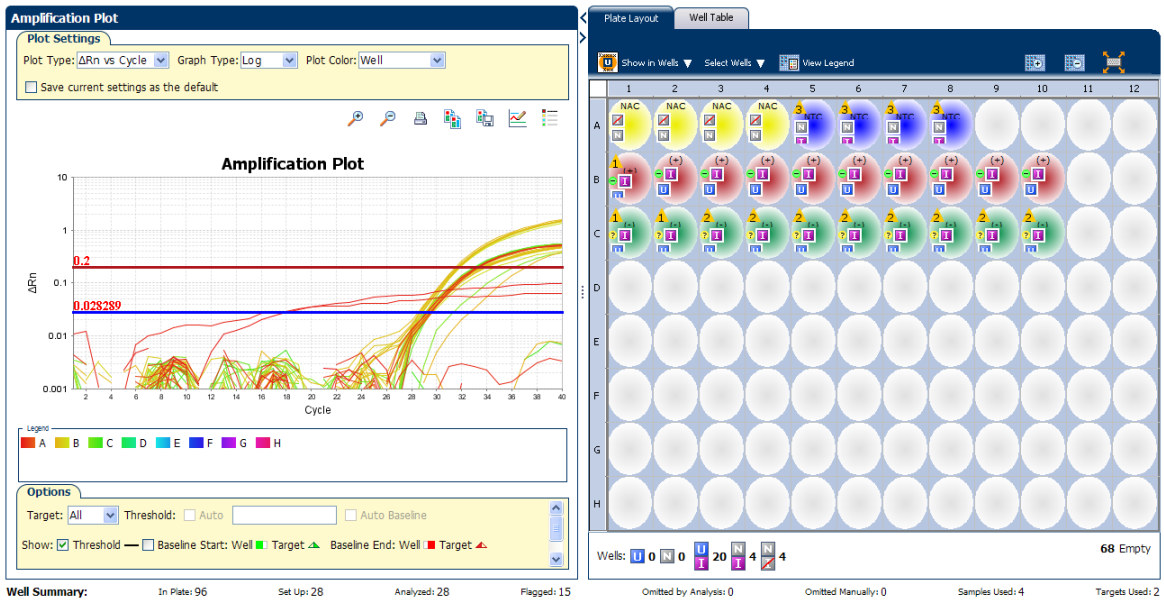
5. View the baseline values:
   a. From the Graph Type drop-down menu, select **Linear**.
   b. Select the **Baseline** check box to show the start cycle and end cycle.
   c. Verify that the baseline is set correctly: The end cycle should be set a few cycles before the cycle number where significant fluorescent signal is detected. In the example experiment, the baseline is set correctly.

Your screen should look like this:



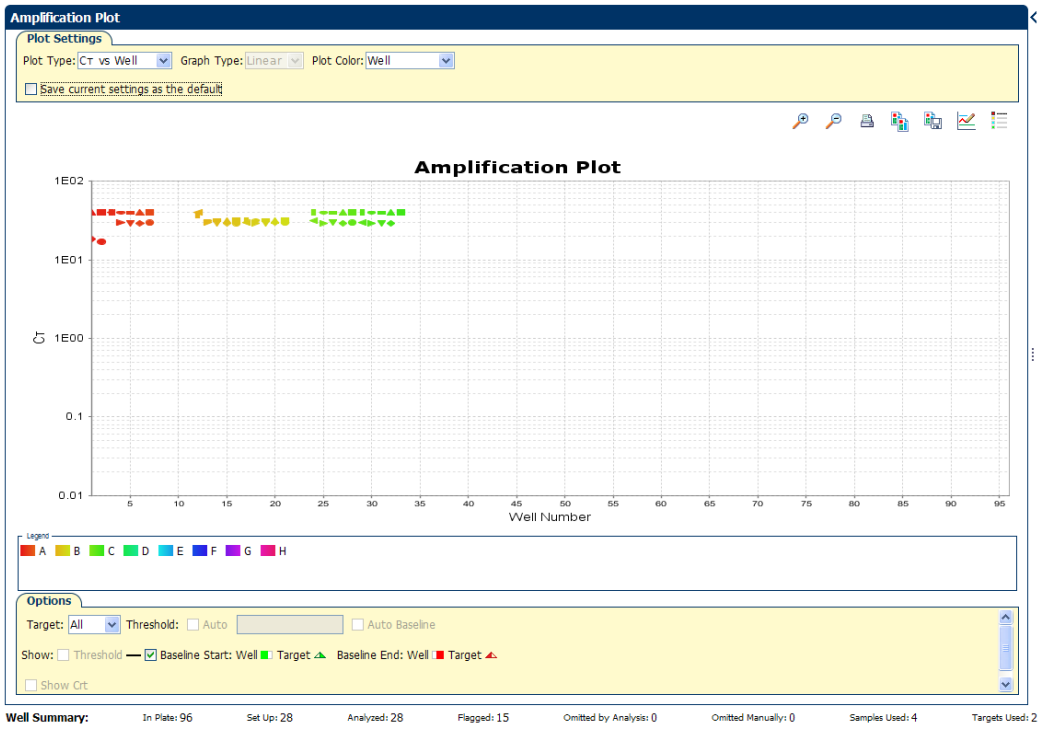
6. View the threshold values:
   a. From the Graph Type drop-down menu, select **Log**.
   b. Select the **Threshold** check box to show the threshold.
   c. Verify that the threshold is set correctly.

Your screen should look like this:



7. Locate any outliers:
   a. From the Plot Type drop-down menu, select **C_T vs Well**.
   b. Look for outliers from the amplification plot. In the example experiment, there are no outliers for IPC.

Your screen should look like this:

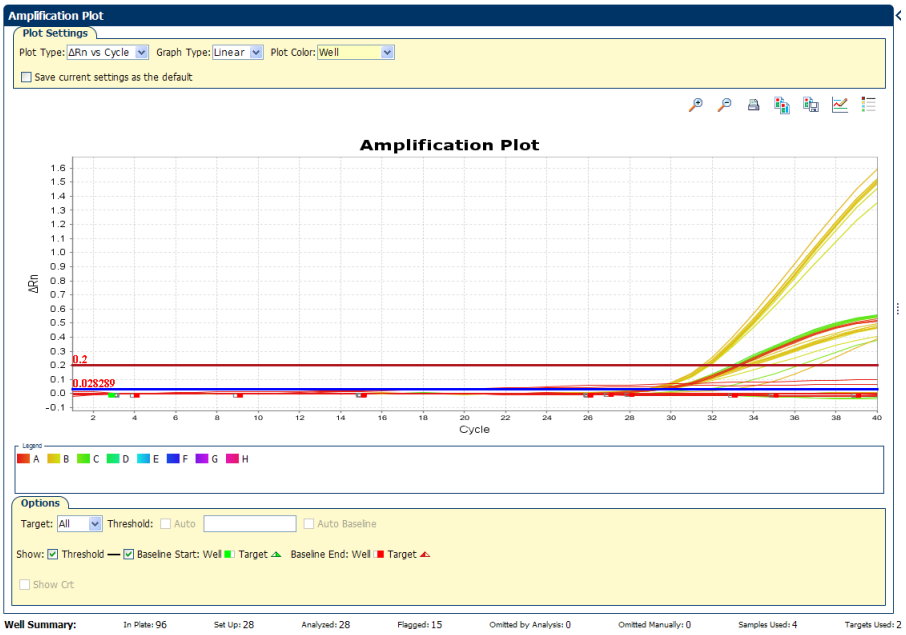


Tips for viewing amplification plots in your own experiments

When you analyze your own Presence/ Absence experiment, look for:

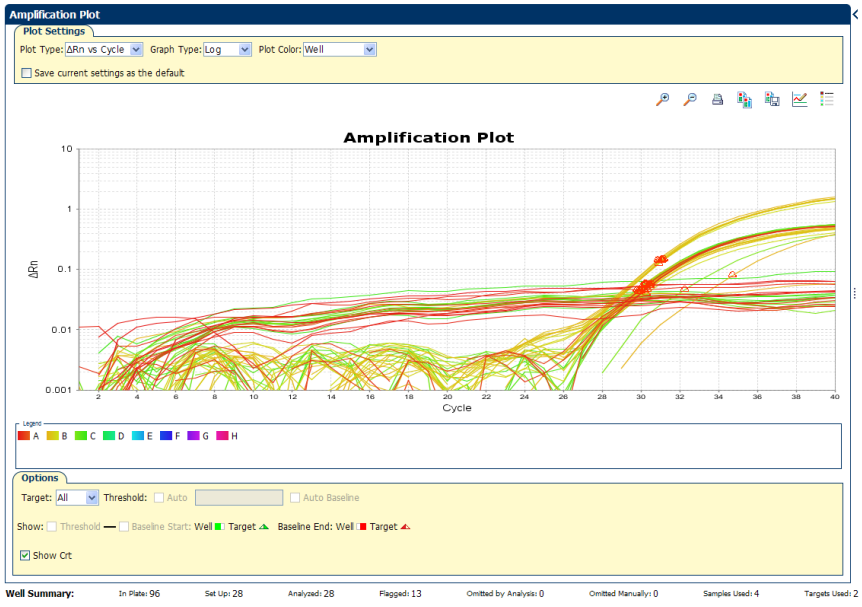
- **Outliers**
- **A typical amplification plot** – The QuantStudio™ 12K Flex Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
  – Plateau phase
  – Linear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

A typical amplification plot should look like this:



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. Life Technologies recommends 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. 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 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

To view the Well Table

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

   Note: If no data are displayed, click **Analyze**.
2. Use the Group By drop-down menu to group wells by a specific category. For the example experiment, group the wells by flag, call, and ΔRn value.

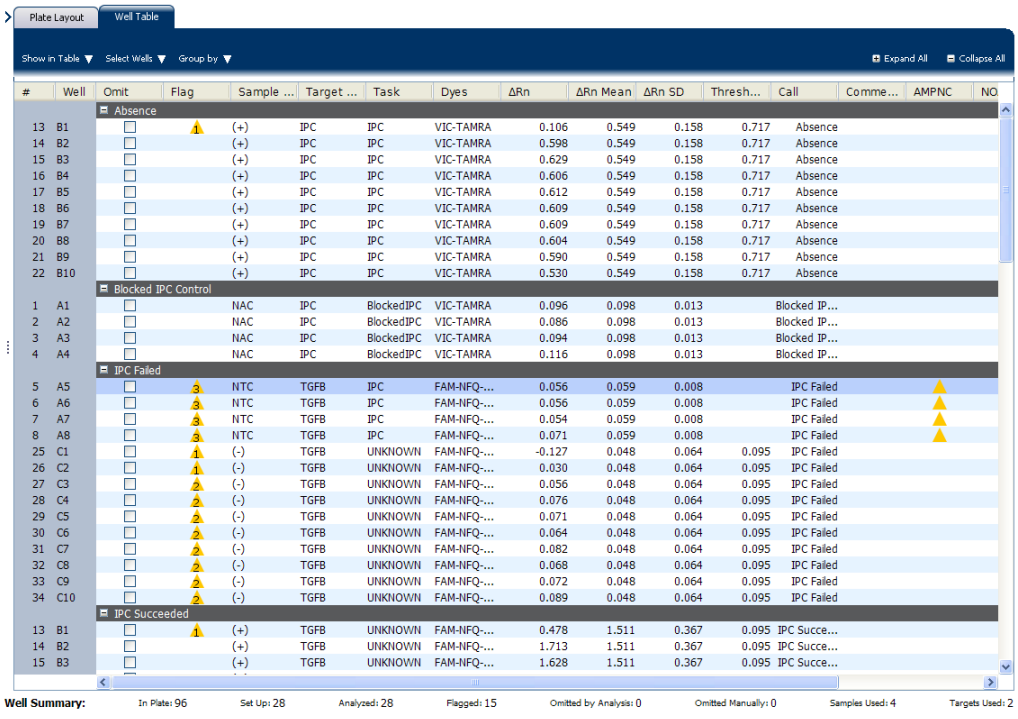
   Note: You can select only one category at a time.

a. From the Group By drop-down menu, select **Flag**:
   - 30 wells are listed under Flagged.
   - 66 wells are listed under Unflagged.

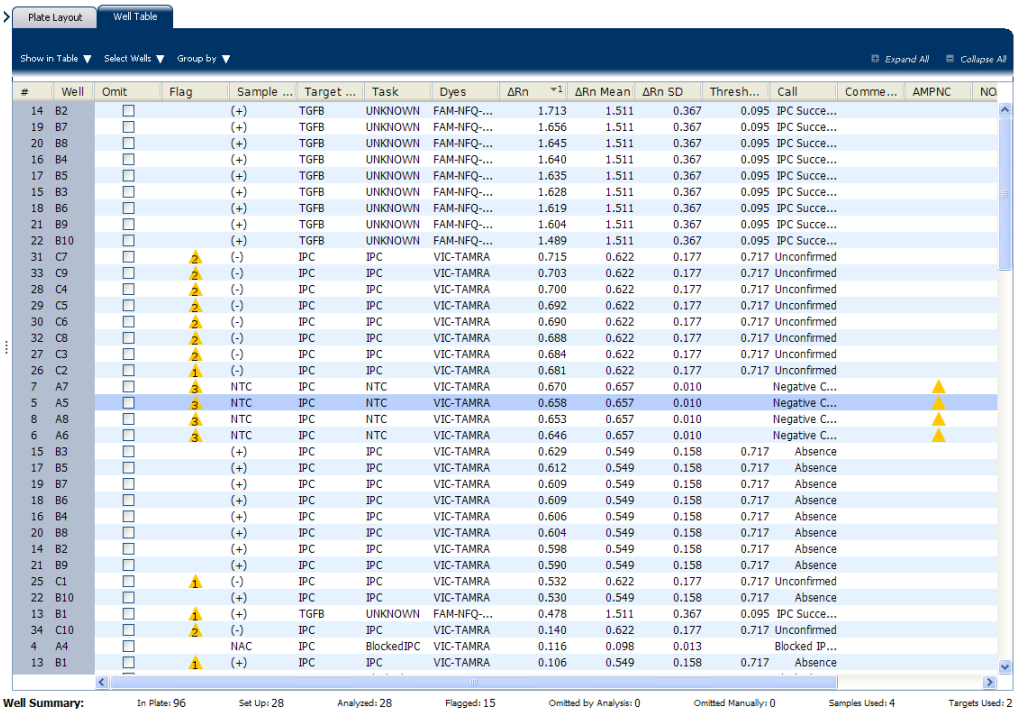
#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	ΔRn	ΔRn Mean	ΔRn SD	Thresh...	Call	Comme...	AMPNC	NO
Flagged Wells															
5	A5	☐	3	NTC	IPC	NTC	VIC-TAMRA	0.658	0.657	0.010		Negative C...		▲	
5	A5	☐	3	NTC	TGFB	IPC	FAM-NFQ-...	0.056	0.059	0.008		IPC Failed		▲	
6	A6	☐	3	NTC	IPC	NTC	VIC-TAMRA	0.646	0.657	0.010		Negative C...		▲	
6	A6	☐	3	NTC	TGFB	IPC	FAM-NFQ-...	0.056	0.059	0.008		IPC Failed		▲	
7	A7	☐	3	NTC	IPC	NTC	VIC-TAMRA	0.670	0.657	0.010		Negative C...		▲	
7	A7	☐	3	NTC	TGFB	IPC	FAM-NFQ-...	0.054	0.059	0.008		IPC Failed		▲	
8	A8	☐	3	NTC	IPC	NTC	VIC-TAMRA	0.653	0.657	0.010		Negative C...		▲	
8	A8	☐	3	NTC	TGFB	IPC	FAM-NFQ-...	0.071	0.059	0.008		IPC Failed		▲	
13	B1	☐	1	(+)	IPC	IPC	VIC-TAMRA	0.106	0.549	0.158	0.717	Absence			
13	B1	☐	1	(+)	TGFB	UNKNOWN	FAM-NFQ-...	0.478	1.511	0.367	0.095	IPC Succe...			
25	C1	☐	1	(-)	IPC	IPC	VIC-TAMRA	0.532	0.622	0.177	0.717	Unconfirmed			
25	C1	☐	1	(-)	TGFB	UNKNOWN	FAM-NFQ-...	-0.127	0.048	0.064	0.095	IPC Failed			
26	C2	☐	1	(-)	IPC	IPC	VIC-TAMRA	0.681	0.622	0.177	0.717	Unconfirmed			
26	C2	☐	1	(-)	TGFB	UNKNOWN	FAM-NFQ-...	0.030	0.048	0.064	0.095	IPC Failed			
27	C3	☐	2	(-)	IPC	IPC	VIC-TAMRA	0.684	0.622	0.177	0.717	Unconfirmed			
27	C3	☐	2	(-)	TGFB	UNKNOWN	FAM-NFQ-...	0.056	0.048	0.064	0.095	IPC Failed			
28	C4	☐	2	(-)	IPC	IPC	VIC-TAMRA	0.700	0.622	0.177	0.717	Unconfirmed			
28	C4	☐	2	(-)	TGFB	UNKNOWN	FAM-NFQ-...	0.076	0.048	0.064	0.095	IPC Failed			
29	C5	☐	2	(-)	IPC	IPC	VIC-TAMRA	0.692	0.622	0.177	0.717	Unconfirmed			
29	C5	☐	2	(-)	TGFB	UNKNOWN	FAM-NFQ-...	0.071	0.048	0.064	0.095	IPC Failed			
30	C6	☐	2	(-)	IPC	IPC	VIC-TAMRA	0.690	0.622	0.177	0.717	Unconfirmed			
30	C6	☐	2	(-)	TGFB	UNKNOWN	FAM-NFQ-...	0.064	0.048	0.064	0.095	IPC Failed			
31	C7	☐	2	(-)	IPC	IPC	VIC-TAMRA	0.715	0.622	0.177	0.717	Unconfirmed			
31	C7	☐	2	(-)	TGFB	UNKNOWN	FAM-NFQ-...	0.082	0.048	0.064	0.095	IPC Failed			
32	C8	☐	2	(-)	IPC	IPC	VIC-TAMRA	0.688	0.622	0.177	0.717	Unconfirmed			
32	C8	☐	2	(-)	TGFB	UNKNOWN	FAM-NFQ-...	0.068	0.048	0.064	0.095	IPC Failed			
33	C9	☐	2	(-)	IPC	IPC	VIC-TAMRA	0.703	0.622	0.177	0.717	Unconfirmed			
33	C9	☐	2	(-)	TGFB	UNKNOWN	FAM-NFQ-...	0.072	0.048	0.064	0.095	IPC Failed			
34	C10	☐	2	(-)	IPC	IPC	VIC-TAMRA	0.140	0.622	0.177	0.717	Unconfirmed			
34	C10	☐	2	(-)	TGFB	UNKNOWN	FAM-NFQ-...	0.089	0.048	0.064	0.095	IPC Failed			
Unflagged Wells															
1	A1	☐		NAC	IPC	BlockedIPC	VIC-TAMRA	0.096	0.098	0.013		Blocked IP...			
1	A1	☐		NAC	TGFB	NTC	FAM-NFQ-...	0.078	0.073	0.004		Negative C...			
2	A2	☐		NAC	IPC	BlockedIPC	VIC-TAMRA	0.086	0.098	0.013		Blocked IP...			

b. From the Group By drop-down menu, select **Call**. Wells are listed in the order:
   - Absence
   - Blocked IPC Control
   - IPC Failed
   - IPC Succeeded
   - Negative Control

- Unconfirmed
- No Call



c. From the Group By drop-down menu, select **None**. In the table, 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:

- **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 QuantStudio™ 12K Flex Software flags, see "Review the flags in the QC Summary" on page 40.
- **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, you review the Multicomponent Plot screen for:

- 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. From 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 screen.

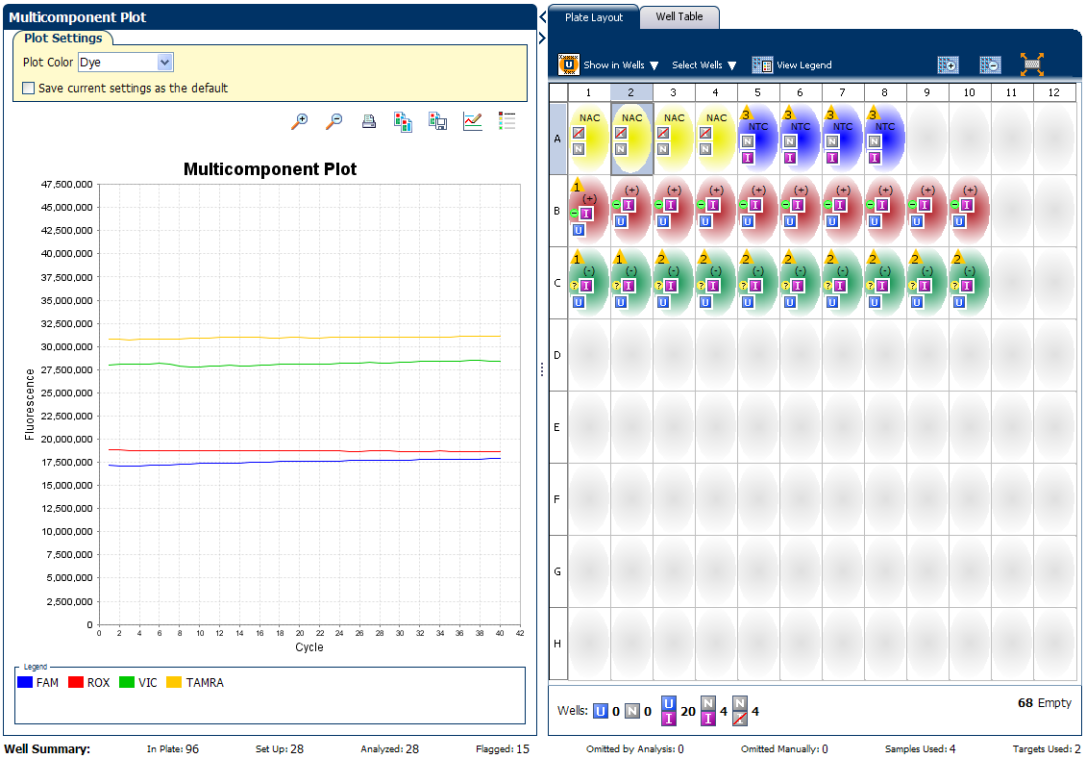
      Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

3. From the Plot Color drop-down menu, select **Dye**.
4. Click  **Show a legend for the plot** (default).

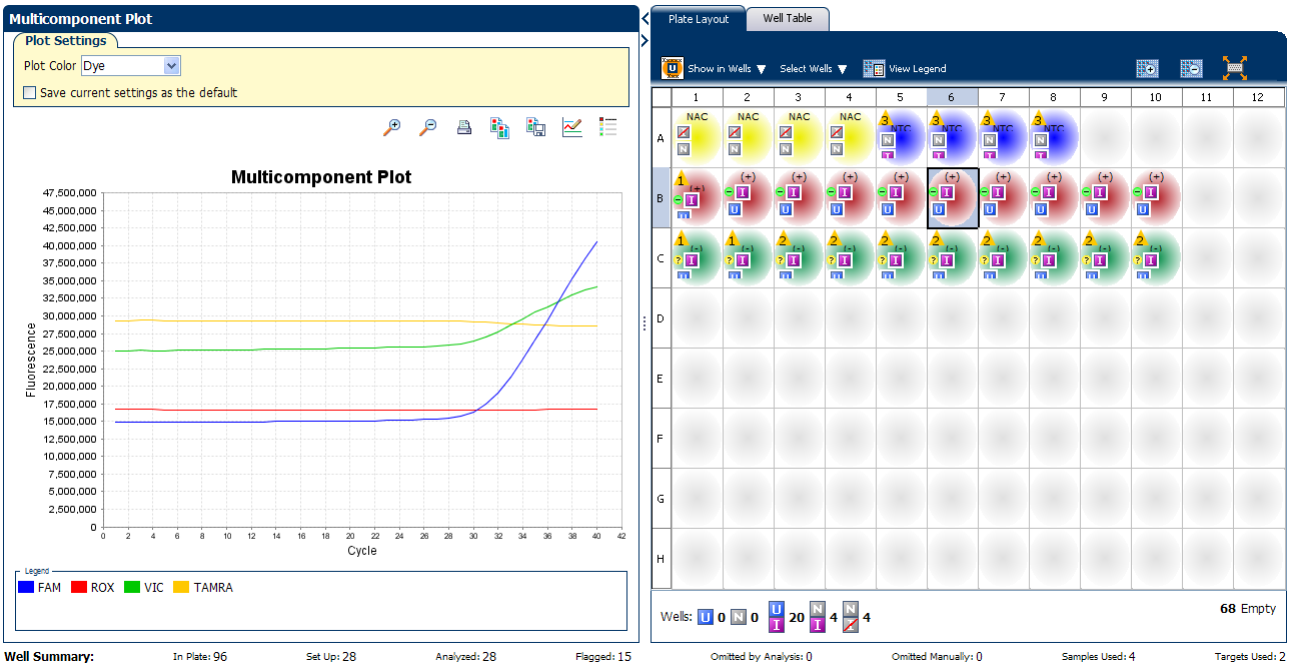
   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

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.

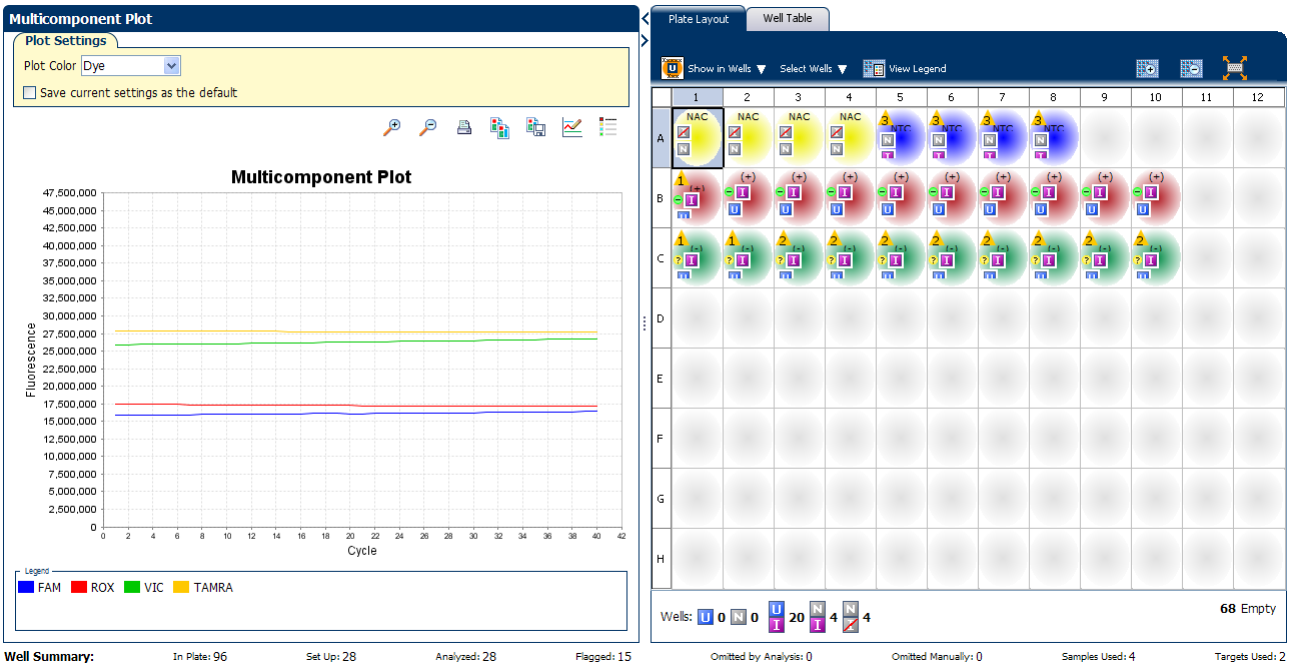
6. Check the VIC dye signal. In the example experiment the VIC dye signal should not amplify for NAC-Blocked IPC wells or if the IPC call for the Unknown-IPC well is IPC Failed.



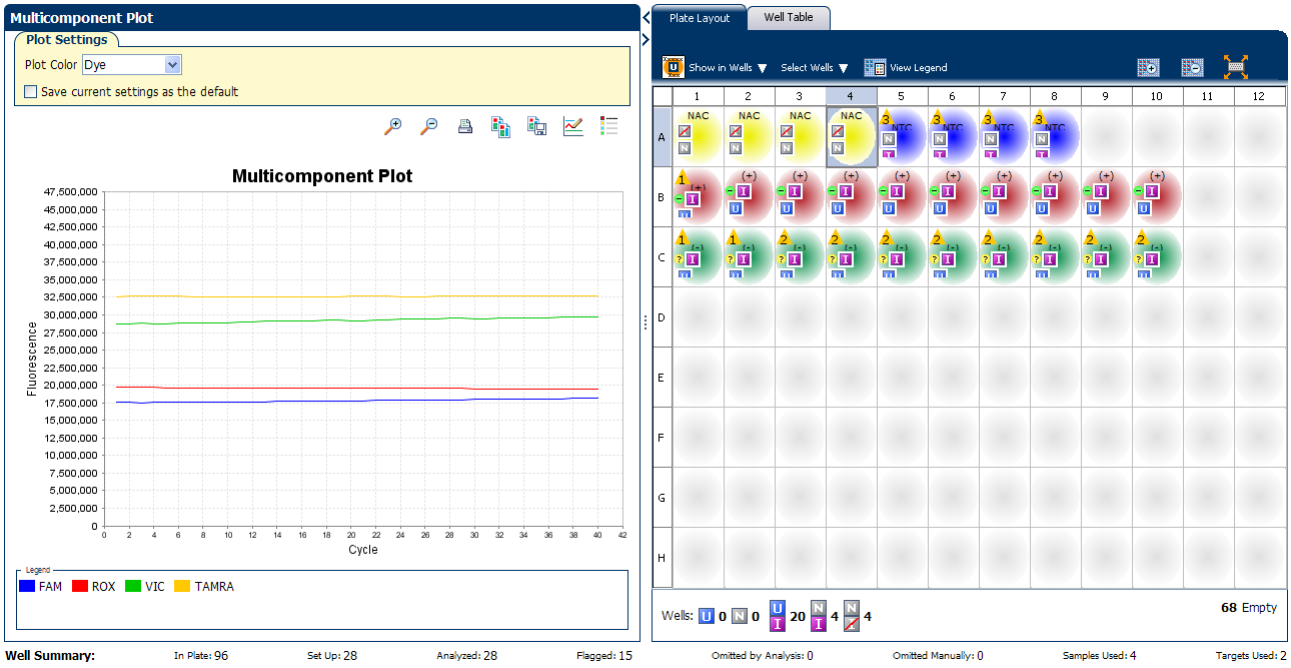
7. Check the FAM dye signal. In the example experiment, for the sample (+), the FAM dye signal increases throughout the PCR process; increase in FAM dye signal indicates normal amplification.



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:

- **Passive reference (ROX)**– The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye (FAM)** – 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

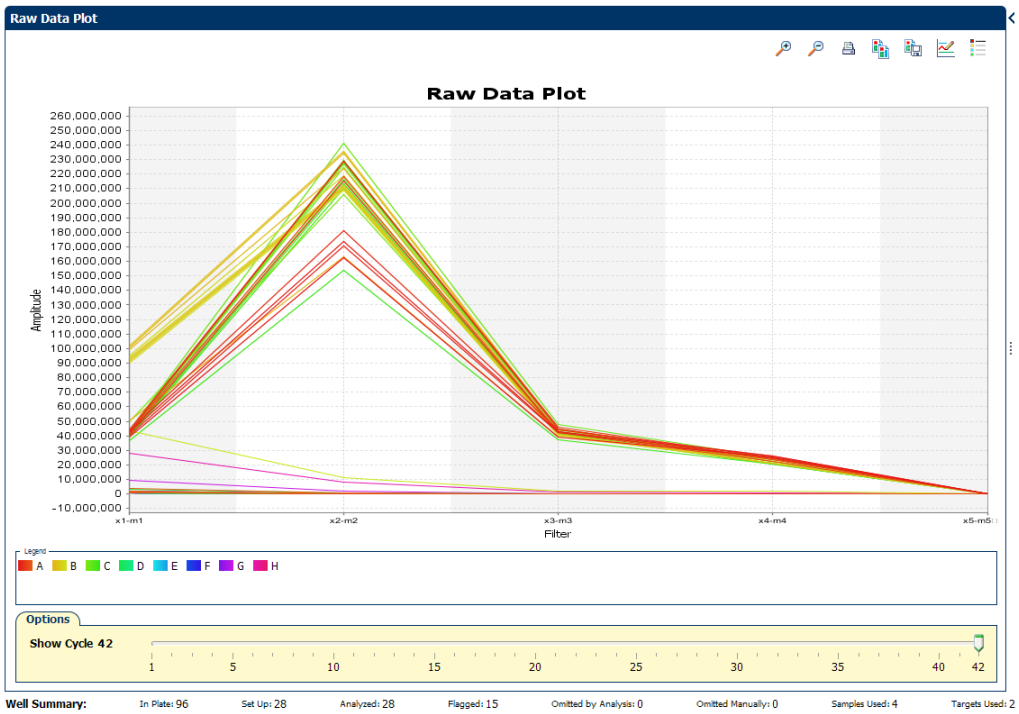
In the Presence/Absence example experiment, review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the Raw Data Plot

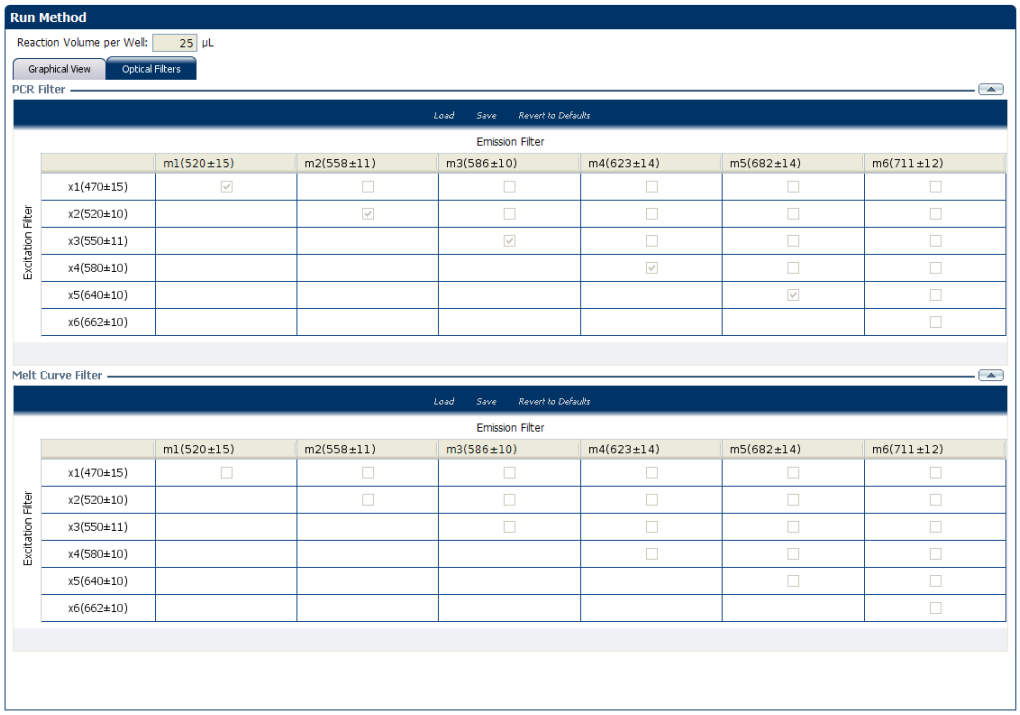
1. From the Experiment Menu pane, select **Analysis** ▸ **Raw Data Plot**.

   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 the Raw Data Plot shown below).
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 corresponding 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 42. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.



The filters used for the example experiment are:



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

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

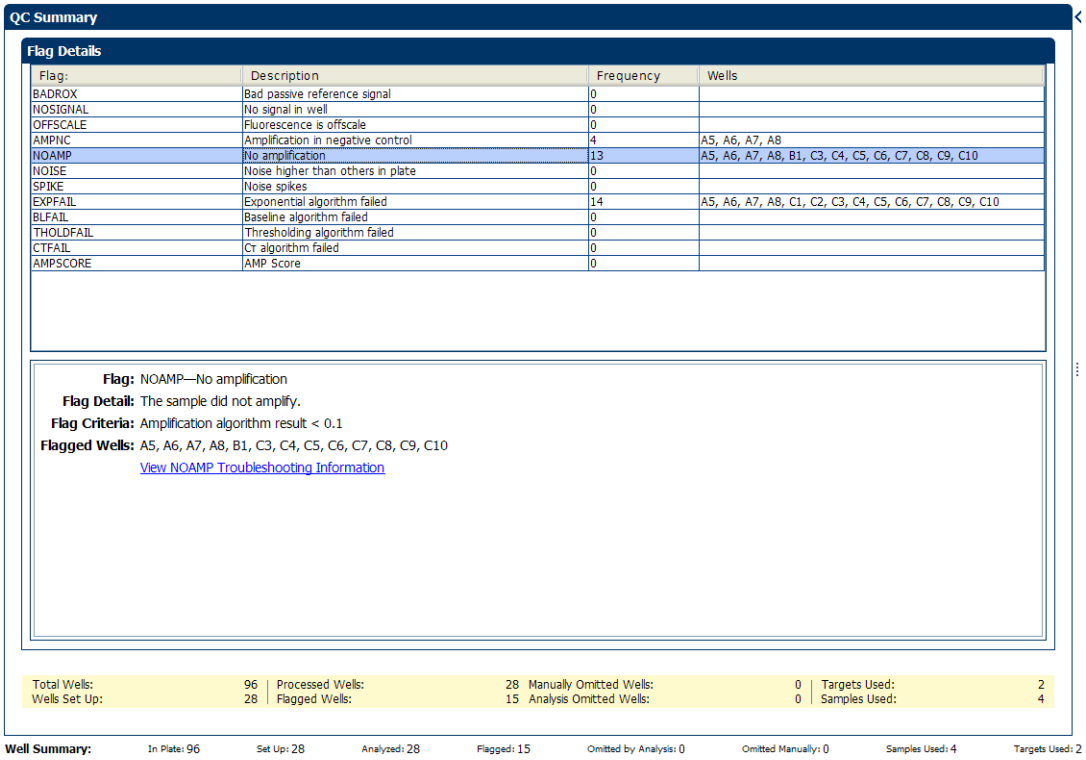
2. Review the Flags Summary

   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment. If the frequency is >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 >0 to display detailed information about the flag. In 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 >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
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

Flag	Description
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

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

Section 5.2 Adjust parameters for re-analysis of your own experiments

Adjust analysis settings

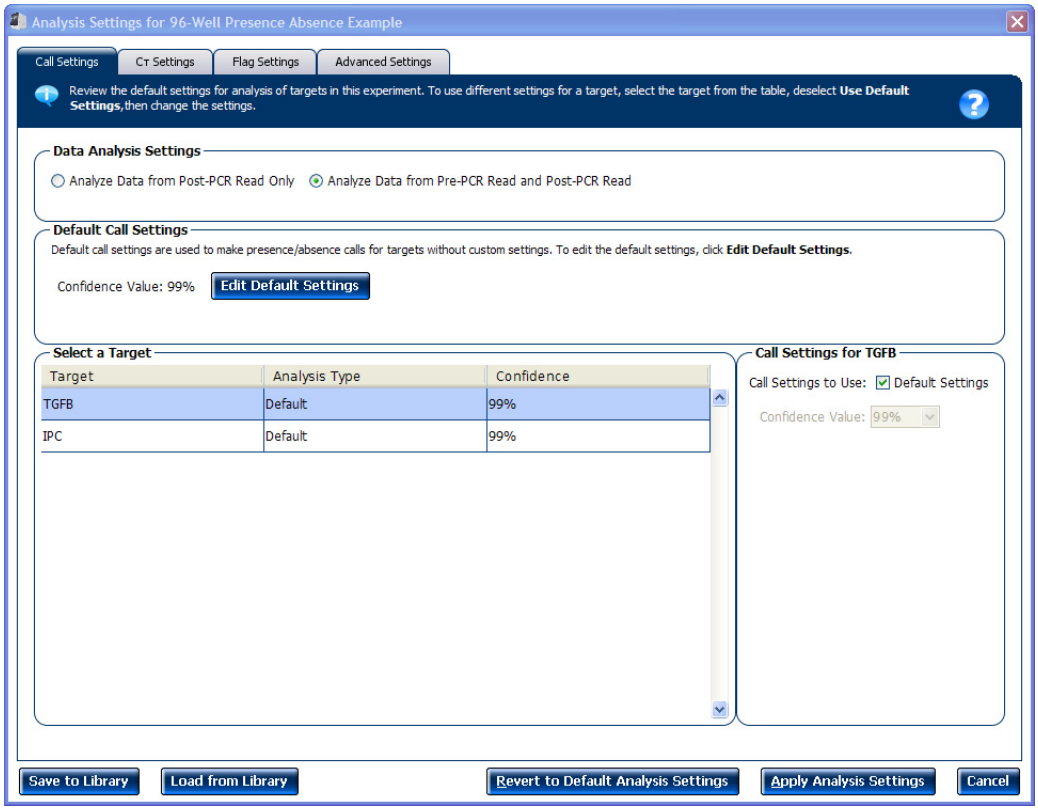
The Analysis Settings dialog box displays the analysis settings for the call, threshold cycle (C_T), flags, and advanced options.

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. 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:
   - Call Settings
   - C_T Settings
   - Flag Settings
   - Advanced Settings

   The Analysis Settings dialog box for a Presence/Absence experiment looks like this:



3. View and, if necessary, change the analysis settings (see "Adjust analysis settings" below).

   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 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.

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:
  - Analyze data from Post-PCR Read only
  - Analyze data from Pre-PCR Read and Post-PCR Read
- Edit the default call settings.
  - Click **Edit Default Settings**, then select the confidence value to use to make presence/absence calls. 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.
  - Select the confidence value to use to make Presence/Absence calls for the selected target(s).

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, Life Technologies recommends:

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: This setting is applicable only to the Baseline Threshold algorithm.

  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:

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

To adjust the flag settings:

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.

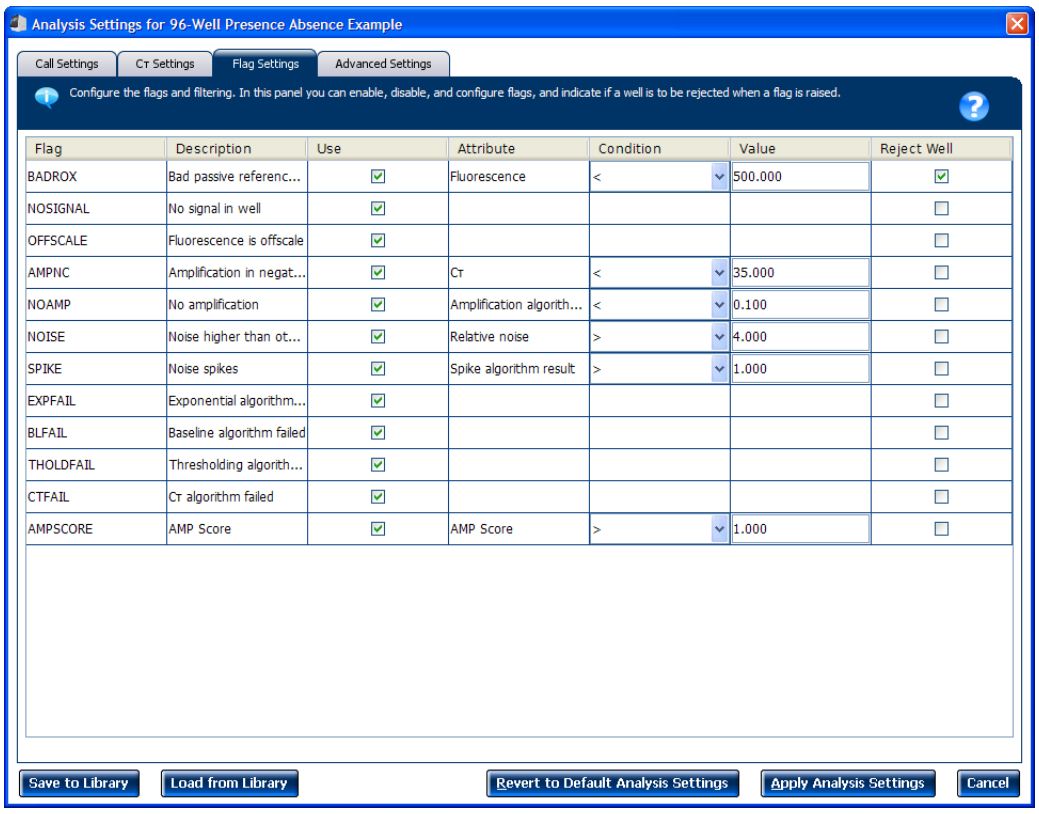
   Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

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.

The Flag Settings tab looks like this:



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.

To use 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** check box.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, then enter the baseline start cycle and baseline end cycle.

For more information

For more information on...	Refer to	Part number
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*.	127AP05-03

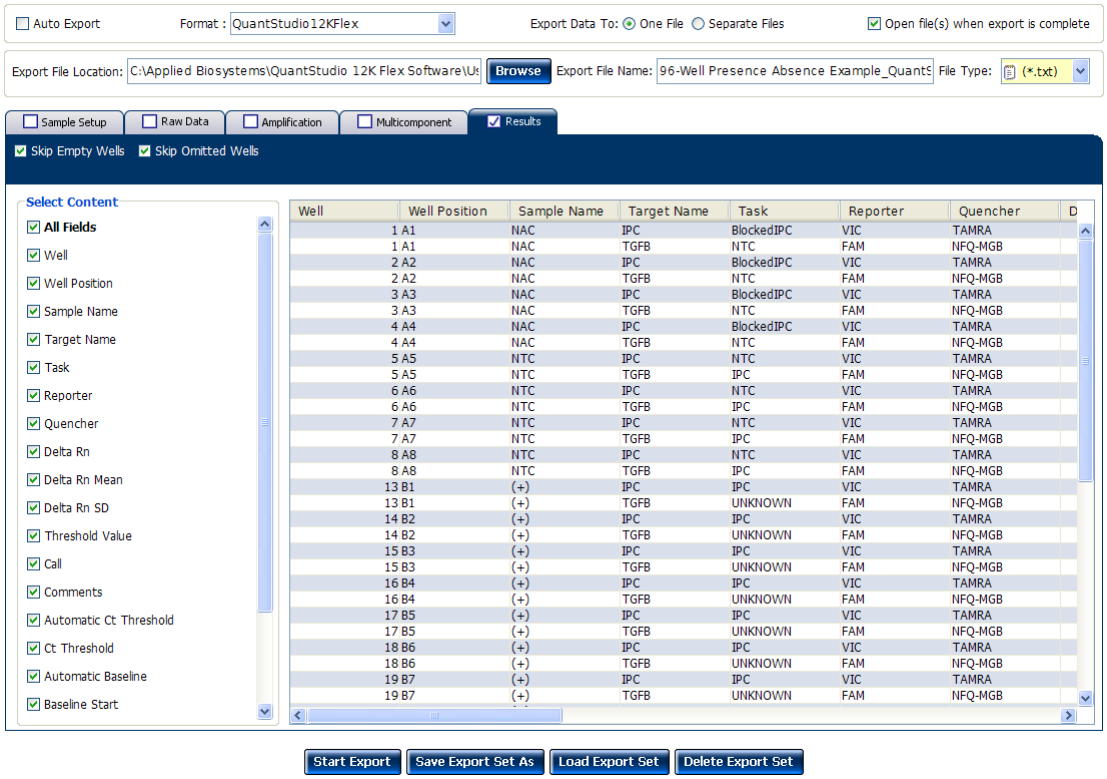
6 Export Analysis Results

1. Open the Presence/Absence example experiment file that you analyzed in Chapter 5.
2. In the Experiment Menu, click  **Export.**

   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.
3. Select **QuantStudio™ 12K Flex format**.
4. Complete the Export dialog box as shown below:

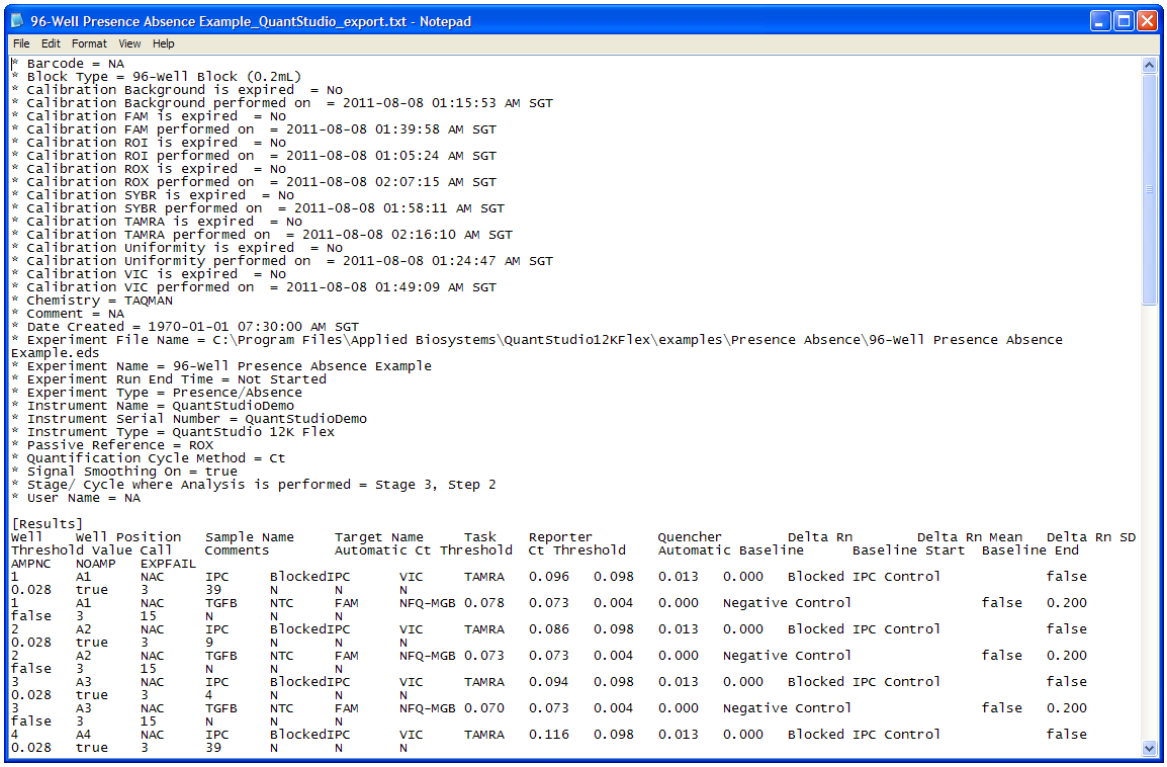
Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	96-Well Presence Absence Example_QuantStudio_export
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments

Your Export screen should look like this:



Well	Well Position	Sample Name	Target Name	Task	Reporter	Quencher
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

Your exported file when opened in Notepad should look like this:



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
```

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GETTING STARTED GUIDE

applied biosystems by life technologies™

Booklet 6 - Running Melt Curve Experiments

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Revision Date March 2012

life technologies™

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1 About Melt Curve Experiments

This chapter covers:

- Overview 5
- About the Melt Curve reactions 5
- About the example experiment 6

IMPORTANT! First-time users of the QuantStudio™ 12K Flex System, please read Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* and Booklet 7, *QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes* of this binder thoroughly. The booklet provides information and general instructions that are applicable to all the experiments described in this binder.

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**.

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 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 unknown melting temperature(s).
- **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 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 System or on another thermal cycler.

2 Design the Experiment

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

This chapter covers:

- Define the experiment properties. 7
- Define targets and samples 8
- Assign targets and samples. 9
- Set up the run method 10
- For more information. 12

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 on Auto Export, refer to Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*.

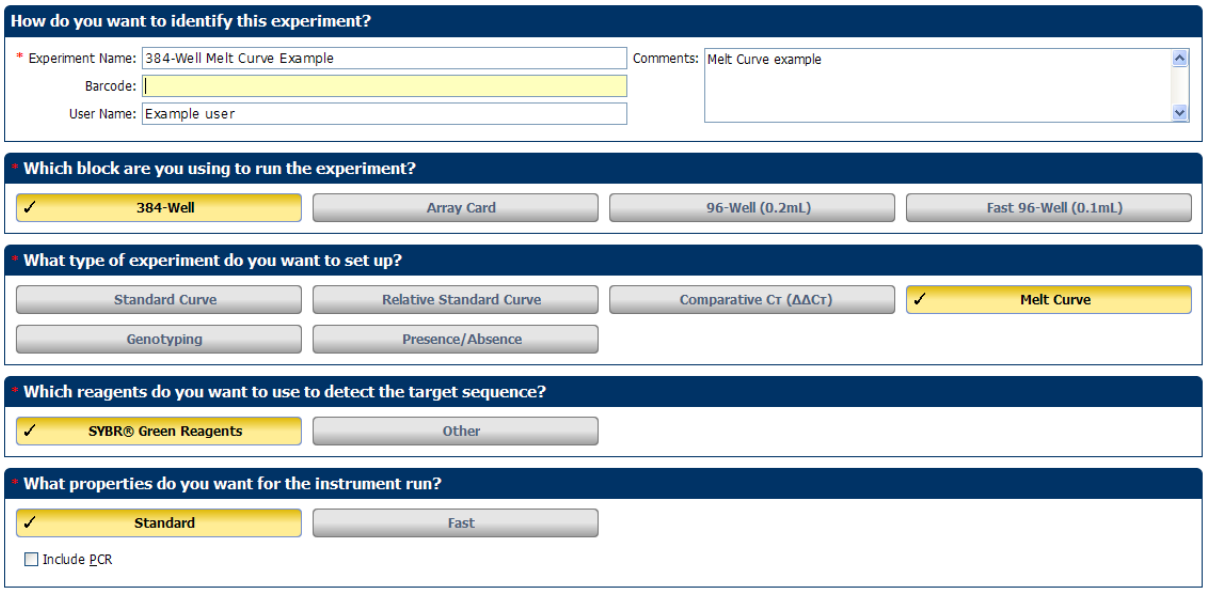
Define the experiment properties

Click **Experiment Setup** ▸ **Experiment Properties** to create a new experiment in the QuantStudio™ Software. Enter:

Field or Selection	Entry
Experiment Name	384-Well Melt Curve Example
Barcode	Leave field empty
User Name	Example User
Comments	Melt Curve example
Block	384-Well
Experiment Type	Melt Curve
Reagents	SYBR® Green Reagents
Ramp speed	Standard
Include PCR	Unchecked

Save the experiment.

Your Experiment Properties screen should look like this:



Define targets and samples

Click **Define** to access the Define screen. Enter:

1. Targets

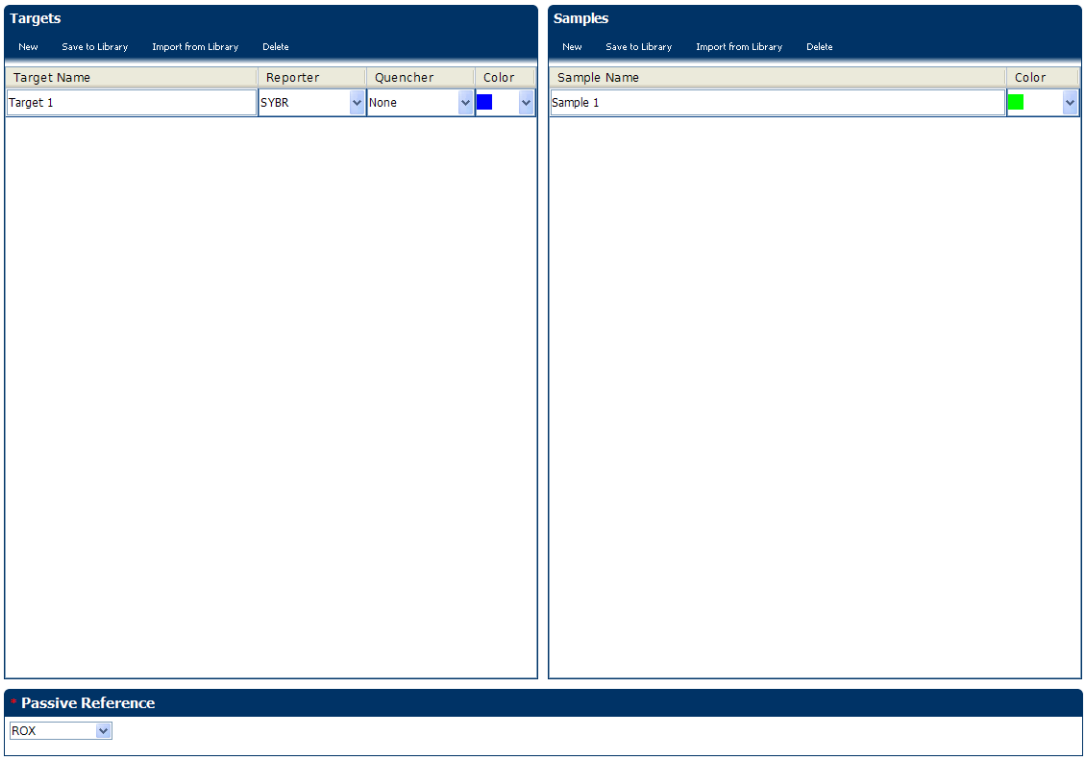
Target Name	Reporter	Quencher	Color
Target 1	SYBR	None	

2. Samples

Sample Name	Color
Sample 1	

3. Dye to be used as a Passive Reference
ROX

Your Define screen should look like this:

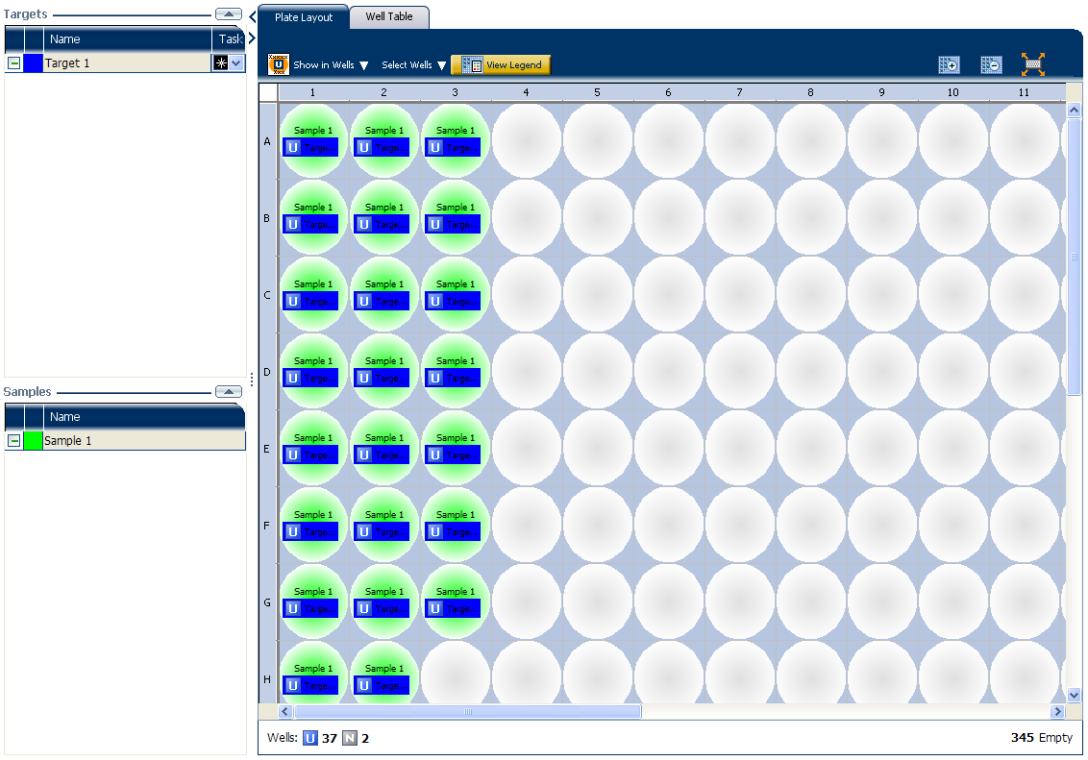


Assign targets and samples

Click **Assign** to access the Assign screen. Enter the targets and samples:

Target Name	Sample	Well Number	Task
SYBR	Sample 1	A1-P2 (Columns 1 and 2), A3-G3 (Column 3)	Unknown

Your Assign screen should look like this:



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:

- 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

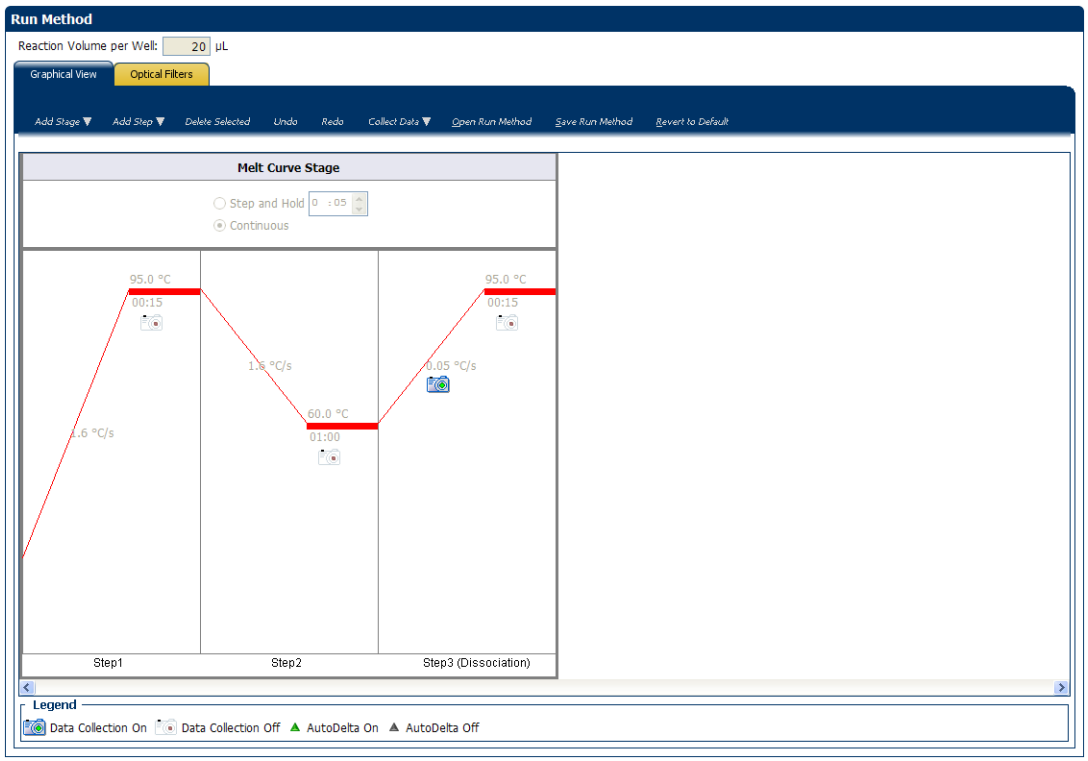
Edit the ramp increment for a melt curve (dissociation) step.

1. Select a melt curve ramp increment method:
   - **Step and Hold** – Increases or decreases the ramp temperature in 0.1 °C increments over the time (duration) for the melt curve ramp.
   - **Continuous (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.

Your Run Method screen should look like this:



For more information

For more information on...	Refer to	Part number
Consumables	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments* Appendix A in Booklet 7, *QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes*	4470050
Using alternative setup	Chapter 2 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

3 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 System or on another thermal cycler.

This chapter covers:

- Assemble required materials 13
- Prepare the sample dilutions 13
- Prepare the reaction mix (“cocktail mix”). 14
- Prepare the reaction plate 14
- For more information. 15

Assemble required materials

- Items listed in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*
- Sample 1
- Example experiment reaction mix components:
  - Power SYBR® Master Mix
  - Target - Assay Mix Forward primer (10µM)
  - Target - Assay Mix Reverse primer (10µM)

Prepare the sample dilutions

The stock concentration of each sample is 100 ng/µL. After you dilute the sample according to the Sample Dilutions Calculations table, the sample will have a concentration of 10 ng/µL. Add 2µL to each reaction.

Sample name	Sample volume (µL)	Diluent volume (µL)	Total volume of diluted sample (µL)
Sample 1 (Amplified PCR Product)	10	90	100

Prepare the reaction mix ("cocktail mix")

The following table lists the universal assay conditions [volume and final concentration for using the Power SYBR® Master Mix (2X)].

Reaction Component	Volume for 1 reaction (µL)	Volume for 40 reactions (µL)
Power SYBR®Green PCR Master Mix (2X)	10	400
Forward primer (10µM)	0.1	4
Reverse primer (10µM)	0.1	4
Water	7.8	312
Total reaction mix volume	18	720

Procedure

1. Label an appropriately sized tube for the reaction mix: Power SYBR® Reaction Mix.
2. Add the required volume of each cocktail 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 cocktail 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. To an appropriately sized tube, add the volumes of reaction mix and sample listed below.

Tube	Unknown reaction	Reaction mix	Reaction mix volume (µL)	Sample	Sample volume (µL)
1	Target 1	Power SYBR® reaction mix	720	Sample 1	80

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 4.

For more information

For more information on...	Refer to...	Part number
Assigning the reaction plate components	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050
Sealing the reaction plate	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

4 Run the Experiment

This chapter explains how run the example experiment on the QuantStudio™ 12K Flex Instrument.

This chapter covers:

- Start the run 17
- Monitor the run 17

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 QuantStudio™ 12K Flex Instrument is in operation.

Start the run

1. Open the Melt Curve example file that you created using instructions in Chapter 2.

   IMPORTANT! The example experiment includes the melt curve analysis of a PCR product from PCR on QuantStudio™ 12K Flex System or another thermal cycler. To run a Melt Curve on the example file you created in Chapter 2, 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

Monitor the example experiment run:

- From the QuantStudio™ Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio™ Software (to monitor an experiment started from another computer or from the QuantStudio™ 12K Flex Instrument touchscreen).
- From the QuantStudio™ 12K Flex Instrument touchscreen.

From the Instrument Console of the QuantStudio™ Software

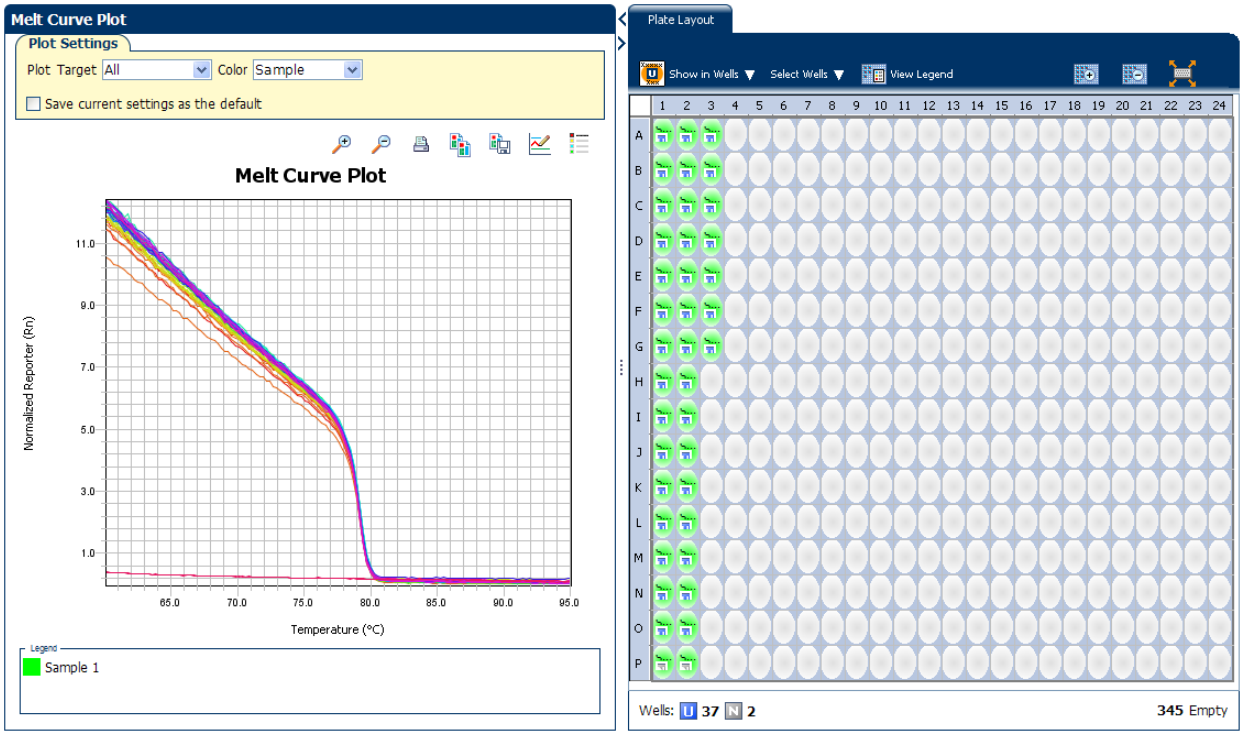
1. In the Instrument Console screen, select the instrument icon.
2. Click **Manage Instrument** or double-click on the instrument icon.
3. On the Manage Instrument screen, click **Monitor Running Instrument** to access the Run screen.

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™ Software for potential problems.

Click **Melt Curve** from the Run Experiment Menu, select the Plate Layout tab, then select the wells to view.

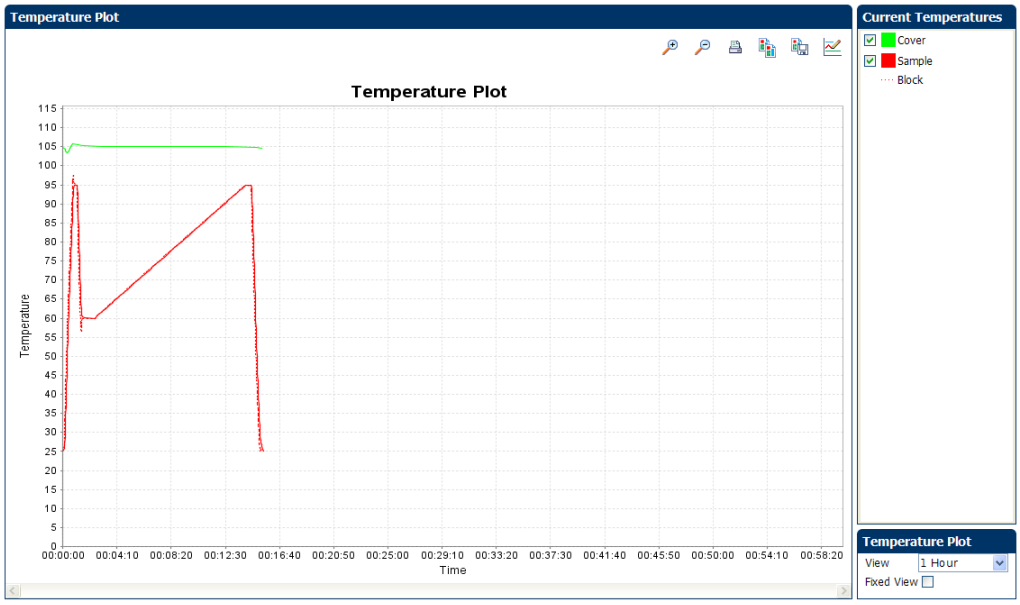
The figure below shows the Melt Curve as it appears at the end of the example experiment.



View the Temperature Plot

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

The figure below shows the Temperature Plot screen as it appears during the example experiment.

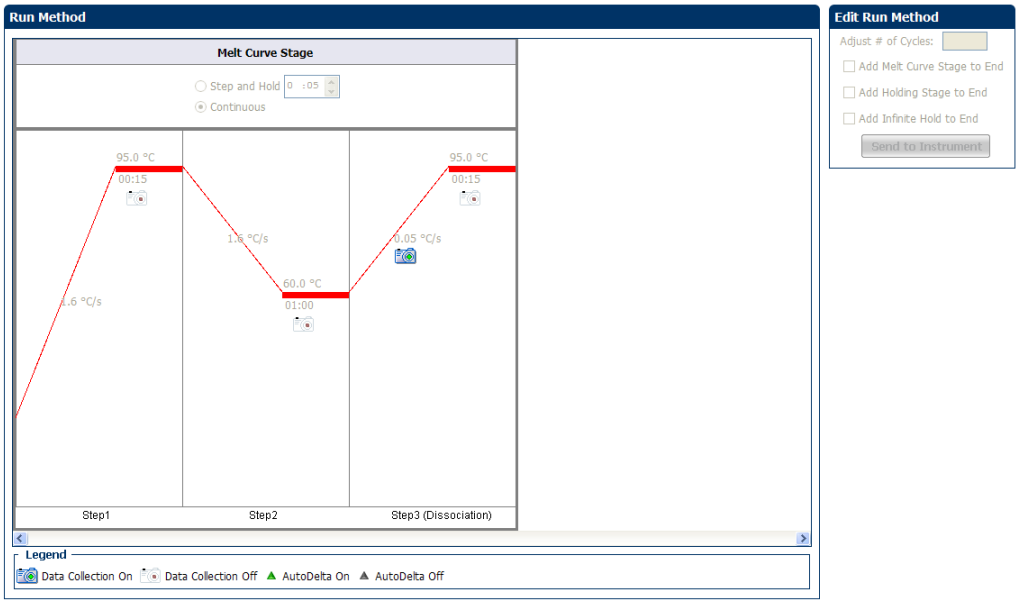


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

View the Run Method

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

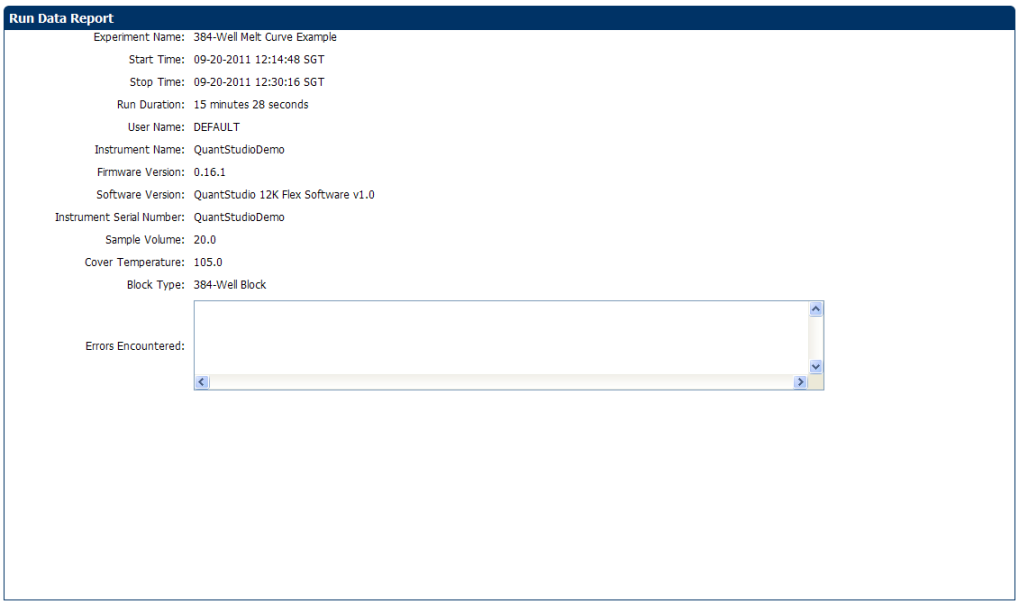
The figure below shows the Run Method screen as it appears in the example experiment.



View run data

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

The figure below shows the View Run Data screen as it appears in the example experiment.

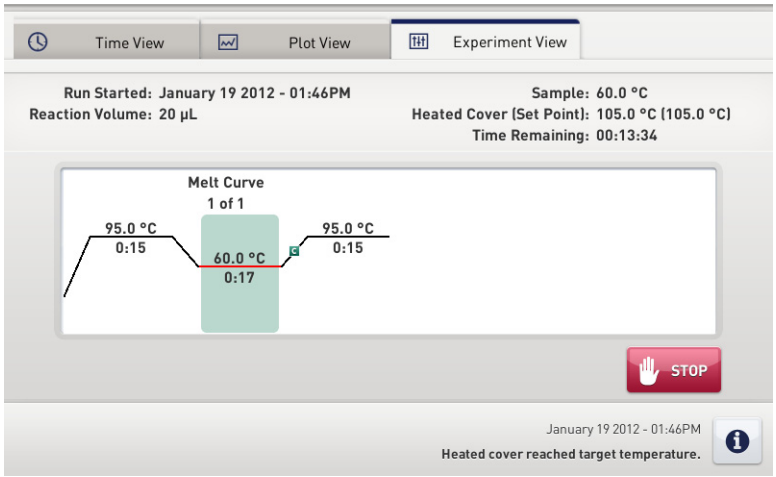


From the QuantStudio™ 12K Flex Instrument touchscreen

You can also view the progress of the run from the touchscreen of the QuantStudio™ 12K Flex Instrument.

The Run Method screen on the **QuantStudio™ 12K Flex Instrument** touchscreen looks like this:

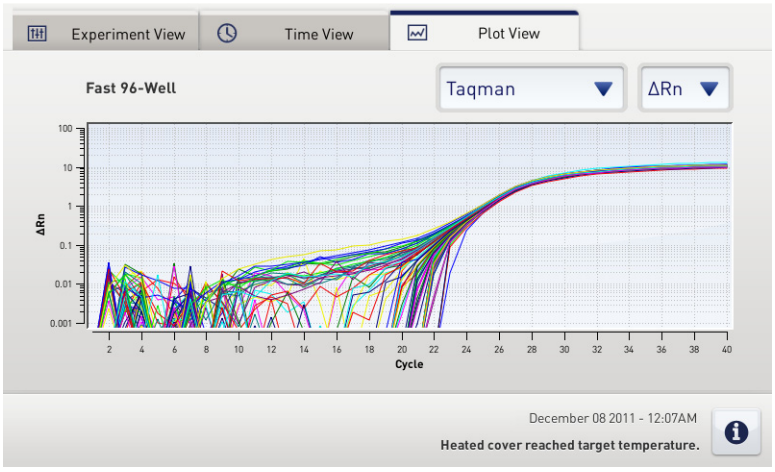
Experiment View



Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

Time View

Plot View



Note: You will see the Plot View only if your experiment includes the PCR process.

5 Review Results and Adjust Experiment Parameters

In Section 5.1 of this chapter you review the analyzed data using several of the analysis screens and publish the data. Section 5.2 of this chapter walks you through the process of modifying experiment parameters to troubleshoot problems with experiment results before rerunning an experiment.

This chapter covers:

Section 5.1 Review Results 25

- Analyze the example experiment. 25
- View the Melt Curve Plot 25
- Identify well problems using the Well Table 26
- Confirm accurate dye signal using the Multicomponent Plot 29
- Determine signal accuracy using the Raw Data Plot 30
- Review the flags in the QC Summary 32
- For more information. 34

Section 5.2 Adjust parameters for re-analysis of your own experiments. 35

- Adjust analysis settings 35
- For more information. 39

Section 5.1 Review Results

Analyze the example experiment

1. Open the example experiment file that you ran in Chapter 4.
2. Click **Analyze**. The software analyzes the data using the default analysis settings.

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

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 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.

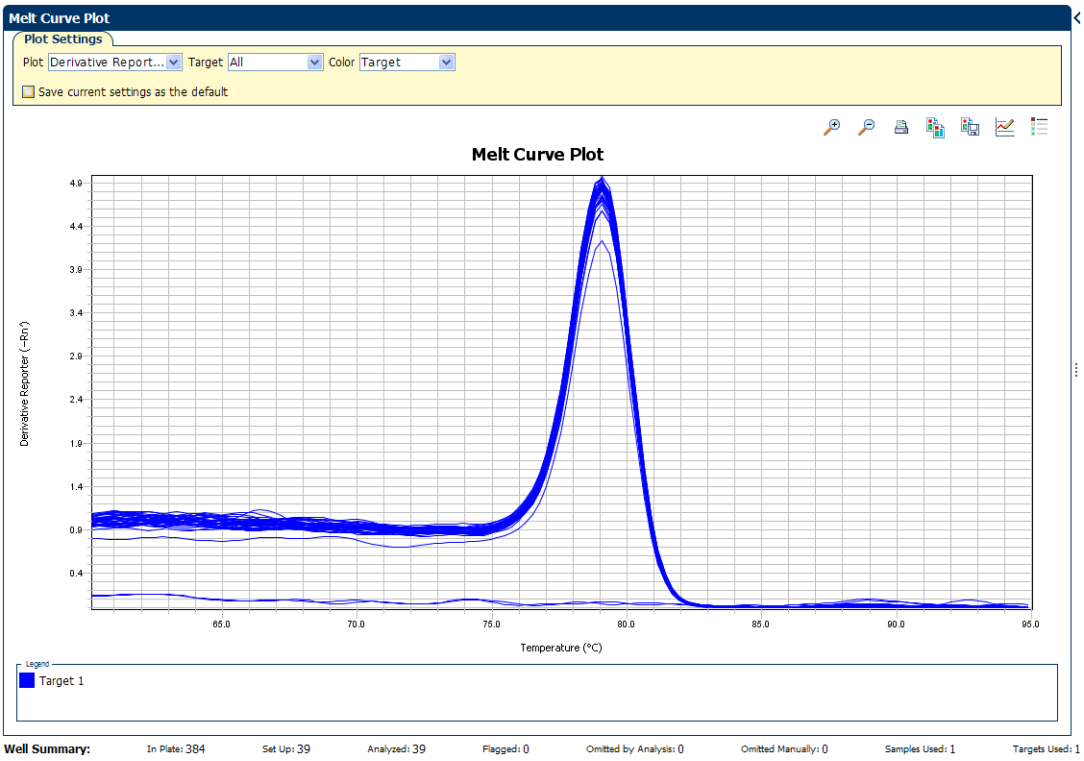
To view and assess the Melt Curve

1. From the Experiment menu pane, select **Analysis ▸ Melt Curve Plot.**

   Note: If no data are displayed, click **Analyze**.
2. Enter the Plot Settings:

Menu	Selection
Plot	Derivative Reporter
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)

The Melt Curve for the example experiment looks like this:



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 35).

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

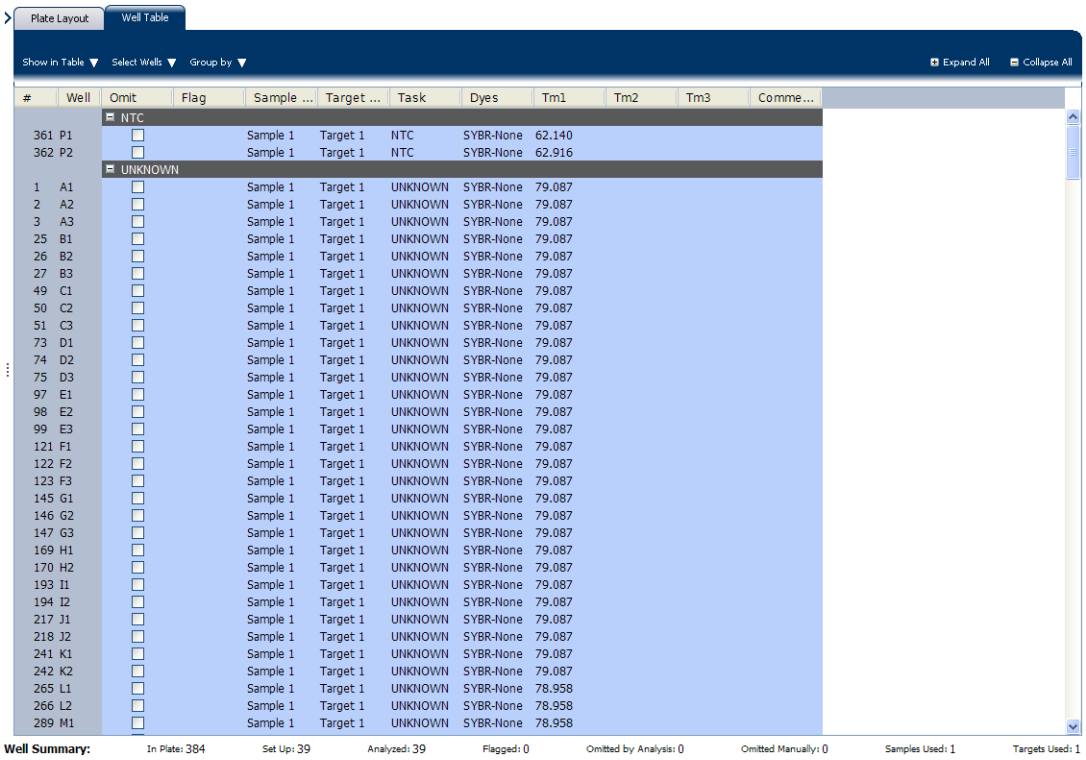
For the example experiment, confirm that no wells of the reaction plate triggered QC flags ⚠.

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. From 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 (⚠).

The figure below shows the well table of the example Melt Curve experiment.



#	Well	Omit	Flag	Sample ...	Target ...	Task	Dyes	Tm1	Tm2	Tm3	Comme...
NTC											
361	P1	☐		Sample 1	Target 1	NTC	SYBR-None	62.140			
362	P2	☐		Sample 1	Target 1	NTC	SYBR-None	62.916			
UNKNOWN											
1	A1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
2	A2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
3	A3	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
25	B1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
26	B2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
27	B3	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
49	C1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
50	C2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
51	C3	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
73	D1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
74	D2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
75	D3	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
97	E1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
98	E2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
99	E3	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
121	F1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
122	F2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
123	F3	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
145	G1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
146	G2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
147	G3	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
169	H1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
170	H2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
193	I1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
194	I2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
217	J1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
218	J2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
241	K1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
242	K2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	79.087			
265	L1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	78.958			
266	L2	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	78.958			
289	M1	☐		Sample 1	Target 1	UNKNOWN	SYBR-None	78.958			

The table below gives the description of each column in the well table.

Column	Description
Well	The position of the well on the reaction plate.
Omit	A check mark indicates that the well has been removed from the analysis.
Flag	A ⚠ indicates that the well triggered the number of flags listed inside the symbol.
Sample Name	The name of the sample.
Target Name	The name of the target evaluated by the well.
Task	The task assigned to the well (Unknown, Negative Control, or Positive Control).
Dyes	The name of the reporter and quencher dyes of the associated sample for the target evaluated by the well.
Tm1	The melting temperature of the target.
Tm2	The second melting temperature (for targets with multiple melting temperatures).
Tm3	The third melting temperature (for targets with multiple melting temperatures).

Column	Description
Comments	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 ⚠ in the Flag column, note the data and the flag(s) triggered by the associated well.
- Select areas of the table or wells of a specified type by:
  - Left-clicking the mouse and dragging across the area you want to select an area of the table.
  - Selecting **Sample**, **Target**, or **Task** from the Select Items menu in the Well Table tab, then selecting the sample, target, or task name from the second Select Items menu 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 menu. You can then collapse or expand the lists either by clicking the **+/-** icon next to individual lists, or by clicking ⊟ Collapse All **Collapse All** or ⊞ Expand All **Expand All**.
- Omit a well from the analysis by selecting the **Omit** check box for that well. To include the well in the analysis, deselect the **Omit** check box.

  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. From 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. From the Plot Color drop-down menu, select **Dye**.

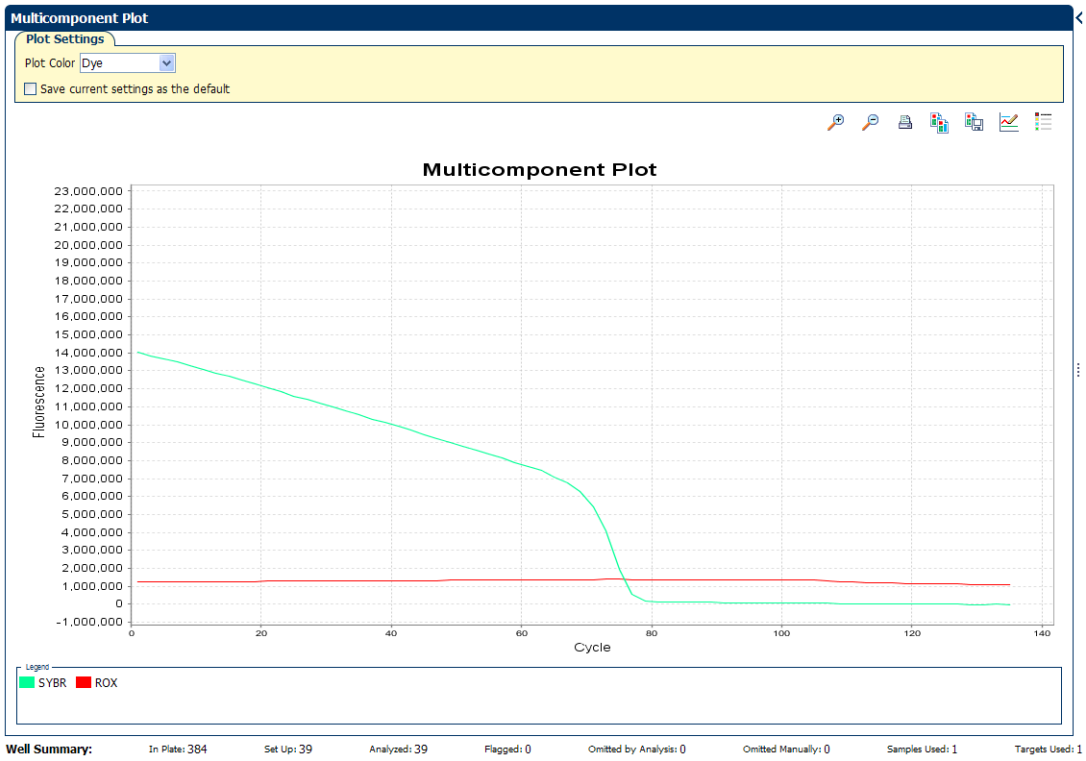
4. Click  **Show a legend for the plot** (default).

   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.

The Multicomponent Plot screen for the example experiment looks like this:



Tips for confirming dye accuracy in your own experiment

When you analyze your own Melt Curve experiment, look for:

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

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

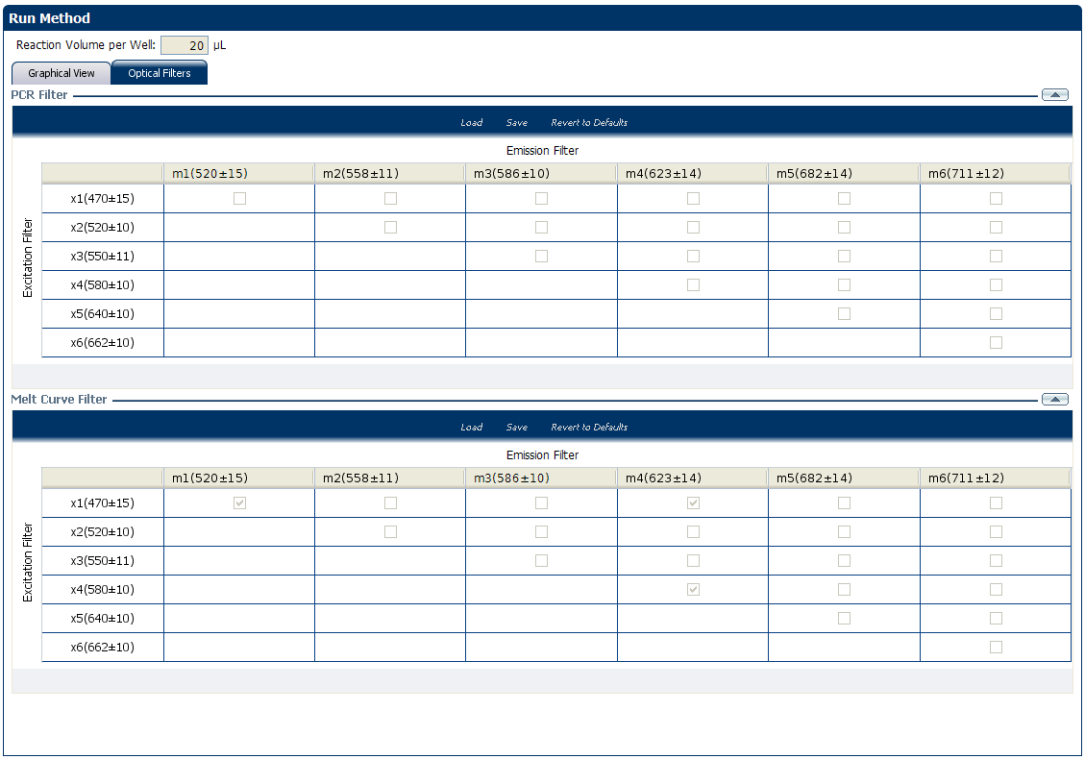
2. Display all 384 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the Plate Layout tab.
3. Click  **Show a legend for the plot** (default). The legend displays the color code for each row of the reaction plate (see the legend in the Raw Data Plot shown below).
4. Click and drag the Show Cycle pointer from cycle 1 to cycle 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.

The Raw Data plot for the example experiment looks like this:

The filters used for the example experiment are:



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

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

2. Review the Flags Summary.

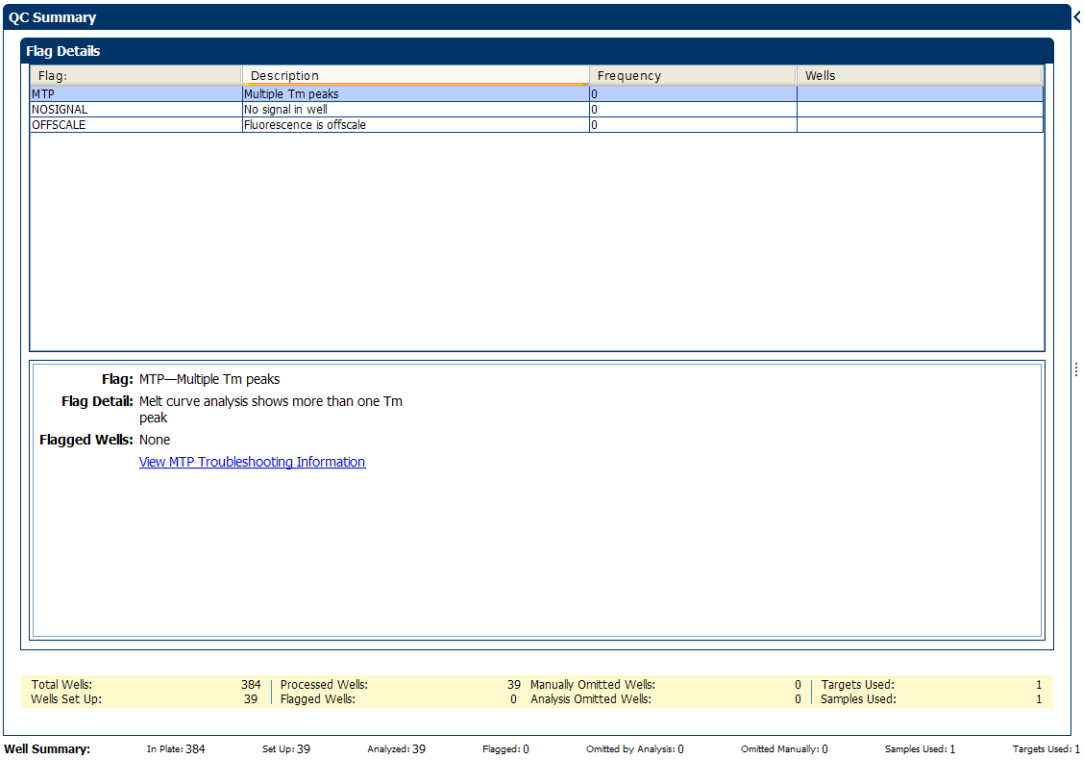
   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment. If the frequency is >0, the flag appears somewhere in the experiment; the well position is listed in the Wells column.

   In the example experiment, there are no flagged wells.

3. In the Flag Details table, click each flag with a frequency >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 >0, click the troubleshooting link to view information on correcting the flag.

   The QC Summary for the example experiment looks like this:



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

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	
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

For more information on...	Refer to...	Part number
Publishing data	Chapter 1 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments*	4470050

Section 5.2 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.

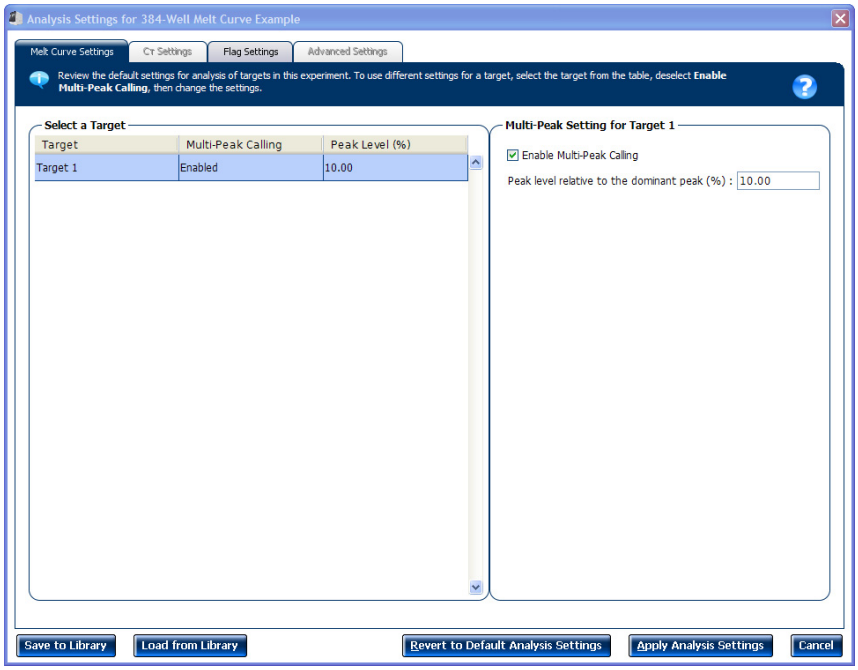
View the analysis settings

1. 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:
   - Melt Curve Settings
   - C_T Settings
   - Flag Settings
   - Advanced Settings

   Note: The C_T Settings and Advanced Settings tabs 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** check box on the Experiment Properties screen to include amplification in your Melt Curve experiment.

   The Analysis Settings dialog box for a Melt Curve experiment looks like this:



3. View and, if necessary, change the analysis settings (see "Adjust analysis settings" below).

   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 in Booklet 1, *Getting Started with QuantStudio™ 12K Flex System Experiments*.

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

You may change the following settings:

Melt Curve Settings

Use this tab to:

- Enable or disable multi-peak calling.
  - Select the **Enable Multi-Peak Calling** check box if you expect to amplify more than 1 PCR product and you want to determine the Tm for more than one peak.
  - Deselect the **Enable Multi-Peak Calling** check box if you expect to amplify 1 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, Life Technologies recommends:

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: This setting is applicable only to the Baseline Threshold algorithm.

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:

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

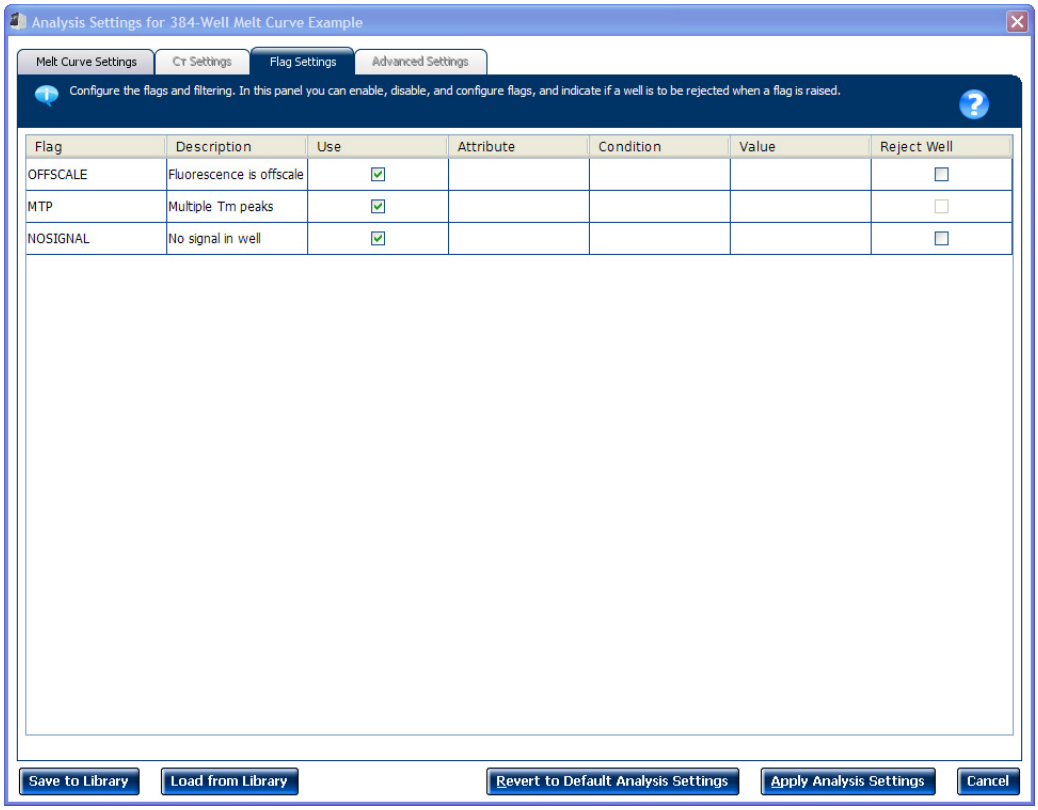
To adjust the flag settings:

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.

The Flag Settings tab looks like this:



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.

To use 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** check box.
3. Define the custom baseline settings:
   - For automatic baseline calculations, select the **Automatic Baseline** check box.
   - To define the baseline, deselect the **Automatic Baseline** check box, then enter the baseline start cycle and baseline end cycle.

For more information

For more information on...	Refer to	Part number
Amplification efficiency	*Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*.	127AP05-03

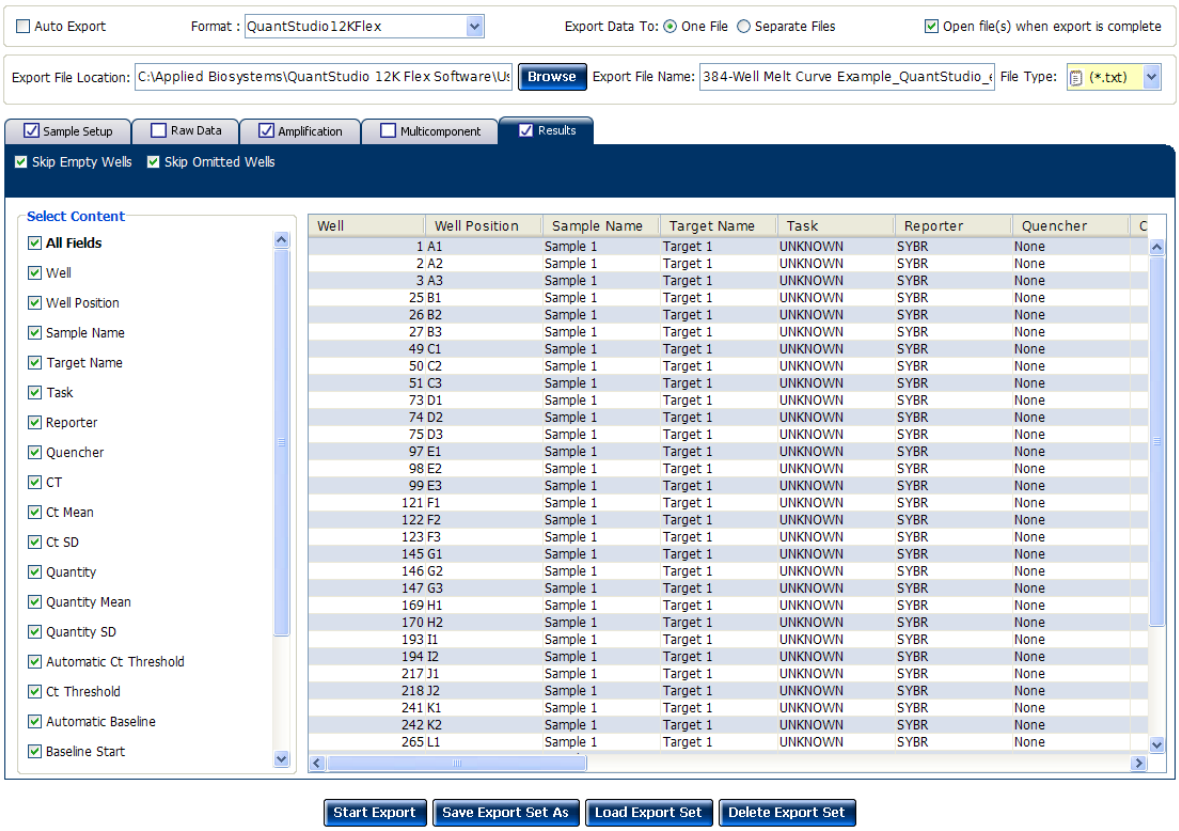
6 Export Analysis Results

1. Open the Melt Curve example experiment file that you analyzed in Chapter 5.
2. In the Experiment Menu, click  **Export.**

   Note: To export data automatically after analysis, select the **Auto Export** check box during experiment setup or before running the experiment. Auto export is unchecked for the example experiment.
3. Select **QuantStudio™ 12K Flex format**.
4. Complete the Export dialog box as shown below:

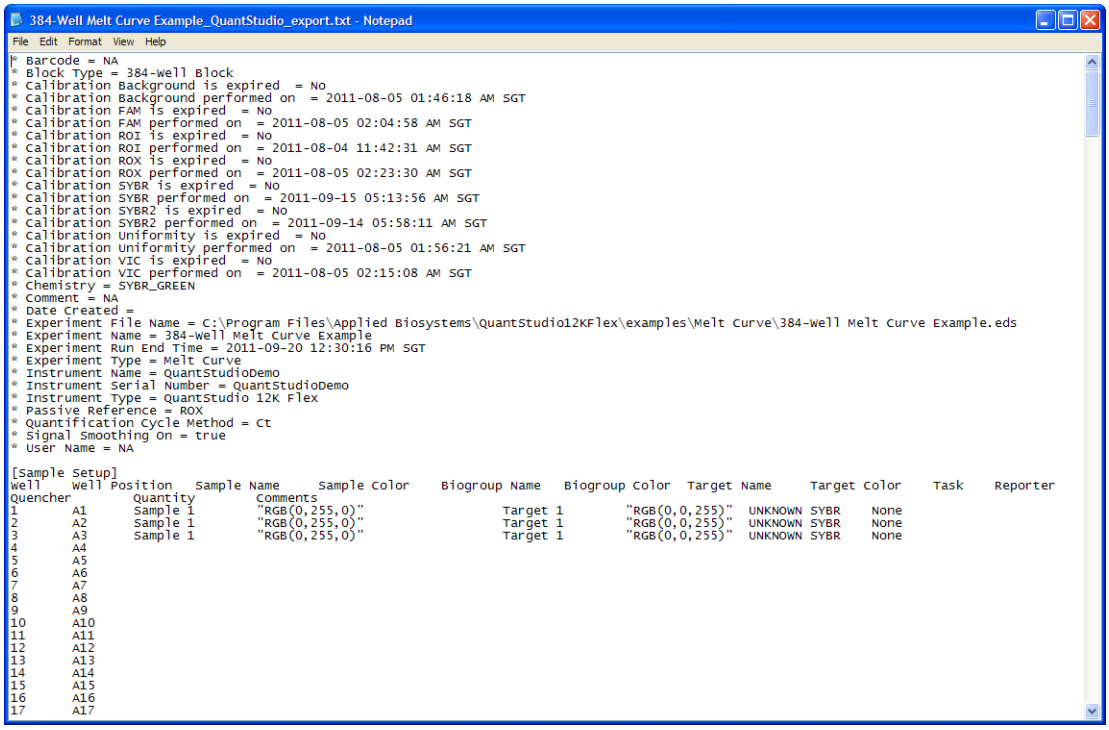
Field or Selection	Entry
Select Data to export/ Select Content	Results
Export Data To	One File
Export File Name	384-Well Melt Curve Example_QuantStudio_export
File Type	*.txt
Export File Location	<drive>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments

Your Export screen should look like this:



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

Your exported file when opened in Notepad should look like this:



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
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GETTING STARTED GUIDE

applied biosystems®
by life technologies™

Booklet 7 - QuantStudio™ 12K Flex System Multi-Well Plates and Array Card Experiments - Appendixes

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life technologies™

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A Ordering Information

This appendix covers:

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Consumables

The consumables listed below are required for calibrating the QuantStudio™ 12K Flex Instrument and for performing experiments with the QuantStudio™ 12K Flex System.

Note: For reagent or consumable shelf-life expiration date, see the package label.

Calibration and verification consumables

The following table shows the reagents and consumables required to calibrate the QuantStudio™ 12K Flex Instrument.

384-well sample block

Consumable	Part number	Shelf-life at environmental temperature	Storage conditions (°C)
384-Well Spectral Calibration Plate with FAM™ Dye	4432271	Use the consumable by the expiration date mentioned on the package	–15 to –25°C
384-Well Spectral Calibration Plate with VIC® Dye	4432278		
384-Well Spectral Calibration Plate with ROX™ Dye	4432284		
384-Well Spectral Calibration Plate with SYBR® Green Dye	4432290		
384-Well Spectral Calibration Plate with TAMRA™ Dye	4432296		
384-Well Spectral Calibration Plate with NED™ Dye	4432302		
384-Well Region of Interest (ROI) and Background Plates	4432320		
384-Well Normalization Plates with FAM™/ROX™ and VIC®/ROX™ Dyes	4432308		
TaqMan® RNase P Fast 384-Well Instrument Verification Plate	4455280		

96-well (0.2 µL) sample block

Consumable	Part number	Shelf-life at environmental temperature	Storage conditions (°C)
96-Well Spectral Calibration Plate with FAM™ Dye	4432327	Use the consumable by the expiration date mentioned on the package	–15 to –25°C
96-Well Spectral Calibration Plate with VIC® Dye	4432334		
96-Well Spectral Calibration Plate with ROX™ Dye	4432340		
96-Well Spectral Calibration Plate with SYBR® Green Dye	4432346		
96-Well Spectral Calibration Plate with TAMRA™ Dye	4432352		
96-Well Spectral Calibration Plate with NED™ Dye	4432358		
TaqMan® RNase P 96-Well Instrument Verification Plate	4432382		
96-Well Region of Interest (ROI) and Background Plates	4432364		
96-Well Normalization Plates with FAM™/ROX™ and VIC®/ROX™ Dyes	4432370		
TaqMan® RNase P 96-Well Instrument Verification Plate	4432382		

Fast 96-well (0.1 µL) sample block

Consumable	Part number	Shelf-life at environmental temperature	Storage conditions (°C)
Fast 96-Well Spectral Calibration Plate with FAM™ Dye	4432389	Use the consumable by the expiration date mentioned on the package	–15 to –25°C
Fast 96-Well Spectral Calibration Plate with VIC® Dye	4432396		
Fast 96-Well Spectral Calibration Plate with ROX™ Dye	4432402		
Fast 96-Well Spectral Calibration Plate with SYBR® Green Dye	4432408		
Fast 96-Well Spectral Calibration Plate with TAMRA™ Dye	4432414		
Fast 96-Well Spectral Calibration Plate with NED™ Dye	4432420		
Fast 96-Well Region of Interest (ROI) and Background Plates	4432426		
Fast 96-Well Normalization Plates with FAM™/ROX™ and VIC®/ROX™ Dyes	4432432		
TaqMan® RNase P Fast 96-Well Instrument Verification Plate	4351979		

Array card sample block

Consumable	Part number	Shelf-life at environmental temperature	Storage conditions (°C)
Array Card Spectral Dye Calibration Kit	4432314	Use the consumable by the expiration date mentioned on the package	–15 to –25°C
Array Card RNase P Instrument Verification Kit	4432265		

Experiment consumables

The following table shows the reagents and consumables required to perform experiments with the QuantStudio™ 12K Flex System.

Consumable		Part number	Shelf-life at environmental temperature	Storage conditions
Array Card Buckets/Clip Set	1st Generation	4337762	Use the consumable by the expiration date mentioned on the package	Room temperature
	2nd Generation	4442571		
TaqMan® Array Micro Fluidic Card Sealer		4331770		
MicroAmp® Optical 384-Well Reaction Plate with Barcode	1000 plates	4343814		
	500 plates	4326270		
	50 plates	4309849		
MicroAmp® Optical 384-Well Reaction Plate, 1000 plates		4343370		
MicroAmp® Optical 96-Well Reaction Plate (0.2µL)	500 plates	4316813		
	10 plates	N8010560		
MicroAmp® Optical 96-Well Reaction Plate with Barcode (0.2µL	500 plates	4326659		
	20 plates	4306737		
MicroAmp® Fast Optical 96-Well Reaction Plate (0.1µL)	10 plates	4346907		
MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1µL)	200 plates	4366932		
	20 plates	4346906		
Centrifuge Buckets, Array Card	1st Generation	4337230		
	2nd Generation	4442573		
Clip, Array Card Centrifuge Adaptor		4334682		
MicroAmp® Optical Adhesive Film	100 films	4311971		
	25 films	4360954		
MicroAmp® Multi-Removal Tool (1 tool)		4313950		
RT-PCR Grade Water		AM9935		
MicroAmp® Optical 8-Cap Strip	300 strips	4323032		
MicroAmp® Optical 8-Tube Strip (0.2µL)	125 strips	4316567		
MicroAmp® Fast 8-Tube Strip (0.1µL)	125 strips	4358293		
MicroAmp® Optical Tube without cap (0.2µL)	2000 tubes	N8010933		
MicroAmp® Fast Reaction Tube with cap (0.1µL)	1000 tubes	4358297		
MicroAmp® 96-Well Tray/Retainer Set (Blue) (for 0.2µL)	10 pairs	4381850		
MicroAmp® 96-Well Tray (Black) (for 0.1µL)	10 plates	4379983		

Consumable		Part number	Shelf-life at environmental temperature	Storage conditions
96-Well Plate Adapter (0.2 µL)	1 piece	4459845	Use the consumable by the expiration date mentioned on the package	Room temperature
Fast 96-Well Plate Adapter (0.1 µL)	1 piece	4459846		
96-Well Tube Adapter (0.2 µL)	1 piece	4462077		
96-Well Tube Adapter (0.1 µL)	1 piece	4462078		
384-Well Plate Adapter	1 piece	4457087		
Array Card Adapter	1 piece	4454166		
MicroAmp® Cap Installing Tool (Handle) (1 tool)		4330015		
MicroAmp® Adhesive Film Applicator (5 applicators)		4333183		

Reagents

The following table lists the reagents that can be ordered for performing experiments with the QuantStudio™ 12K Flex System.

Recommended reagent kits

To perform	Reagent	Part Number
Reverse Transcription	SuperScript® VILO™ cDNA Synthesis Kit	4453650
TaqMan® PCR	TaqMan® Fast Advanced Master Mix	4444557
	TaqMan® GTXpress™ Master Mix	4401892
	TaqMan® Fast Virus 1-Step Master Mix	4444432
	TaqMan® Gene Expression Master Mix	4369016
	TaqMan® Genotyping Master Mix	4371355
	TaqMan® Universal Master Mix II, with UNG	4440038
	TaqMan® RNA-to-CT™ 1-Step Kit	4392938
SYBR® Green PCR	Fast SYBR® Green Master Mix	4385612
	Power SYBR® Green PCR Master Mix	4367659
	Power SYBR® Green RNA-to-CT™ 1-Step Kit	4389986

General-use materials and consumables

The following general-use materials and consumables are required to calibrate, maintain, and perform experiments with the QuantStudio™ 12K Flex System. Unless indicated otherwise, all materials shown below are available from major laboratory suppliers (MLS). The materials are applicable to all sample blocks.

Material/Consumable	Source
Bleach, 10% solution	MLS
Lint-free lab tissues	MLS
Cotton or nylon swabs and lint-free cloths	MLS
Centrifuge with buckets appropriate for your consumable type	MLS
Ethanol, 95% solution	MLS
Glasses, safety	MLS
Gloves, powder-free	MLS
Permanent marker or pen	MLS
Pipettors: 100-µL and 200-µL (with pipette tips)	MLS
Screwdriver, flathead	MLS

Documentation and Support

Related documentation

The following related documents are shipped with the system:

Document	PN	Description
Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Maintenance and Administration Guide	4470689	Explains how to use and maintain the Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Intended for laboratory staff responsible for the use and maintenance of the QuantStudio™ 12K Flex Instrument.
Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Multi-well Plates and Array Card Experiments User Guide	4470050	Contains five individual booklets that explain how to perform the six different experiments on the QuantStudio™ 12K Flex Instrument The experiments include Standard Curve, Relative Standard Curve and Comparative C_T, Genotyping, Presence/ Absence and Melt Curve. Each Getting Started Guide booklet functions as both: • A tutorial, using example experiment data provided with the QuantStudio™ 12K Flex Software. • A guide for your own experiments. Intended for laboratory staff and principal investigators who perform experiments using the QuantStudio™ 12K Flex System.
Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Quick Reference Guide	4470688	Explains how to install and maintain the QuantStudio™ 12K Flex Instrument Intended for laboratory staff responsible for the use and maintenance of the QuantStudio™ 12K Flex Instrument.
Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System Site Preparation Guide	4470654	Explains how to prepare your site to receive and install the QuantStudio™ 12K Flex Instrument Intended for personnel who schedule, manage, and perform the tasks required to prepare your site for installation of the QuantStudio™ 12K Flex Instrument.

Document	PN	Description
QuantStudio™ 12K Flex Software Help	NA	Explains how to use the QuantStudio™ 12K Flex Software to: • Set up, run, and analyze experiments. • Monitor a networked QuantStudio™ 12K Flex Instrument. • Calibrate the QuantStudio™ 12K Flex Instrument. • Verify the performance of QuantStudio™ 12K Flex Instrument with an RNase P run. • Intended for: – Laboratory staff and principal investigators who perform experiments using the QuantStudio™ 12K Flex System. – Laboratory staff responsible for the installation and maintenance of the QuantStudio™ 12K Flex Instrument.

Note: For additional documentation, see "How to obtain support" on page 13.

Other related documents

Documents related to Genotyping experiments

Document	PN
Allelic Discrimination Pre-Developed TaqMan® Assay Reagents Quick Reference Card	4312212
Custom TaqMan® Genomic Assays Protocol	4367671
Custom TaqMan® SNP Genotyping Assays Protocol	4334431
Ordering TaqMan® SNP Genotyping Assays Quick Reference Card	4374204
Pre-Developed TaqMan® Assay Reagents Allelic Discrimination Protocol	4312214
TaqMan® Drug Metabolism Genotyping Assays Protocol	4362038
TaqMan® SNP Genotyping Assays Protocol	4332856

Documents related to Presence/ Absence experiments

Document	PN
DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol	4343586
NucPrep® Chemistry: Isolation of Genomic DNA from Animal and Plant Tissue Protocol	4333959
PrepMan® Ultra Sample Preparation Reagent Protocol	4318925

Documents related to Relative Standard Curve and Comparative C_T experiments

Document	PN
Amplification Efficiency of TaqMan® Gene Expression Assays Application Note	127AP05
Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol	4375575
Custom TaqMan® Gene Expression Assays Protocol	4334429
Primer Express® Software Version 3.0 Getting Started Guide	4362460
TaqMan® Gene Expression Assays Protocol	4333458
User Bulletin #2: Relative Quantitation of Gene Expression	4303859

Documents related to Standard Curve experiments

Document	PN
Amplification Efficiency of TaqMan® Gene Expression Assays Application Note	127AP05
Custom TaqMan® Gene Expression Assays Protocol	4334429
Primer Express® Software Version 3.0 Getting Started Guide	4362460
TaqMan® Gene Expression Assays Protocol	4333458
User Bulletin #2: Relative Quantitation of Gene Expression	4303859

How to obtain support

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At the Life Technologies web site, you can:

- Access worldwide telephone and fax numbers to contact Life Technologies Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, SDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

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 by doing one of the following:

- Click  in the toolbar of the QuantStudio™ 12K Flex Software window.
- Select **Help ▸ QuantStudio™ 12K Flex Software Help**.
- Press **F1**.

You can use the Help system to find topics of interest by:

- Reviewing the table of contents
- Searching for a specific topic
- Searching an alphabetized index

You can also access PDF versions of all documents in the QuantStudio™ 12K Flex Software document set from the Help system.

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