USER GUIDE



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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Roadmap

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- BOOKLET 3 Running Relative Standard Curve and Comparative C_T Experiments
 - PART I: Running Relative Standard Curve Experiments
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Roadmap

GETTING STARTED GUIDE



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
А	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.

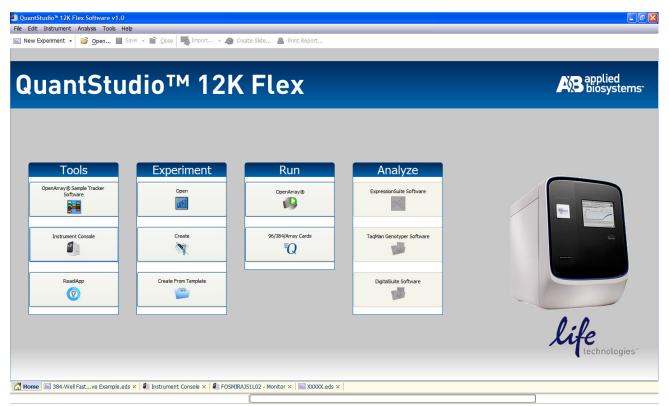
How to access an example experiment

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 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 properties 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 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. name and type Enter a name that is descriptive and easy to remember. You can enter up to ٠ 100 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 QuantStudioTM 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.
- 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:

How do you want to identify this experime	nt?		
Experiment Name: 384-Well Fast Standard Curve B Barcode: User Name:	Example.eds	Comments: Standard Curve example	 ×
* Which block are you using to run the expe	eriment?		
✓ 384-Well	Array Card	96-Well (0.2mL)	Fast 96-Well (0.1mL)
* What type of experiment do you want to s	set up?		
✓ Standard Curve	Relative Standard Curve	Comparative Cr (ΔΔCr)	Melt Curve
Genotyping	Presence/Absence		
* Which reagents do you want to use to det	ect the target sequence?		
✓ TaqMan® Reagents	SYBR® Green Reagents	Other	
* What properties do you want for the instr	ument run?		
Standard	✓ Fast		

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.
- 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:

Targets			Samples		
New Save to Library Import from Library D	elete			New Save to Library Import from Library Delete	
Target Name	Reporter	Quencher	Color	Sample Name C	olor
RNaseP	FAM 🗸	NFQ-MGB	· 🗾 🗸	Pop1	~
				Pop2	~
				<u> </u>	
Biological Replicate Groups					
New Delete					
Biological Group Name Color		Comments			
* Passive Reference					
ROX					

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						
	Ν	Negative Control						
	I							
Relative Standard Curve	U	Unknown						
	S	Standard						
	Ν	Negative Control						
Comparative CT	U	Unknown						
	Ν	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						
	Ν	No Template Control						
		•						
Presence/ Absence	U	Unknown						
	I	Internal Positive Control						
	Ν	Negative Control						
	X	Blocked Internal Positive Contro						
Melt Curve	U	Unknown						
	Ν	Negative Control						

3. Assign Samples.

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

- **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:

Targets	< Plate	Layout	Well	Table																				
Name Task Quantity		show in We	ells ▼ ⊆	ielect Well	ls 🔻 🖥	Uiew	Legend															Ð	8 🕽	~
RNaseP Nov		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	A 🔳	S	S	S	S	S	Pop1	Hon1	Hon1	Pop1	Hon1	Pop1	Hon1	Pop1	Pop1	Pop/	Pop/	Pop/	Pop/	Pop/	Hop/	Hop/	Hop/	Pop/
	в 🗵	S	S	S	S	5	Hon1	Hon1	Hon1	Hop1	Hon1	Hop1	Hon1	Hon1	Pop1	Pop/	Pop/	Pop/	Pop/	Pop/	Hop/	Rop/	Rop/	Pop/
	c 🛛	S	S	S	S	S	Hon1	Pop1	Hon1	Pop1	Pop1	Pop1	Pop1	Pop1	Hon1	Pon/	Pon/	Pop/	Pop/	Pop/	Pop/	Pop/	Pop/	Pop/
	D	S	S	S	S	S	Pop1	Pon1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop/	Hop/	Pop/						
mples 🖂	E 🛛	S	S	S	S	S	Hon1	Hon1	Hon1	Hon1	Hon1	Han	Hon1	Hon1	Hon1	Pop/	Pop/	Pop/	Pop/	Pop/	Kop/	Kon/	Rop/	Pop/
Name	F 🛛	S	5	S	S	S	Pop1	Hop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop/	Hop/	Pop/						
Pop1	G 🛛	S	5	S	S	S	Pop1	Pop1	Pop1	Hop1	Pop1	Pop1	Pop1	Pop1	Pon1	Pop/	Pop/	Pop/	Pop/	Pop/	Kop/	Kon/	Pop/	Pop/
	н 🛛	S	S	S	S	5	Hop1	Pop1	Hon1	Pop1	Pop1	Pop1	Hon1	Pop1	Pon1	Pop/	Pop/	Pop/	Pop/	Pop/	Pop/	Kon/	Pop/	Kop/
	I	S	5	5	S	5	Hon1	Pop1	Hon1	Pop1	Pop1	Pop1	Hon1	Hon1	Hon1	Pop/	Pop/	Pon/						
	J 🛛		S	S	S	S	Hop1	Pop1	Hon1	Pop1	Hon1	Pop1	Pop1	Hon1	Hon1	Pop/	Pop/	Pop/						
	К 🖪	_	S	S	S	S	Hop1	Hop1	Hon1	Pop1	Hop1	Pop1	Hon1	Hop1	Hon1	Hon/	Pop/	Pop/	Pop/	Pop/	Kop/	Kon/	Pop/	Kop/
ological Groups			S	5	S	S	Hon1	Pop1	Hon1	Hon1	Hon1	Pop1	Hop1	Pop1	Pon1	Pon/	Pop/	Pop/	Pop/	Pop/	Pop/	Pop/	Rop/	Pop/
Biological Group	M 🖸		S	5	S	S	Mon1	Hop1	Hon1	Pop1	Hop1	Pop1	Pop1	Pop1	Hon1	Pon/	Pon/	Pop/	Pon/	Pop/	Rop/	Hop/	Kon/	Pop/
	N N		S									Voo1	_	Pop1		Pon/	Pon/	Pop/		Pon/	Pop/			Pop/
				S	S	S	Hop1	Pop1	Pop1	Pop1	Pop1	Popl	Pop1	Hop1	Pop1	Pop/	_	Pop/	Pop/		Rop/	Pop/	Pop/	Pop/
	0		S	S	S	S	Hon1	Pop1	Hon1	Pop1	Pop1	in li	Hon1	m	in l		Pop/	Pop/		Pop/		171	Hop/	
	P 🖾	S	S	S	S	S	Hon1	Pop1	Hon1	Pop1	Pop1	Pop1	Pop1	Pop1	Pon1	Pop/	Pon/	m /	Pop/	Pop/	Pop/	Pop/	Pop/	Pop/
	Wells:	U 288	80 🔁	N 16																			(D Empty

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	- (°	<i>f</i> ∗ Well					
	А	В	С	D	E	F	G	Н
1	Well	Sample	liological 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
----------	-------	-----	----------	-------------

Isame Target 1 ✓ Y Target 1 ✓ Y RNaseP U/ 1 A1 SK RNaseP U////////////////////////////////////	🔍 Define and Set Up Standards	Plate	Layout	Well Table						
Name Name <th< th=""><th>Targets ></th><th></th><th></th><th></th><th></th><th></th><th></th><th></th><th></th><th></th></th<>	Targets >									
Weil Sample Biological Groups # Weil Sample Biological Group Take Clank Clank <thclank< th=""> <thclank< th=""> <thclank< <="" th=""><th>Name Task Quantity</th><th>Show i</th><th>n Table 🔻</th><th>Select Wells 🐧</th><th>Group by 🔻</th><th></th><th></th><th></th><th></th><th></th></thclank<></thclank<></thclank<>	Name Task Quantity	Show i	n Table 🔻	Select Wells 🐧	Group by 🔻					
Image Image Image Image Image Image 1 A1 SK RNaseP UIKNOWN FAM-HQ 3 A3 SK RNaseP UIKNOWN FAM-HQ 3 A3 SK RNaseP UIKNOWN FAM-HQ 4 A4 SK RNaseP UIKNOWN FAM-HQ 5 A5 SK RNaseP UIKNOWN FAM-HQ 5 A5 SK RNaseP UIKNOWN FAM-HQ 6 A5 SK RNaseP UIKNOWN FAM-HQ 9 A9 SK RNaseP UIKNOWN FAM-HQ 9 A9 SK RNaseP UIKNOWN FAM-HQ 11 A11 SK RNaseP UIKNOWN FAM-HQ 12 A12 SK RNaseP UIKNOWN FAM-HQ 13 A13 SK RNaseP UIKNOWN FAM-HQ 14 A14 SK RNaseP UIKNOWN FAM-HQ 14<		#	Well	Sample	Biologic	Target	Task	Dves	Quantity	Comme
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Define the runUse 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.

- 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.

🛗 AutoDelta Settings	×			
AutoDelta Settings For Cycling Stage				
AutoDelta Temperature: + 💌 0.01 👗				
Valid AutoDelta Temperature Range: -2.27 to 0.12				
AutoDelta Time: + 💌 0:35				
Starting Cycle: 1				
Save Setting Cancel				

- **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.

Experiment	Print	Export	Display Format	SMTP	Settings
Non-OpenArray	OpenAri	ay® Block Run	Global Notification Se	ettings	Startu
Select the default	folders, the default	block type, and wheth	er to show optical filters in the r	un method.	
Data Folder:	d Biosystems	QuantStudio 12K	CFlex Software\User Files	s\experiments	Brows
Import Folder:	d Biosystems	QuantStudio 12K	Flex Software\User Files	<pre>s\experiments</pre>	Brows
Block Type:	384-Well Blo	ck		~	•
Decimal Places to Sho	w: 3				
Show optical filte	ers for run method	i l			

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

- Run Method ion Volume per Well: Graphical View Optical Filters Filter ion Filte m1(520±15) m4(623±14) m5(682±14) m6(711±12) m2(558±11) m3(586±10) x1(470±15) atter. x2(520±10) x3(550±11) x4(580±10) x5(640±10) x6(662±10) elt Curve Filter ion Filte m4(623±14) m5(682±14) m6(711±12) m1(520±15) m2(558±11) m3(586±10) x1(470±15) itter x2(520±10) x3(550±11) x4(580±10) x5(640±10) x6(662±10)
- 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*TM 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.

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.

(Optional) Use libraries when designing your own experiments 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	A1	 MicroAmp[®] Optical 384-Well Reaction Plate MicroAmp[®] Optical Adhesive Film 	30	5-20
Array Card	A1 A1 A1 A1 A1 A1 A1 A1 A1 A1 A1 A1 A1 A	Applied Biosystems Array Card	1	1

to select a full method me

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

1

1

Sample block	Consumabl	le	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

 \checkmark 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 μ L) reaction plates or reaction tubes and tube strips sealed with caps.

Reagent detectionApplied Biosystems TaqMan® ReagentsprocessDescription

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

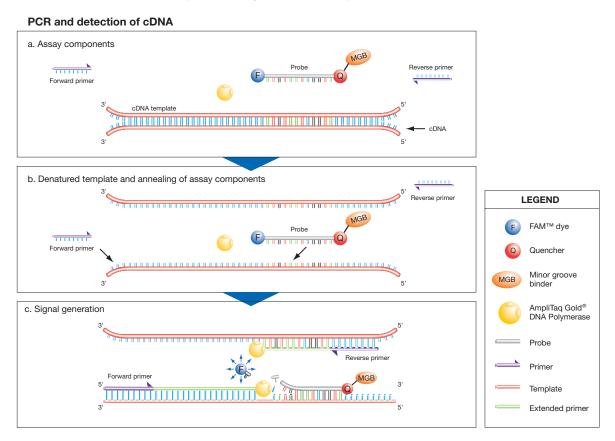
Advantages

- 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.





Applied Biosystems SYBR[®] Green reagents Description

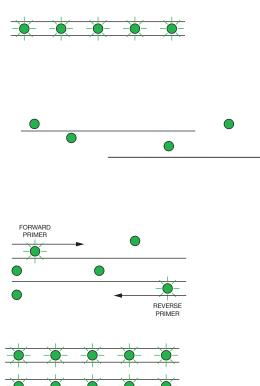
SYBR Green reagents use SYBR[®] Green I dye, a double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles.

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.

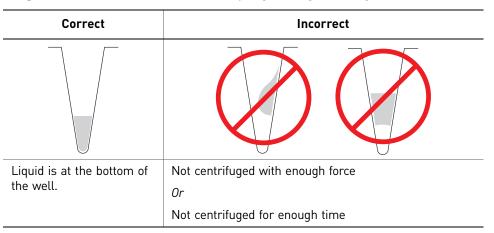
Materials required while preparing the dilutions

- 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.



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

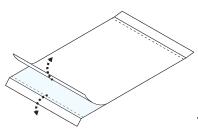
1. Load the reaction plate using the plate layout described in "Assign targets, samples, and biological replicate groups" on page 14.

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

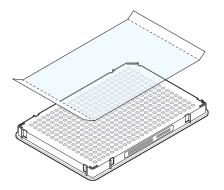
Note: You can also use the MicroAmp[®] Optical 8-Cap Strip to seal the 96-well reaction plates.

- **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.

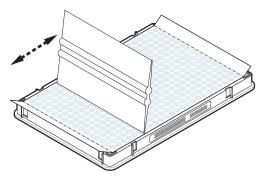


IMPORTANT! Improper peeling of the optical adhesive film may result in haziness, but it will not affect results. Haziness disappears when the film comes into contact with the heated cover in the instrument.

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.



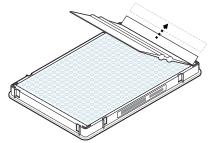
5. Applying firm pressure, move the applicator slowly across the film, horizontally and vertically, to ensure good contact between the film and the entire surface of the reaction plate.



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 endtabs from the dotted line. Improper peeling of the end-tab can cause sticking of plate on the heated cover assembly.

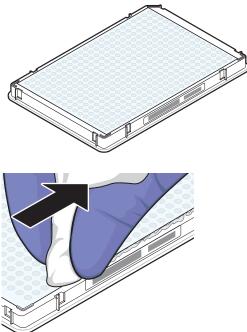
7. To ensure a tight, evaporation-free seal, repeat Applying firm pressure, move the applicator slowly across the film, horizontally and vertically, to ensure good



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

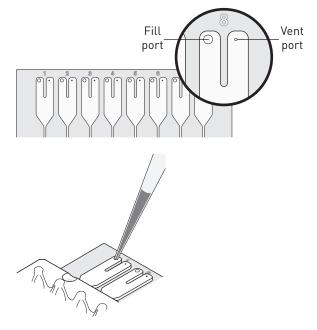
 array card
 IMPORTANT! Wear powder-free gloves while preparing the Arrays.

- 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.

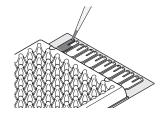
For each transfer:

- 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.

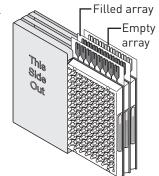


d. Dispense the fluid so that it sweeps in and around the fill reservoir toward the vent port. Pipette fluid into the fill reservoir, but **do not** go past the first stop of pipettor plunger when pipetting the reagents into the array card, or you may blow the solution out of the port.



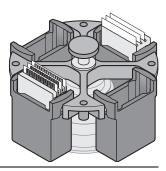
IMPORTANT! Do not allow the tip to contact and possibly damage the coated foil beneath the fill port.

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.

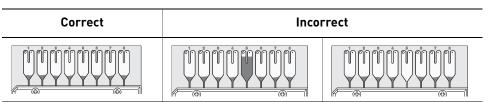


IMPORTANT! You must run the centrifuge with all four buckets in place and each of the two carriers filled with the array card. Place empty array cards (4-pack PN 4334812 and 1-pack PN 4351471) into unfilled slots.

- **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.



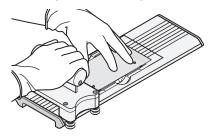
9. If necessary, centrifuge the array card(s) for an additional minute to fill any unfilled wells. Do not exceed three 1-minute runs or centrifuge the array card for longer than 1 minute at a time.

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

Alignment pins

Seal the array card(s)

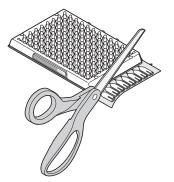
- 1. With the carriage (roller assembly) of the TaqMan[®] Array Micro Fluidic Card Sealer in the Start position, place a filled array card into the fixture with the foil side up so that the fill reservoirs are the farthest away from the carriage.
- **2.** Press down on all four corners of the array card 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.

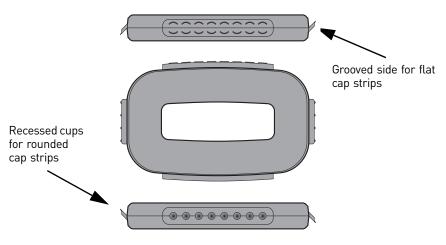
Capping and uncapping the 96well reaction tubes and tube strips **Note:** Make sure that you secure the caps on the tubes and tube-strips tightly to avoid sample evaporation.

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)

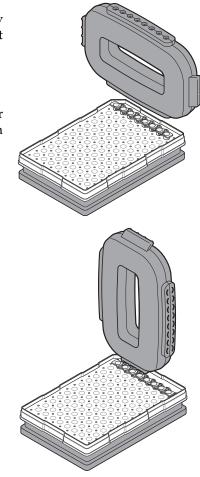
1. Grasp the Cap Installing Tool so that the grooved side is exposed.

- 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:

- 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

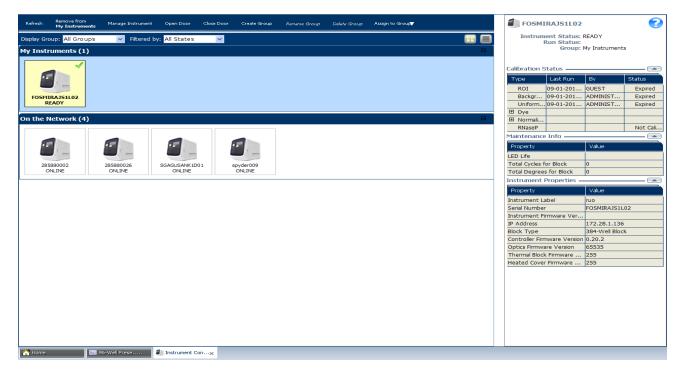
To start an experiment:

- 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.

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

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 Δ 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.

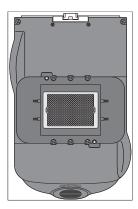
To monitor the instrument status:

- 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.

lcon	Instrument status
FOSMIRAJSILO2 READY	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.)
0	Unavailable
*	Incompatible firmware version
8	No longer connected to the network

lcon	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

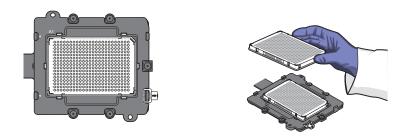
IMPORTANT! Wear powder-free gloves when you handle the **reaction plate or array card**.

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.

 Touch and 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.

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

- **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 0.2μ L tubes and 0.1μ L 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* $Biosystems QuantStudio^{TM}$ 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.

Run Status	
	START RUN
	fosmirajs1l01
	SGAGUSANK1D01
	engg8

From the QuantStudio[™] 12K Flex Instrument touchscreen

- 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.

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 Add and Delete buttons to add and delete a stage or step to the thermal profile.
 - C_{And Melt Curve} Add Melt Curve button to add a melt curve to the thermal profile.
 - **b** save 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 **F** start Run **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
experimentNote: 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

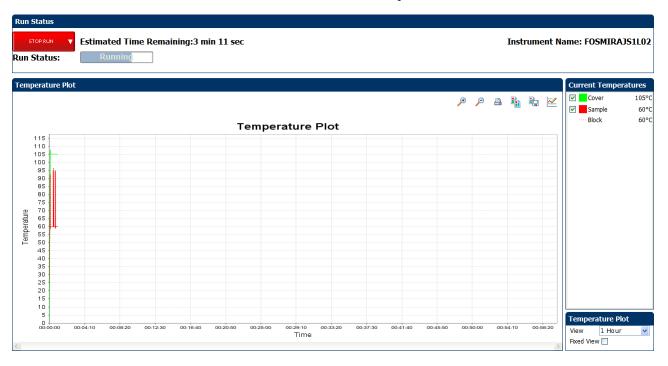
- 1. In the Instrument Console screen, select the icon of the instrument that you are using to run the experiment.
- 2. Click Manage Instrument.
- 3. On the Instrument Manager screen, click Monitor Running Instrument.

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.

То	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	Select Temperature Plot.
for the run in real time	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	Select or deselect Enable Notifications.
Notification Settings	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 realtime 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 QuantStudioTM 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.

То	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	Select Fixed View.
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. 	

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.

То	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.

1

То	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 QuantStudioTM 12K Flex Software Help (click \bigcirc 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:

Run Data Report	
Experiment Name:	384-Well Fast Standard Curve Example
Start Time:	08-06-2011 12:33:30 SGT
Stop Time:	08-06-2011 13:15:47 SGT
Run Duration:	42 minutes 17 seconds
User Name:	DEFAULT
Instrument Name:	QuantStudioDemo
Firmware Version:	0.13.1
Software Version:	QuantStudio 12K Flex Software v1.0
Instrument Serial Number:	QuantStudioDemo
Sample Volume:	20.0
Cover Temperature:	105.0
Block Type:	384-Well Block
Errors Encountered:	~

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.

То	Action
Display a graphical view of the run	Touch 🖽 Experiment View.
Show the Amplification Plot for the run	Touch the <i>I</i> Plot View , then touch <i>I</i> Experiment View to return to the Run Method screen.

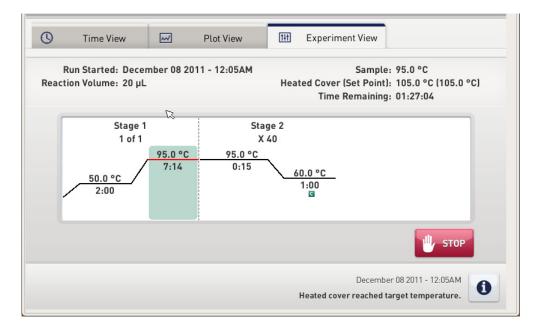
Getting Started with QuantStudio[™] 12K Flex System Multi-Well Plates and Array Card Experiments

1

То	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 QuantStudioTM 12K Flex Instrument touchscreen is shown in the following graphics:

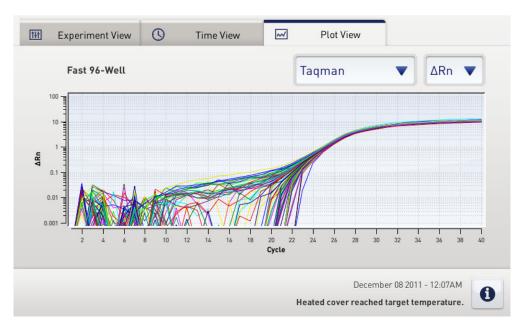
Experiment View



Time View

Sample: 59.5 °C Heated Cover (Set Point): 105.0 °C (105.0 °C)
Heated Cover (Set Point): 105.0 °C (105.0 °C)
Stage / Step / Cycle: 2 / 2 / 5
1:22 • • Elapsed Time

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	То
Rn 🔻	Change the data displayed on the y axis. Select either Rn (normalized reporter) or Δ Rn (baseline-corrected normalized reporter).

1

	Touch	То				
	FAM V	Change the reporter dye displayed in the plot. Only dyes used in your experiment are shown.				
	0	View the run events that occurred during the run. Touch () again to close the event list				
Unload the instrument	5	tudio [™] 12K Flex Instrument displays the Main Menu screen, plate from the instrument and transfer the experiment data to the sis.				

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.				
	 Touch an 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 Close Door to retract the plate adapter back into the instrument.				
	If the QuantStudio TM 12K Flex Instrument does not eject the plate, remove the plate as follows:				
	a. Power off the QuantStudio ^{TM} 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				
	 In the QuantStudio[™] 12K Flex Software, select Instrument ➤ Instrument Console. 				
	 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.				

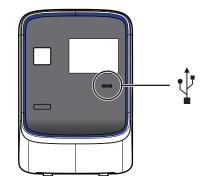
4. In the Instrument Manager, click Manage Files.

- 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:

 $< drive>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments\where, <math>< drive>$ is the computer hard drive on which the QuantStudioTM 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 **III 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 **1** 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.
- Copy the example experiment file to:

 Applied Biosystems\QuantStudio 12K Flex Software\experiments\

1

1

where $\langle drive \rangle$ is the computer hard drive on which the QuantStudioTM 12K Flex Software is installed. The default installation drive for the software is the C: drive.

Review experiment results

About analysis
resultsImmediately 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 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:

🦺 Analysis Settings Libi	rary			×			
Select analysis settings or delete analysis settings. Apply a filter to reduce the number of analysis settings displayed.							
Enter a filter query, the	n click "Apply Filter."	~~					
IF Analysis Settings	× = ×		Apply	Filter Remove Filter			
Delete Delete All							
Analysis Settings	Experiment Type	Comments	Created On	Last Modified			
SC-22	Standard Curve		12-08-2011 16:32:14 SGT	12-08-2011 16:32:22 SGT			
GT-56	Standard Curve		12-08-2011 16:32:31 SGT	12-08-2011 16:32:41 SGT			
			E <u>x</u> it A	nalysis Settings Library			

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
calibrationEach 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

- 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.

File Edit Instrument	Analysis	Tools	Help						
New Experiment 🔹		ılysis Sett ılyze	tings		-	⊆lose	🆏 Import	- 纋	Create Slide.
Experiment Menu	Ove	Override Calibration 🕨			Use	Calibration From	Anothe	er File	
						Reve	ert To Original C	alibratio	n

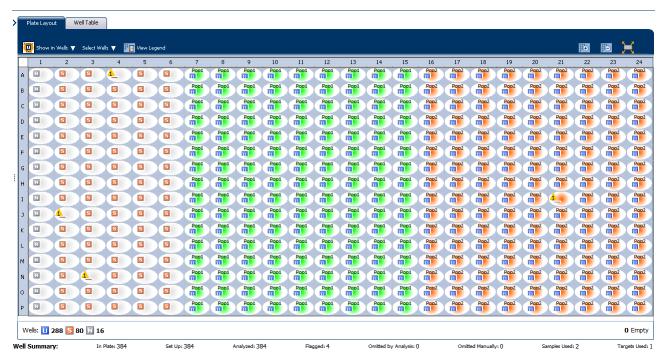
The experiment file will display analysis results as per the calibration data of the QuantStudio[™] 12K Flex Instrument that the experiment was run on.

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.

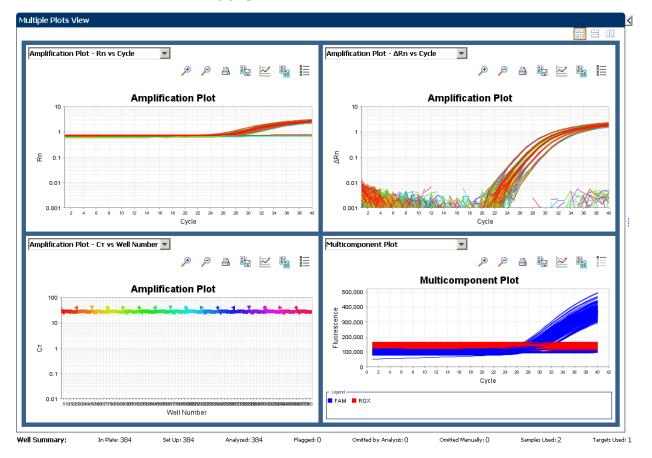
The plate layout for a Standard Curve experiment is shown in the following graphic:



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.
- 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

To edit plot properties

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

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 ion 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 currentYou can change the Plot Settings for the different analysis plots, and save them as
defaults.settings as defaultdefaults.

Select the **Save current settings as the default** check box on the respective plot screens under the Analysis Experiment Menu.

(Plot Settings
	Plot Type: д n vs Cycle 💌 Graph Type: Log 💌 Plot Color: Well 🔍
	Save current settings as the default

To publish the analyzed data

То	Click
Save a plot as an image file	Ú.
Print a plot	B
Copy a plot to the clipboard	1
Print a report	📇 Print Report
Export data	

То	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 QuantStudioTM 12K Flex Software to the external application, CopyCaller[®] Software if it is installed on your computer before the QuantStudioTM 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.

Preferences					×
Non-OpenArray	OpenArray® Block Run		Global Notification Sett	ings	Startup
Experiment	Print	Export	Display Format	SMTP	Settings
O Use Last File Location	1				
● Use Default Folder	ns\QuantStu	dio 12K Flex Softv	vare\User Files\experiment	s Bro	owse
			Restore Default	s App	ly
					OK Cancel

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:

oort File Location: C:\Applied Biosyste	ems\QuantStudio	12K Flex Software\U	Browse Export File	Name: 384-Well	Fast Standard C	urve Example_Qu	a File Type: 🍇 (*.	xls)
Sample Setup Raw Data Skip Empty Wells Skip Omitted Wi	Amplification		Results					
Select Content								
	Well	Well Position	Sample Name	Target Name	Task	Reporter	Quencher	•
All Fields	<u>^</u>	1 A1		RNaseP	NTC	FAM	NFQ-MGB	
		2 A2		RNaseP	STANDARD	FAM	NFQ-MGB	29
Vell Vell		3 A3		RNaseP	STANDARD	FAM	NFQ-MGB	28
Vel Position		4 A4		RNaseP	STANDARD	FAM	NFQ-MGB	2
		5 A5		RNaseP	STANDARD	FAM	NFQ-MGB	2
Sample Name		6 A6		RNaseP	STANDARD	FAM	NFQ-MGB	2
		7 A7	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Target Name		8 A8	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
✓ Task		9 A9	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
✓ T dSK		10 A10	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
 Reporter 		11 A11	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	20
		12 A12	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	20
Quencher		13 A13	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	20
	-	14 A14	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	27
🗹 СТ		15 A15	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Ct Mean		16 A16	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
CC Mean		17 A17	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Ct SD		18 A18	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
		19 A19	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	20
Quantity		20 A20	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
		21 A21	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Quantity Mean		22 A22	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Ouantity SD		23 A23	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
E gaanacy oo		24 A24	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Automatic Ct Threshold		25 B1		RNaseP	NTC	FAM	NFQ-MGB	
		26 B2		RNaseP	STANDARD	FAM	NFQ-MGB	2
Ct Threshold		27 B3		RNaseP	STANDARD	FAM	NFQ-MGB	28
Automatic Baseline		28 B4		RNaseP	STANDARD	FAM	NFQ-MGB	2
Mutomatic baseline		29 B5		RNaseP	STANDARD	FAM	NFQ-MGB	2
		30 B6		RNaseP	STANDARD	FAM	NFQ-MGB	25

Start Export Save Export Set As Load Export Set Delete Export Set

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

384-Well Fast Standard Curve Example_QuantStudio_export.tx	t - Notepad	
File Edit Format View Help		
Example.eds * Experiment Name = 384-well Fast Standard Curve Ex * Experiment Run End Time = 2011-08-06 13:15:47 PM * Experiment Type = Standard Curve * Instrument Name = QuantStudioDemo * Instrument Serial Number = QuantStudioDemo * Instrument Type = QuantStudio 12K Flex * Passive Reference = ROX Quantification Cycle Method = Ct * Stage/ Cycle where Analysis is performed = Stage * User Name = NA	58 AM SGT 31 AM SGT 30 AM SGT 01:56:21 AM SGT 38 AM SGT iosystems\QuantStudio12KFlex\examples\Standard Curve\384-Well Fast Standard Curve ample SGT	
[sample setup] Well Well Position Sample Name Sample Colo Quencher Quantity Comments	Biogroup Name Biogroup Color Target Name Target Color Task Repor	ter
Quencher Quantity Comments 1 A1 RNa 2 A1 RNa 3 A3 RNa 4 A4 RNa 5 A5 RNa 6 A6 RNa 7 A7 Pop1 "RGB(0,255,0)" 8 A8 Pop1 "RGB(0,255,0)" 10 A10 Pop1 "RGB(0,255,0)" 11 A11 Pop1 "RGB(0,255,0)" 12 A12 Pop1<"RGB(0,255,0)"	SEP "RGB(176,23,31)" STANDARD FAM NFQ-MGB "1,250.000" SEP "RGB(176,23,31)" STANDARD FAM NFQ-MGB "2,500.000" SEP "RGB(176,23,31)" STANDARD FAM NFQ-MGB "5,000.000" SEP "RGB(176,23,31)" STANDARD FAM NFQ-MGB "5,000.000" SEP "RGB(176,23,31)" STANDARD FAM NFQ-MGB "10,000.000"	×

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.

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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.

 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.
 Select File > Save As Template.
 Enter a file name, select a location for the template, then click Save and ² Close. You can use that experiment as a template for similar experiments.
 From the Home screen, click ² Create From Template.

experiment using a	2. Locate and select the template file, then click Open .
template	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

- **2.** Select a pre-existing template from the templates list on the View Templates screen.
- **3.** Touch **O View** to see the run profile before you start a run.
- **4.** After confirming the template setup is correct, touch **s** 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.
- Log in to the QuantStudio[™] 12K Flex Software and, from the Home screen, click Q 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.

2

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

🐌 Run 96/384/Array Cards	×
2 Load the reaction plate into the instrument. Select the instrument and enter the setup files, then click Start Run.	
Select Instrument	
Enter Experiment Name and Location * Experiment Name: 2011-12-08 175832 Barcode (Optional): Comments (Optional): User Name (Optional): Image: Comments (Optional):	
Select Experiment Template * Experiment Template File: Browse	
Samples	
Sample You may import a plate setup file or a sample definition text file. Alternatively, you may directly edit the sample names in the table to the left, or copy and paste sample names from a spreadsheet.	
Experiment Setup	

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

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

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 predefined experiment or folder buttons.

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

QuickStart from the QuantStudio[™] 12K Flex Instrument touchscreen

- 1. Touch **III** 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.

In	nport Plate Setup	×
	Select the plate setup file to import, then click Start Import .	0
	Select File:	Browse
		Start Import Cancel

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

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.

	A	В	С	D	E	F	G	Н
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		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

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

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.

2

Well Table 📃 🍾 Define and Set Up Sta Plate Lavout Targets -• Task Quantity 🛄 Show in Wells 🔻 Sel View Lege Name t Wells 🔻 Target 1 Sample: Custom Property: Sample 1 Lustom Pr ID: Age: Sex: Weight: HairColor: Smoker: 1 22 Samples --Female 25 Name black Yes Sample 1 Sample 2 Ŀ. Sample 2 Sample 3 Sample 4 Sample 5 Sample 6 Sample 7 Г Sample 8 Sample 9 Sample 10 Biological Groups • Wells: 🔟 0 <u>S</u> 0 <u>N</u> 0 364 Empty

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	Select	Ст	Group By ▼	 CT SD	Quantit	(Quant	ity Qua	ontity	Common	te I	D	Ac	10	Sex	Wo	ight	Expand All HairColor	Smoker
#	A1		CI	CIMea	CISD	Quantity	y Quant	ity Qua	anuty	commen	1		22		Female	25		black	Yes
2	A2										2		25		Male	26			No
3	AG										3		45		Female	50		blonde	Yes
4	A4										4		31		Male	33		red	Yes
5	A5										5		29		Female	46			No
6	A6										6		26		Male	35			No
7	A7										7		31		Female	33			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
	A14										14		35		Male	33		black	No
15	A15										15		36		Female	46		black	Yes
16	A16										16		36		Male	35			Yes
	A17										17		37		Female	33		blonde	No
	A18										18		37		Male	67			No
19	A19										19		38		Female	55		grey	Yes
	A20										20		38		Male	44		black	No
	A21																		
22	A22																		
	A23																		
	A24																		
	B1																		
26	B2																		
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20 29	B5																		
	B6																		
	B7																		
32	B8																		
	B9																		
	B10																		
	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:

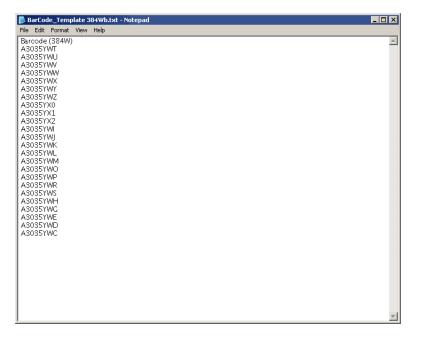
Batch Experiment S	etup Utility	
- For multi-well pla - For OpenArray®	elect the barcode, file naming convention, and export location; then click Create Experiments. te experiments, array card experiments, or experiments that use sample integration, select an *.edt file. experiments, select an *.edt file or the folder that contains *.spf or *.tpf files. additional input files (*.alf, *.txt).	•
1. Input Files		
* Experiment Te	mplate File (*.edt):	Browse
01		
* Setup File F	iolder (*.spf,*.tpf):	Browse
Assay Info	rmation File (*.aif):	Browse
Plate	Setup File (*.txt):	Browse
2. Barcode(s) and N	aming Convention	
Create Experiment	Files Using: O Barcode:	Browse
	• Specify number of files: 25	
File Name Format:	Attribute Include Custom Name Field:	
	Custom Name Field	
	Plate Barcode III Move Up File Name Preview: Custom Name Field_ID	
	Filename from SPF/TPF	
	Move Down	
3. Sample Files Fold		
	Browse 🔿 Match by Plate Barcode 💿 Match by ID	
Expected Sample	File Name: Custom Name Field_ID.csv Validate	
4. Export Location		
* Export setup file	s to:	Browse
	Create Experiments	Cancel
	Create Experiments	

- **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)).

2

- **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.

ReadiApp	
Select your Experiment:	
TaqMan® Array MicroRNA Cards	TaqMan® Gene Signature Array Cards
TaqMan® Copy Number Assays (CNV)	Custom TaqMan® Array Cards
TaqMan® SNP Genotyping Assays	TaqMan® Gene Expression Assays
	TaqMan® Drug Metabolism Assays
	Cancel

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



Booklet 2 - Running Standard Curve Experiments

Publication Part Number 4470050 Rev. A Revision Date March 2012



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About Standard Curve Experiments

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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 • 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

components

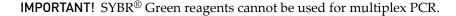
When performing real-time PCR, choose between:

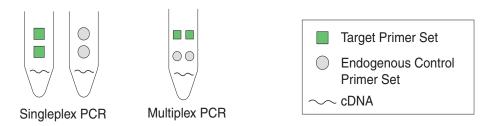
- Singleplex and multiplex PCR (page 6) *and*
- 1-step and 2-step RT-PCR (page 6)

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.





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 FAMTM 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).

Design the Experiment

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

This chapter covers:

2

Define the experiment properties.	. 9
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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*TM 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:

How do you want to identify this exp	periment?					
* Experiment Name: 384-Well Fast Standar	d Curve Example	Comments: Standard Curve example				
Barcode:						
User Name: Example User			<u>v</u>			
* Which block are you using to run t	he experiment?					
✓ 384-Well	Array Card	96-Well (0.2mL)	Fast 96-Well (0.1mL)			
* What type of experiment do you w	rant to set up?					
Standard Curve	Relative Standard Curve	Comparative CT (ΔΔCT)	Melt Curve			
Genotyping	Presence/Absence					
* Which reagents do you want to use	e to detect the target sequence?					
✓ TaqMan® Reagents	SYBR® Green Reagents	Other				
* What properties do you want for t	he instrument run?					
Standard	✓ Fast					

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

Targets				Samples			
New Save to Library Import from Library	Delete			New Save to Library	Import from Library	Delete	
Target Name	Reporter	Quencher	Color	Sample Name			Color
RNaseP	FAM 🗸	NFQ-MGB	* * *	Pop1			– •
				Pop2			<u> </u>
Biological Replicate Groups							
New Delete							
Biological Group Name Color		Comments					
Biological Group Name Color		Comments					
Biological Group Name Color		Comments					
Biological Group Name Color		Comments					
Biological Group Name Color		Comments					
Biological Group Name Color	1	Comments					
Biological Group Name Color	1	Comments					
Biological Group Name Color	E.	Comments					
Biological Group Name Color		Comments					
		Comments					
Biological Group Name Color Passive Reference Rox		Comments					

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:

Define and Set Up Standards	
Select a target from the list of targets in the reaction plate. Define the standard curve, select wells for the sta	andards, then click Apply. Repeat for each standard curve in the reaction plate, then click Close to return to plate setup.
Select a target	* = Required
Select the target for this standard curve: RNaseP 🗸	
Define the standard curve	= Required Standard Curve Preview (5 Recommended)
* # of Replicates: 16	1E4
* Starting Quantity: 1250.0	(Enter the highest or lowest standard quantity for the standard curve.) 5E3 2.5E3
* Serial Factor: 2×	▼ (Select a value from 1:10 to 10×.) 1.25E3
5 Points X 16 Replicates = 80 Re	aquired Wells
elect and arrange wells for the standards	
rrange standards in: 🔘 Columns 🛛 Nows	
se Wells: 🔿 Automatically Select Wells for Me 💿 Let Me Select Wells	
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 2	23 24 80 Required Wells / 80 Selected Wells
	A2,A3,A4,A5,A6,B2,B3,B4,B5,B6,C2,C3,C4,C5,C6,D2,D3,D4,D5,D6,E2,E3,E4,E5,E6,F2,F3,F4,F5,F6,G2,
	G3,G4,G5,G6,H2,H3,H4,H5,H6,I2,I3,I4,I5,I6,J2,J3,J4,J5,J6,K2,K3,K4,K5,K6,L2,L3,L4,L5,L6,M2,M3,M4, M5,M6,N2,N3,N4,N5,N6,O2,O3,O4,O5,O6,P2,P3,P4,P5,P6
	Apply Reset Clos

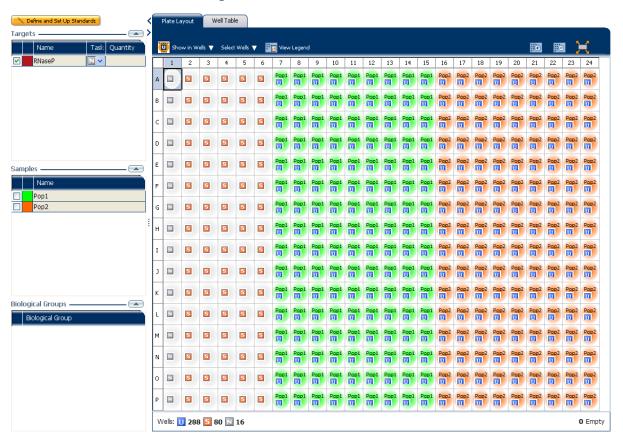
2. Assign targets and samples.

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

Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Standard Curve Experiments

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:



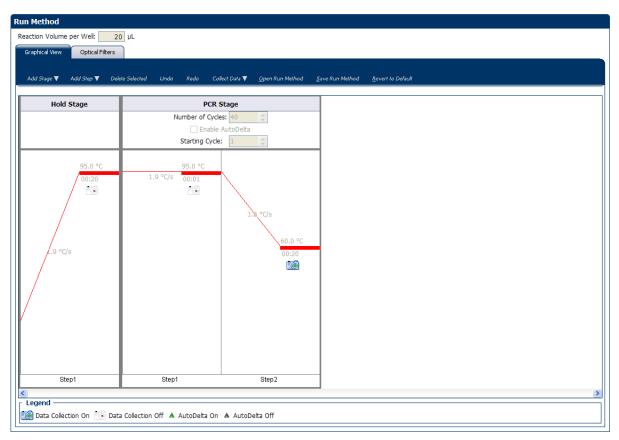
Set up the run method

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

Your Run Method screen should look like this:



2

For more information

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



Chapter 2 Design the Experiment *For more information*

Prepare the Reactions

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

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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/ μ L. After dilution, the sample Pop1 has a concentration of 6.6 ng/ μ L and Pop 2 has a concentration of 3.3 ng/ μ L. Add 2 μ L 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

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:

З

- **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.
- 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

The reaction plate for the Standard Curve example experiment contains:

A MicroAmp[®] Optical 384-Well Reaction Plate

reaction plate components

Example

experiment

- Reaction volume: 10 µL/well
- 288 Unknown wells U
- 80 Standard wells S
- 16 Negative Control wells

The plate layout looks like this:

F	Plate Lay	rout	Well Tab	le																				
Ĩ	🗾 Show	v in Wells '	🗸 Selec	t Wells 🔻) 	/iew Leger	nd															٠	o	X
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A		S	S	S	S	S	Pop1	Pop2	Pop2															
в	N	S	S	S	S	S	Pop1	Pop2	Pop2															
с	N	S	S	S	S	S	Pop1	Pop2	Pop2															
D	N	S	S	S	S	S	Pop1	Pop2	Pop2															
E	N	S	S	S	S	S	Pop1	Pop2	Pop2															
F	N	S	S	S	S	S	Pop1	Pop2	Pop2															
G	N	S	S	S	S	S	Pop1	Pop2	Pop2															
н	N	S	S	S	S	S	Pop1	Pop2	Pop2															
I	N	S	S	S	S	S	Pop1	Pop2	Pop2															
J	N	S	S	S	S	S	Pop1	Pop2	Pop2															
к	N	S	S	S	S	S	Pop1	Pop2	Pop2															
L	N	S	S	S	S	S	Pop1	Pop2	Pop2															
м	N	S	S	S	S	S	Pop1	Pop2	Pop2															
N	N	S	S	S	S	S	Pop1	Pop2	Pop2															
0	N	S	S	S	S	S	Pop1	Pop2	Pop2															
Р	N	S	S	S	S	S	Pop1	Pop2	Pop2															
W	ells: 🕕	288 💽	80 🛛	16																				0 Empty

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	рор1	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, <i>Getting Started with QuantStudio™ 12K</i> Flex System Multi-Well Plate and Array Card Experiments	4470050
Sealing the reaction plate	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 12K</i> Flex System Multi-Well Plate and Array Card Experiments	4470050

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

This chapter covers:

- 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

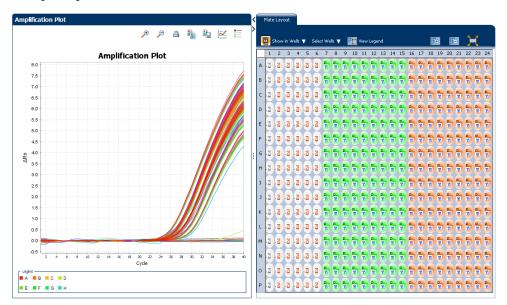
- **1.** In the Instrument Console screen, select the instrument icon.
- 2. Click Manage Instrument or double-click on the instrument icon.
- **3.** On the Manage Instrument screen, click **Monitor Running Experiment** to access the Run screen.

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.



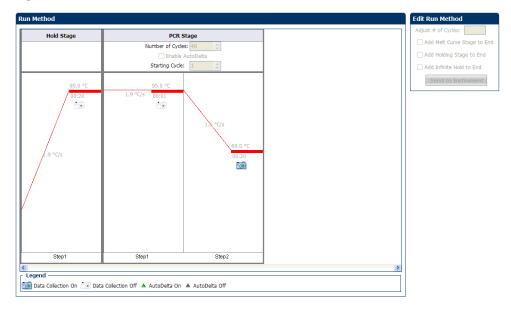
Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Standard Curve Experiments

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.

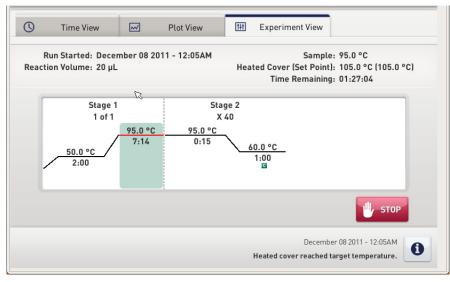
Run Data Report	
Experiment Name:	384-Well Fast Standard Curve Example
Start Time:	08-06-2011 12:33:30 SGT
Stop Time:	08-06-2011 13:15:47 SGT
Run Duration:	42 minutes 17 seconds
User Name:	DEFAULT
Instrument Name:	QuantStudioDemo
Firmware Version:	0.13.1
Software Version:	QuantStudio 12K Flex Software v1.0
Instrument Serial Number:	QuantStudioDemo
Sample Volume:	20.0
Cover Temperature:	105.0
Block Type:	384-Well Block
	<u> </u>
Errors Encountered:	
	×

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

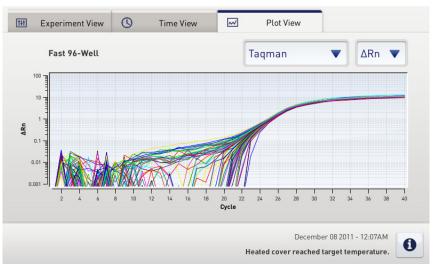


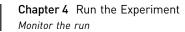
Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

Time View



Plot View



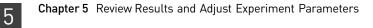


Review Results and Adjust Experiment Parameters

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:

Sect	tion 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
Sec	tion 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.
- 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
- R² value (correlation coefficient)
- C_T values

To view and assess the Standard Curve Plot

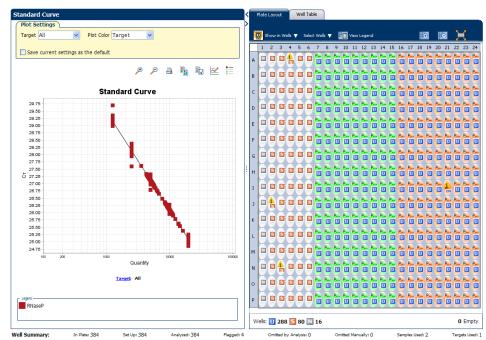
- From the Experiment Menu pane, select Analysis > Standard Curve.
 Note: If no data are displayed, click Analyze.
- **2.** Display all 384 wells in the Standard Curve Plot screen by clicking the upper left corner of the plate layout in the **Plate Layout** tab.
- **3.** Enter the Plot Settings:

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

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).

ihow i	n Table 🔻	Select Wells	▼ Group by	•									🗄 Expand All	🗉 Collap
¥	Well	Omit	Flag	Sample	. Target .	Task	Dyes	Ст	Ст Mean	CT SD	Quantity	Quantit	Quantit NOI	SE
		🗏 Pop1 - F	RNaseP - UNKN	NOWN										
7	A7			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.110	27.096	0.140	5,015.352	5,090.081	565.712	
8	A8			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,387.923	5,090.081	565.712	
9	A9			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,645.138	5,090.081	565.712	
10	A10			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,181.675	5,090.081	565.712	
11	A11			Pop1	RNaseP	UNKNOWN	FAM-NFQ	26.921	27.096	0.140	5,708.598	5,090.081	565.712	
12	A12			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,412.582	5,090.081	565.712	
13	A13			Pop1	RNaseP	UNKNOWN	FAM-NFQ	26.953	27.096	0.140	5,584.070	5,090.081	565.712	
14	A14			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.034	27.096	0.140	5,284.767	5,090.081	565.712	
15	A15			Pop1	RNaseP	UNKNOWN	FAM-NFQ	26.838	27.096	0.140	6,041.067	5,090.081	565.712	
31	B7			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.079	27.096	0.140	5,124.039	5,090.081	565.712	
32	B8			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.167	27.096	0.140	4,824.094	5,090.081	565.712	
33	B9			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.057	27.096	0.140	5,200.990	5,090.081	565.712	
34	B10			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.203	27.096	0.140	4,706.999	5,090.081	565.712	
35	B11			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.021	27.096	0.140	5,330.722	5,090.081	565.712	
36	B12			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.097	27.096	0.140	5,059.768	5,090.081	565.712	
37	B13			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.099	27.096	0.140	5,052.877	5,090.081	565.712	
38	B14			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.011	27.096	0.140	5,366.522	5,090.081	565.712	
39	B15			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.103	27.096	0.140	5,041.529	5,090.081	565.712	
55	C7			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.045	27.096	0.140	5,242.704	5,090.081	565.712	
56	C8			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.182	27.096	0.140	4,775.204	5,090.081	565.712	
57	C9			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.222	27.096	0.140	4,646.954	5,090.081	565.712	
58	C10			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.153	27.096	0.140	4,871.199	5,090.081	565.712	
59	C11			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.253	27.096	0.140	4,548.860	5,090.081	565.712	
60	C12			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.217	27.096	0.140	4,664.264	5,090.081	565.712	
61	C13			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.228	27.096	0.140	4,628.641	5,090.081	565.712	
62	C14			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.272	27.096	0.140	4,492.085	5,090.081	565.712	
63	C15			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.329	27.096	0.140	4,320.416	5,090.081	565.712	
79	D7			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.036	27.096	0.140	5,277.137	5,090.081	565.712	
80	D8			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.101	27.096	0.140	5,048.890	5,090.081	565.712	
81	D9			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.142	27.096	0.140	4,909.571	5,090.081	565.712	
82	D10			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.198	27.096	0.140	4,722.698	5,090.081	565.712	
83	D11			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,146.530	5,090.081	565.712	
84	D12			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.266	27.096	0.140	4,508.289	5,090.081	565.712	
	D13			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27,096	0.140	5,651,525	5,090,081	565.712	

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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 (10⁵ to 10⁶ 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.
- **R² values (correlation coefficient)** The R² 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 R² 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

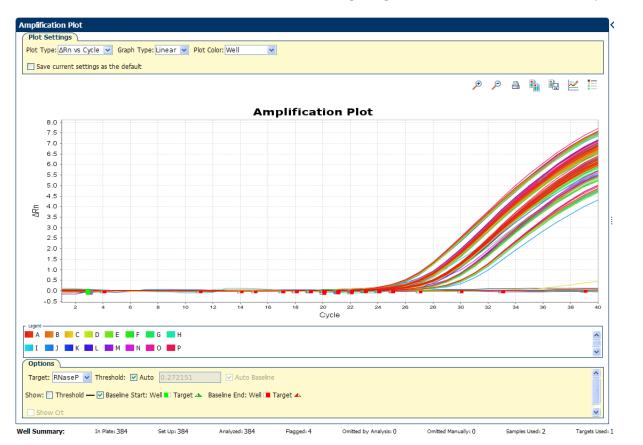
>	Plate	Layout		Well Tab	ble																				
ĕ	😳 Show in Wells 🔻 Select Wells 🔻 🗰 View Legend 😥 👔																								
	1		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A				5	4	5	5	Pop1	Pop1	Pop1	Pop1	Pop1	(Pop1	Pop1	(Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
в				5	5	8	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
с				5	5	5	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
D				5	5	5	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
Е				5	5	8	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
F				5	5	5	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
G				8	5	8	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
: н				5	5	S	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
I				8	8	8	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	1. 	Pop2	Pop2	Pop2
3		1		5	5	5	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
к				8	8	8	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
L				8	8	5	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
м			i)	5	8	5	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
N				4	8	8	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
0				5	5	S	5	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
Р				8	5	B	8	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop1	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2	Pop2
v	Volk		0	80 N	16																				0 Empty
		mary:	0		Plate: 384	1	Set Up	: 384	A	nalyzed: 3	84	Flag	ged: 4	(Omitted by	y Analysis:	0	Omitt	ed Manua	lly: O	Sa	mples Use	d: 2		gets Used: 1

3. In the Amplification Plot screen, enter:

Menu	Select
Plot Type	Δ Rn vs Cycle
Plot Color	Well (default)

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

- **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.



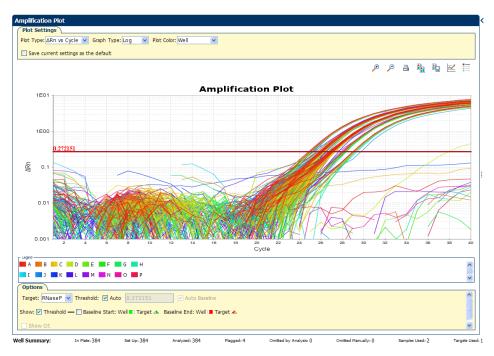
5. View the threshold values.

Menu	Select
Graph Type	Log
Target	RNaseP

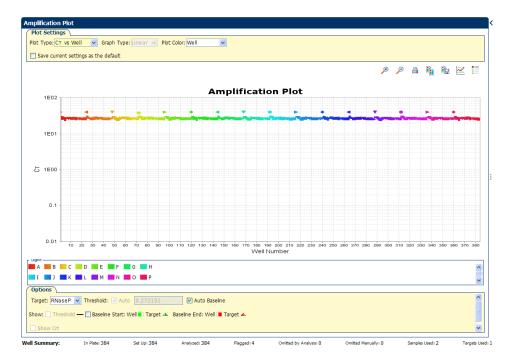
a. Select the Threshold check box to show the threshold.

35

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.



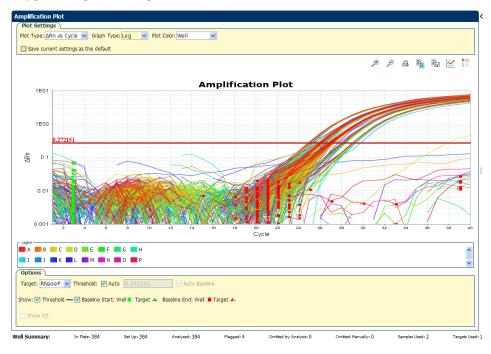
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Tips for analyzing your own experiments

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

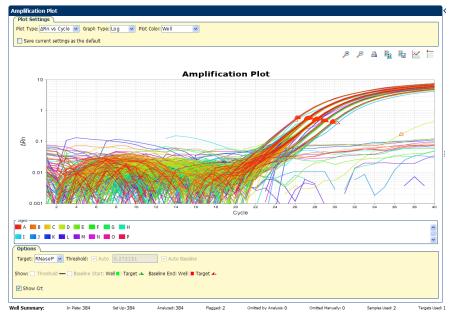
- Outliers
- A typical amplification plot The QuantStudio[™] 12K Flex Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
 - Plateau phase
 - Linear phase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

A typical amplification plot should look like this:

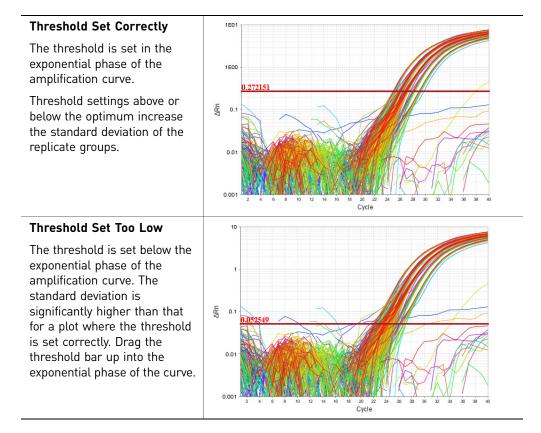


IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudio[™] 12K Flex Software. Life Technologies recommends that you examine the Amplification Plot screen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



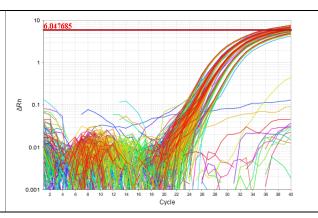
• Correct threshold values:



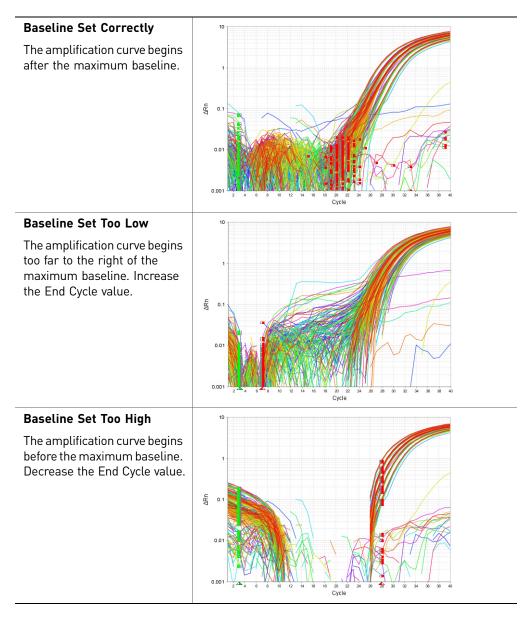
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Threshold Set Too High

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:



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

b

The purpose of viewing the well table is to identify:

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

View the well table1. 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.

±	Well	Select Wells	Target Name		at	Task	Dune	Ст	Ст Mean	(= CD	Ouantity	Ouantit			C
			Sample Name				Dyes			CT SD	Quantity	Quantit	Quantit	NUISE	
1	A1		Task		P	NTC	FAM-NFQ								
2	A2		Replicate		2	STANDARD	FAM-NFQ		29.140	0.178	1,250.000				
3	A3				P	STANDARD	FAM-NFQ		28.113	0.181	2,500.000				
-	A5 A6		Dye		P	STANDARD	FAM-NFQ		26.132		10,000.000				
6			Flag		P	STANDARD	FAM-NFQ		25.056	0.098		E 000 001	565 710		
7	A7		Ст		P	UNKNOWN	FAM-NFQ		27.096	0.140	5,015.352	5,090.081			
8 9	A8 A9		NOISE		P	UNKNOWN	FAM-NFQ		27.096	0.140	5,387.923	5,090.081			
					P	UNKNOWN	FAM-NFQ		27.096	0.140	5,645.138	5,090.081			
10	A10		Well Position (Rov	· ·	P D	UNKNOWN	FAM-NFQ		27.096	0.140	5,181.675	5,090.081			
11	A11		Well Position (Colu	umn)	P	UNKNOWN	FAM-NFQ		27.096	0.140	5,708.598	5,090.081			
12	A12 A13		✓ None		r	UNKNOWN	FAM-NFQ		27.096	0.140	5,412.582	5,090.081			
13						UNKNOWN	FAM-NFQ		27.096	0.140	5,584.070	5,090.081			
14	A14		Pop1	RNase		UNKNOWN	FAM-NFQ		27.096	0.140	5,284.767	5,090.081			
15	A15		Pop1	RNase		UNKNOWN	FAM-NFQ		27.096	0.140	6,041.067	5,090.081			
16 17	A16 A17		Pop2	RNase RNase		UNKNOWN	FAM-NFQ		26.101		10,849.413				
18	A17 A18		Pop2 Pop2	RNase		UNKNOWN	FAM-NFQ FAM-NFQ		26.101 26.101	0.159	11,484.034	10,053.434			
10	A10 A19		Pop2 Pop2	RNase		UNKNOWN	FAM-NFQ		26.101	0.159		10,053.434			
20	A19 A20		Pop2 Pop2	RNase		UNKNOWN	FAM-NFO		26.101	0.159	10,997.522				
20	A20		Pop2 Pop2	RNase		UNKNOWN	FAM-NFQ		26.101	0.159					
21	A21 A22		Pop2 Pop2	RNase		UNKNOWN	FAM-NFQ		26.101	0.159		10,053.434			
22	A23		Pop2 Pop2	RNase		UNKNOWN	FAM-NFQ		26.101	0.159	10,793.909				
23	A23		Pop2 Pop2	RNase			FAM-NFO		26.101	0.159					
25	B1		POPZ	RNase		NTC	FAM-NFQ			0.139	10,000.025	10,000.404	1,142.032		
25	B2			RNase		STANDARD	FAM-NFQ		. 29.140	0.178	1,250.000				
20	82 83			RNase		STANDARD	FAM-NFQ		29.140	0.178	2,500.000				
28	B4			RNase		STANDARD	FAM-NFQ		20.113	0.131	5,000.000				
29	B5			RNase		STANDARD	FAM-NFQ		26.132		10,000.000				
30	B6			RNase		STANDARD	FAM-NFQ		25.056	0.098					
31	B7	H	Pop1	RNase		UNKNOWN	FAM-NFQ		27.096	0.140	5,124.039	5,090.081	565.712		
32	B8		Pop1	RNase		UNKNOWN	FAM-NFQ		27.096	0.140	4,824.094	5,090.081			
33	B9	H	Pop1	RNase		UNKNOWN	FAM-NFQ		27.096	0.140	5,200.990	5,090.081			
34	B10		Pop1	RNase		UNKNOWN	FAM-NFQ		27.096	0.140	4,706.999	5.090.081			
35	B11		Pop1	RNase		UNKNOWN	FAM-NFQ		27.096	0.140	5,330.722	5,090.081	565.712		
	B12		Pop1	RNase			FAM-NFQ		27.096	0.140	5,059.768	5,090.081			
		<							2.1050		,	.,			

The well table looks like this:

ihew i	n Table 🔻	Select Wells	Group by '	•									🖲 Expand	Al E	Collaps
¢	Well	Omit	Flag	Sample	Target	. Task	Dyes	Ст	CT Mean	CT SD	Quantity	Quantit	Quantit	OISE	C
_			NaseP - UNKN												
7	A7			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,015.352	5,090.081	565.712		
8	A8			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,387.923	5,090.081	565.712		
9	A9			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,645.138	5,090.081	565.712		
10	A10			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,181.675	5,090.081	565.712		
11	A11			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,708.598	5,090.081	565.712		
12	A12			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,412.582	5,090.081	565.712		
13	A13			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,584.070	5,090.081	565.712		
14	A14			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,284.767	5,090.081	565.712		
15	A15			Pop1	RNaseP	UNKNOWN	FAM-NFQ	26.838	27.096	0.140	6,041.067	5,090.081	565.712		
31	B7			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,124.039	5,090.081	565.712		
32	B8			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,824.094	5,090.081	565.712		
33	B9			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.057	27.096	0.140	5,200.990	5,090.081	565.712		
34	B10			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.203	27.096	0.140	4,706.999	5,090.081	565.712		
35	B11			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.021	27.096	0.140	5,330.722	5,090.081	565.712		
36	B12			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.097	27.096	0.140	5,059.768	5,090.081	565.712		
37	B13			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.099	27.096	0.140	5,052.877	5,090.081	565.712		
38	B14			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.011	27.096	0.140	5,366.522	5,090.081	565.712		
39	B15			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.103	27.096	0.140	5,041.529	5,090.081	565.712		
55	C7			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.045	27.096	0.140	5,242.704	5,090.081	565.712		
56	C8			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.182	27.096	0.140	4,775.204	5,090.081	565.712		
57	C9			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.222	27.096	0.140	4,646.954	5,090.081	565.712		
58	C10			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.153	27.096	0.140	4,871.199	5,090.081	565.712		
59	C11			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.253	27.096	0.140	4,548.860	5,090.081	565.712		
60	C12			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.217	27.096	0.140	4,664.264	5,090.081	565.712		
61	C13			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.228	27.096	0.140	4,628.641	5,090.081	565.712		
62	C14			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.272	27.096	0.140	4,492.085	5,090.081	565.712		
63	C15			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.329	27.096	0.140	4,320.416	5,090.081	565.712		
79	D7			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.036	27.096	0.140	5,277.137	5,090.081	565.712		
80	D8			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.101	27.096	0.140	5,048.890	5,090.081	565.712		
81	D9			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.142	27.096	0.140	4,909.571	5,090.081	565.712		
82	D10			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.198	27.096	0.140	4,722.698	5,090.081	565.712		
83	D11			Pop1	RNaseP	UNKNOWN	FAM-NFQ	27.073	27.096	0.140	5,146.530	5,090.081	565.712		
84	D12			Pop1	RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,508.289	5,090.081	565.712		
85	D13			Pop1	RNaseP	UNKNOWN	-		27.096	0.140	5,651.525		565,712		

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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.

ihow ii	n Table 🔻	Select Wells 🔻	Gro										🗄 Expand A	🖬 Col	apse
¥	Well	Omit		Target Name Sample Name	e	t Task	Dyes	Ст	Ст Mean	CT SD	Quantity	Quantit	Quantit No	NSE	OU
		💻 Pop1 - RNa		Task											
7	A7				Ρ	UNKNOWN	FAM-NFQ		27.096	0.140	5,015.352	5,090.081			
8	A8		<u> </u>	Replicate	P	UNKNOWN	FAM-NFQ		27.096	0.140	5,387.923	5,090.081	565.712		
9	A9			Dye	Ρ	UNKNOWN	FAM-NFQ		27.096	0.140	5,645.138	5,090.081			
10	A10			Flag	N.	UNKNOWN	FAM-NFQ		27.096	0.140	5,181.675	5,090.081			
11	A11			Ст	- T	UNKNOWN	FAM-NFQ		27.096	0.140	5,708.598	5,090.081	565.712		
12	A12			NOISE	P	UNKNOWN	FAM-NFQ		27.096	0.140	5,412.582	5,090.081	565.712		
13	A13				Ρ	UNKNOWN	FAM-NFQ		27.096	0.140	5,584.070	5,090.081	565.712		
14	A14			Well Position (Row)	P	UNKNOWN	FAM-NFQ		27.096	0.140	5,284.767	5,090.081	565.712		
15	A15			Well Position (Column)	Ρ	UNKNOWN	FAM-NFQ		27.096	0.140	6,041.067	5,090.081	565.712		
31	B7			None	P	UNKNOWN	FAM-NFQ		27.096	0.140	5,124.039	5,090.081	565.712		
32	B8				P	UNKNOWN	FAM-NFQ		27.096	0.140	4,824.094	5,090.081	565.712		
33	B9				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,200.990	5,090.081	565.712		
34	B10				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,706.999	5,090.081	565.712		
35	B11				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,330.722	5,090.081	565.712		
36	B12				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,059.768	5,090.081	565.712		
37	B13				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,052.877	5,090.081			
38	B14				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,366.522	5,090.081	565.712		
39	B15				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,041.529	5,090.081			
55	C7				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,242.704	5,090.081	565.712		
56	C8				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,775.204	5,090.081			
57	C9				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,646.954	5,090.081	565.712		
58	C10				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,871.199	5,090.081			
59	C11			Pop1 RM	laseP	UNKNOWN	FAM-NFQ	27.253	27.096	0.140	4,548.860	5,090.081	565.712		
60	C12				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,664.264	5,090.081	565.712		
61	C13			Pop1 RM	laseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,628.641	5,090.081	565.712		
62	C14				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,492.085	5,090.081	565.712		
63	C15				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,320.416	5,090.081	565.712		
79	D7			Pop1 RM	laseP	UNKNOWN	FAM-NFQ	27.036	27.096	0.140	5,277.137	5,090.081	565.712		
80	D8				laseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,048.890	5,090.081	565.712		
81	D9			Pop1 RM	laseP	UNKNOWN	FAM-NFQ	27.142	27.096	0.140	4,909.571	5,090.081	565.712		
82	D10			Pop1 RM	laseP	UNKNOWN	FAM-NFQ	27.198	27.096	0.140	4,722.698	5,090.081	565.712		
83	D11			Pop1 RM	laseP	UNKNOWN	FAM-NFQ	27.073	27.096	0.140	5,146.530	5,090.081	565.712		
84	D12			Pop1 RM	laseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,508.289	5,090.081	565.712		
85	D13			Pop1 RM	laseP	UNKNOWN	FAM-NFQ	26.935	27.096	0.140	5,651.525	5,090.081	565.712		

To group by C_T value

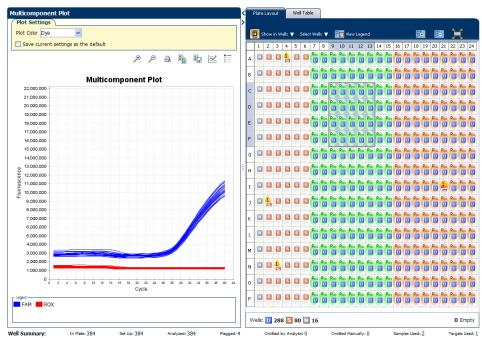
From the Group By drop-down menu, select C_T . The software groups the wells by C_T value: low, medium, high, and undetermined. In the example experiment, the C_T values are within the expected range (>8 and <35).

>	Plate	e Layout	Well Table												
													🗉 Expand A		
	Show	in Table 🗸	Select Wells 🔻										Expand A	di 🛛 🖃 Collapse All	
	#	Well	Omit	Target Na	me e	t Task	Dyes	Ст	CT Mean	CT SD	Quantity	Quantit	Quantit N	OISE OU	1
	-	Wein	Low (Cr les	Sample Na	me 🎽	C.I.I. TUSK	Dyes	CI	er mean	CT 3D	Quantity	Quarteren	Quantata. N		a
			E Medium (Cr	Task										i i i	
	2	A2		Replicate	p	STANDARD	FAM-NFO	20.012	29.140	0.178	1,250.000				
	3	A3		Dye	p	STANDARD	FAM-NFO		28,113	0.181	2,500.000				
	4	A4			P	STANDARD	FAM-NFO		27.133	0.130	5,000.000				2
	5	AS		Flag		STANDARD	FAM-NFQ		26.132		10,000.000				
	6	AG		🗸 Ст	NP	STANDARD	FAM-NFO		25.056		20,000.000				
	7	AZ		NOISE	1/5	UNKNOWN	FAM-NFQ		27.096	0.140	5,015.352	5,090.081	565,712		
	8	A8		Well Positio	on (Row)	UNKNOWN	FAM-NFQ		27.096	0.140	5,387.923	5,090.081			
	9	A9			on (Column)	UNKNOWN	FAM-NFQ		27.096	0.140	5,645.138	5,090.081			
	10	A10			pri (column)	UNKNOWN	FAM-NFQ		27.096	0.140	5,181.675	5,090.081			
	11	A11		None	p	UNKNOWN	FAM-NFQ		27.096	0.140	5,708.598	5,090.081	565.712		
	12			Po	p1 RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,412.582	5,090.081			
	13		i i	Po		UNKNOWN	FAM-NFQ		27.096	0.140	5,584.070	5,090.081			
	14	A14		Po		UNKNOWN	FAM-NFQ		27.096	0.140	5,284.767	5,090,081			
	15	A15		Po	p1 RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	6,041.067	5,090.081	565.712		
	16	A16		Po	p2 RNaseP	UNKNOWN	FAM-NFQ	25.981	26.101	0.159	10,849.413	10,053.434	1,142.832		
	17	A17		Po	p2 RNaseP	UNKNOWN	FAM-NFQ	25.897	26.101	0.159	11,484.034	10,053,434	1,142.832		
	18	A18		Po	p2 RNaseP	UNKNOWN	FAM-NFQ		26.101	0.159	9,891.872	10,053.434	1,142.832		
	19	A19		Po	p2 RNaseP	UNKNOWN	FAM-NFQ		26.101	0.159	9,519.561	10,053.434	1,142.832		
	20	A20		Po	p2 RNaseP	UNKNOWN	FAM-NFQ	25.961	26.101	0.159	10,997.522	10,053.434	1,142.832		
	21	A21		Po	p2 RNaseP	UNKNOWN	FAM-NFQ	25.788	26.101	0.159	12,373.389	10,053.434	1,142.832		
	22	A22		Po	p2 RNaseP	UNKNOWN	FAM-NFQ	26.171	26.101	0.159	9,523.480	10,053.434	1,142.832		
	23	A23		Po	p2 RNaseP	UNKNOWN	FAM-NFQ	25.988	26.101	0.159	10,793.909	10,053.434	1,142.832		
	24	A24		Po	p2 RNaseP	UNKNOWN	FAM-NFQ	26.099	26.101	0.159	10,008.625	10,053.434	1,142.832		
	26	B2			RNaseP	STANDARD	FAM-NFQ	28.981	29.140	0.178	1,250.000				
	27	B3			RNaseP	STANDARD	FAM-NFQ	28.022	28.113	0.181	2,500.000				
	28	B4			RNaseP	STANDARD	FAM-NFQ	27.076	27.133	0.130	5,000.000				
	29	B5			RNaseP	STANDARD	FAM-NFQ	25.960	26.132	0.100	10,000.000				
	30	B6			RNaseP	STANDARD	FAM-NFQ	25.018	25.056	0.098	20,000.000				
	31	B7		Po	p1 RNaseP	UNKNOWN	FAM-NFQ	27.079	27.096	0.140	5,124.039	5,090.081	565.712		
	32	B8		Po	p1 RNaseP	UNKNOWN	FAM-NFQ	27.167	27.096	0.140	4,824.094	5,090.081	565.712		
	33	B9		Po	p1 RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	5,200.990	5,090.081	565.712		
	34			Po	p1 RNaseP	UNKNOWN	FAM-NFQ		27.096	0.140	4,706.999	5,090.081	565.712		
	35	B11		Po	p1 RNaseP	UNKNOWN	FAM-NFQ	27.021	27.096	0.140	5,330.722	5,090.081	565.712		
			<											>	1
w	ell Su	mmary:	In Plate:	384	5et Up: 384	Analyzed: 384	Flagged: 4	4	Omitted by Analysis:	0 0	mitted Manually:	0 Si	amples Used: 2	Targets Used:	1

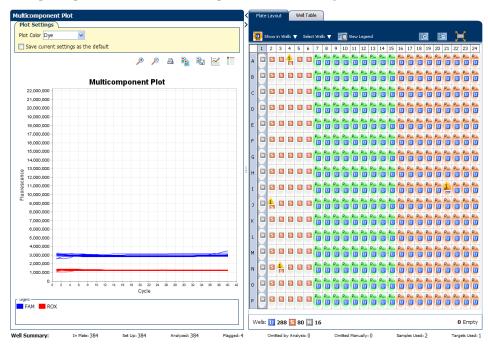
Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Standard Curve Experiments

Tips for analyzing Replicate – The software groups the wells by replicate: negative controls, standards, and samples. Look in the Quantity columns to make sure the quantity your own values for each replicate group are similar indicating tight C_T precision. experiments • 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: • ROXTM dye (passive reference) • FAMTM dye (reporter) Spikes, dips, and/or sudden changes Amplification in the negative control wells View the 1. From the Experiment Menu pane, select **Analysis** • **Multicomponent Plot**. Multicomponent Note: If no data are displayed, click Analyze. Plot 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

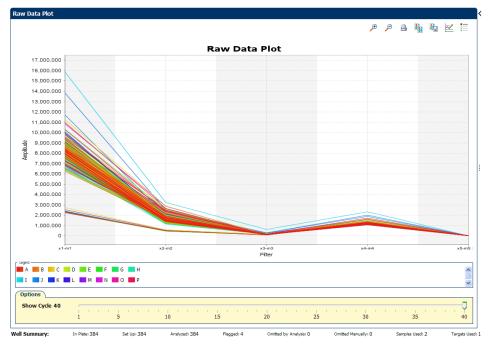
When you analyze your own standard curve experiment, look for:

- **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	 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:

R Filt							
				Load Save Revert to	Defaults		
				Emission Filter			
		m1(520±15)	m2(558±11)	m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
	x1(470±15)	×					
	x2(520±10)						
	x3(550±11)			V			
	x4(580±10)				V		
	x5(640±10)					V	
	X3(040±10)						
lt Cu	x6(662±10) x6(662±10)						
lt Cu	x6(662±10)			Load Save Revert to	Defaultz		
lt Cu	x6(662±10)			Emission Filter			
t Cu	x6(662±10) arve Filter ———	m1(520±15)	m2(558±11)	Emission Filter m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
t Cu	x6(662±10)	m1(520±15)	m2(558±11)	Emission Filter			
	x6(662±10) arve Filter ———			Emission Filter m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
	x6(662±10) urve Filter x1(470±15)			Emission Filter m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
t Cu	x6(662±10) rve Filter x1(470±15) x2(520±10)			Emission Filter m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
	x6(662±10) rve Filter x1(470±15) x2(520±10) x3(550±11)			Emission Filter m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)

Tips for determining signal accuracy in your own experiments

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

- Characteristic signal growth
- No abrupt changes or dips

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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.

lag 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	0		
NOAMP	No amplification	0		
IOISE	Noise higher than others in plate	1	I21	
SPIKE	Noise spikes	0		
NOSIGNAL	No signal in well	0		
DUTLIERRG	Outlier in replicate group	3	A4, J2, N3	
EXPFAIL	Exponential algorithm failed	0		
BLFAIL	Baseline algorithm failed	0		
THOLDFAIL	Thresholding algorithm failed	0		
CTFAIL	CT algorithm failed	0		
AMPSCORE	AMP Score	0		

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
F	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
Se	econdary 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, <i>Getting Started with QuantStudio™ 12K Flex</i> 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

1. From the Experiment Menu pane, select Analysis.

settings

- 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:

nalysis Settings f	for 384-Well Fast Standard	Curve Example		
т Settings Fla	g Settings Advanced Settings	Standard Curve Settin	ngs	
	ault settings for analysis of targets the change the settings.	in this experiment. To use difl	ferent settings for a target, se	elect the target from the table, deselect Use Default
analysis have been Stage 2, Step 2 - Default Cr Set	d stage to use for CT analysis. Only collected are displayed. 2 v tings			Algorithm Settings Select the algorithm to calculate Cr. Baseline Threshold v
-	: are used to calculate the Cτ for ta Ο Baseline Start Cycle: AUTO	-		-
- Select a Target	t			Ст Settings for RNaseP
Target	Threshold	Baseline Start	Baseline End	CT Settings to Use: Default Settings
RNaseP	0.272151	3	28	Automatic Threshold
				Threshold: 0.272151
				Automatic Baseline
				Baseline Start Cycle: 3 2 End Cycle: 28 2
				<u> </u>
ve to Library	Load from Library		Revert to Default An	alysis Settings Apply Analysis Settings Ca

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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.

Flag	Description	Use	Attribute	Condition	Value	Reject Well
MPNC	Amplification in negat	. 💌	Ст	< 🗸	35.000	
ADROX	Bad passive referenc	V	Bad passive referenc	> •	0.600	
FFSCALE	Fluorescence is offscale					
IGHSD	High standard deviati		CT standard deviation	> •	0.500	
IOAMP	No amplification	V	Amplification algorith	< 🗸	0.100	
IOISE	Noise higher than ot	V	Relative noise	> •	4.000	
SPIKE	Noise spikes	v	Spike algorithm result	> •	1.000	
IOSIGNAL	No signal in well	V				
OUTLIERRG	Outlier in replicate gr					
EXPFAIL	Exponential algorithm.	. 🗸				
BLFAIL	Baseline algorithm faile					
THOLDFAIL	Thresholding algorith					
CTFAIL	CT algorithm failed	~				
AMPSCORE	AMP Score		AMP Score	> •	1.000	

The Flag Settings tab looks like this:

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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:

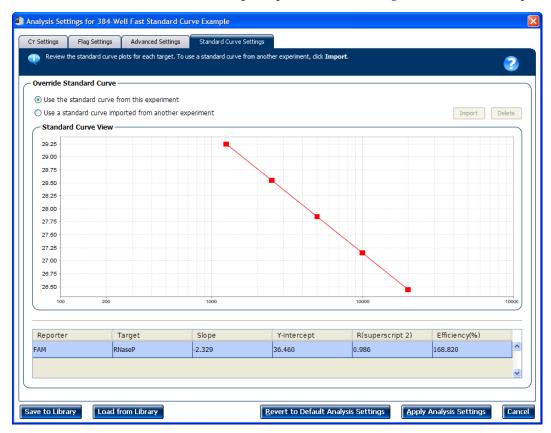
- 1. Select one or more well-target combinations in the table.
- 2. Deselect the Use C_T Settings Defined for Target check box.
- **3.** Define the custom baseline settings:
 - For automatic baseline calculations, select the **Automatic Baseline** check box.
 - To define the baseline, deselect the **Automatic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

- 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	Ст	Ст Mean	CT SD	Quantity	Quantit	Quantit	NOISE	υο
366 P6	i i	, lug		RNaseP		FAM-NFQ		25.056		20,000.000	quantitati	quantien	110102	
300 P0		- STANDARD -		UNDOCF	STANDARD	1 APPAR Q	24.001	25.050	0.090	20,000.000				
3 A3		o minorino		RNaseP	STANDARD	FAM-NFO	28,102	28.113	0.181	2,500.000				
27 B3				RNaseP	STANDARD	FAM-NFO		28.113	0.181	2,500.000				
51 C3				RNaseP	STANDARD	FAM-NFQ		28.113	0.181	2,500.000				
75 D3				RNaseP	STANDARD	FAM-NFQ		28.113	0.181					
99 E3				RNaseP	STANDARD	FAM-NFQ		28.113	0.181					
123 F3				RNaseP		FAM-NFQ		28.113	0.181					
147 G3				RNaseP	STANDARD	FAM-NFQ		28.113	0.181					
171 H3				RNaseP	STANDARD	FAM-NFQ		28.113	0.181					
195 I3				RNaseP	STANDARD	FAM-NFQ		28.113	0.181					
219 J3				RNaseP	STANDARD	FAM-NFO		28.113	0.181					
243 K3				RNaseP		FAM-NFQ		28.113	0.181					
267 L3				RNaseP		FAM-NFQ		28.113	0.181					
207 LD 291 M3				RNaseP	STANDARD	FAM-NFQ		28.113	0.181	· · · · · · · · · · · · · · · · · · ·				
315 N3		1		RNaseP	STANDARD	FAM-NFQ		28.113	0.181					
339 03		-		RNaseP	STANDARD	FAM-NFQ		28.113	0.181	2,500.000				
363 P3				RNaseP	STANDARD	FAM-NFQ		28.113	0.181					
	RNaseP	- STANDARD -		andocr	STANDARD	1714111 Q	20.101	20.115	0.101	2,500.000				
4 A4		1		RNaseP	STANDARD	FAM-NFQ	26,788	27.133	0.130	5,000.000				
28 B4		-		RNaseP	STANDARD	FAM-NFO		27.133	0.130					
52 C4				RNaseP	STANDARD	FAM-NFQ		27.133	0.130					
76 D4				RNaseP	STANDARD	FAM-NFQ		27.133	0.130					
100 E4				RNaseP		FAM-NFQ		27.133	0.130					
124 F4				RNaseP		FAM-NFQ		27.133	0.130					
148 G4				RNaseP	STANDARD	FAM-NFQ		27.133	0.130					
172 H4				RNaseP	STANDARD	FAM-NFQ		27.133	0.130					
6 I4				RNaseP	STANDARD	FAM-NFQ		27.133	0.130					
0 34				RNaseP	STANDARD	FAM-NFQ		27.133	0.130					
4 K4				RNaseP		FAM-NFQ		27.133	0.130					
8 L4				RNaseP	STANDARD	FAM-NFQ		27.133	0.130					
2 M4				RNaseP	STANDARD	FAM-NFQ		27.133	0.130					
6 N4				RNaseP	STANDARD	FAM-NFQ		27.133	0.130					
0 04				RNaseP	STANDARD	FAM-NFQ		27.133	0.130					
4 P4				RNaseP		FAM-NFQ		27.133	0.130					

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**.

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For more information

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

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	<pre><drive>:\Applied Biosystems\QuantStudio 12K Flex Software\User Files\experiments</drive></pre>

Your Export screen should look like this:

Auto Export Forr	mat : QuantSt	LUUIDIZKFIEX	~	Export Data 10	o: 💿 One File 🔘 Se	sparace riles	[♥] Open n	le(s) when export is	compi
ort File Location: C:\Applied E	3iosystems∖Q	uantStudio 12k	CFlex Software\U	Browse Export File	Name: 384-Well	Fast Standard C	urve Example_Qu	a File Type: 📋 (*.t	txt)
Sample Setup 🗌 Raw Dat	:a 🚺 🗹 Amp	lification	Multicomponent	Results					
Skip Empty Wells 🗹 Skip Om	itted Wells								
Select Content		Well	Well Position	Sample Name	Target Name	Task	Reporter	Quencher	
All Fields	~		1 A1		RNaseP	NTC	EAM	NFO-MGB	
			2 A2		RNaseP	STANDARD	FAM	NFQ-MGB	2
Vel Vel			3 A3		RNaseP	STANDARD	FAM	NFO-MGB	2
Well Position			4 A4		RNaseP	STANDARD	FAM	NFQ-MGB	2
Veli Posicion			5 A5		RNaseP	STANDARD	FAM	NFQ-MGB	2
Sample Name			6 A6		RNaseP	STANDARD	FAM	NFQ-MGB	2
			7 A7	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Target Name			8 A8	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Task			9 A9	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Task			10 A10	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Reporter			11 A11	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
			12 A12	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
2 Quencher	=		13 A13	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
СТ			14 A14	Pop1	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
			15 A15 16 A16	Pop1 Pop2	RNaseP RNaseP	UNKNOWN	FAM FAM	NFQ-MGB NFO-MGB	2
Ct Mean			17 A17	Pop2 Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	4
7			18 A18	Pop2 Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Ct SD			19 A19	Pop2	RNaseP	UNKNOWN	EAM	NFQ-MGB	2
Quantity			20 A20	Pop2	RNaseP	UNKNOWN	EAM	NFO-MGB	
Quanticy			21 A21	Pop2	RNaseP	UNKNOWN	FAM	NFO-MGB	
Quantity Mean			22 A22	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	
			23 A23	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Quantity SD			24 A24	Pop2	RNaseP	UNKNOWN	FAM	NFQ-MGB	2
Automatic Ct Threshold			25 B1		RNaseP	NTC	FAM	NFQ-MGB	
			26 B2		RNaseP	STANDARD	FAM	NFQ-MGB	2
Ct Threshold			27 B3		RNaseP	STANDARD	FAM	NFQ-MGB	2
			28 B4		RNaseP	STANDARD	FAM	NFQ-MGB	2
Automatic Baseline			29 B5 30 B6		RNaseP	STANDARD	FAM FAM	NFQ-MGB NFO-MGB	2
					RNaseP	STANDARD			

Start Export Save Export Set As Load Export Set Delete Export Set

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

384-Well Fast Standard Curve Example_QuantStudio_export.txt - Not		
File Edit Format View Help		
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 Serial Number = QuantStudioDemo * Instrument Serial Number = QuantStudioDemo * Instrument Serial Number = QuantStudioLemo * GuantStudioLemo * QuantStification Cycle Method = Ct * Stage/ Cycle Neirod = Ct * Stage/ Cycle Neirod = Ct * Stage/ Cycle Neirod = Stage 2, St * User Name = NA	I SGT I SGT I SGT I SGT I SGT stems\QuantStudio12KFlex\examples\Standard Curve\384-well Fast Standard Curve	
[Sample Setup] well well Position Sample Name Sample Color Quencher Quantity Comments RNaseP 1 Al RNaseP 2 A2 RNaseP 3 A3 RNaseP 5 A5 RNaseP 6 A6 "RGB(0,255,0)" 8 A8 Pop1<"RGB(0,255,0)"	Biogroup Name Biogroup Color Target Name Target Color Task Repo "RcB(176,23,31)" NTC FAM NFQ-MGB "1,250.000" "RcB(176,23,31)" STANDARD FAM NFQ-MGB "1,000.000" "RcB(176,23,31)" STANDARD FAM NFQ-MGB "1,000.00.000" "RcB(176,23,31)" UNKNOWN FAM NFQ-MGB "1,000.00.000" "RcB(176,23,31)" UNKNOWN FAM NFQ-MGB NFQ-MGB	rter

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GETTING STARTED GUIDE



Booklet 3 - Running Relative Standard Curve and Comparative C_T Experiments

Publication Part Number 4470050 Rev. A Revision Date March 2012



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PART I Running Relative Standard Curve Experiments

About Relative Standard Curve Experiments

This chapter covers:

- About the example experiment 11

IMPORTANT! First-time users of the QuantStudioTM 12K Flex System, please read Booklet 1, *Getting Started with QuantStudioTM 12K Flex System Multi-Well Plate and Array Card Experiments* and Booklet 7, *QuantStudioTM 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.

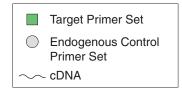
IMPORTANT! SYBR[®] Green reagents cannot be used for multiplex PCR.



Singleplex PCR

Multiplex PCR

00



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 QuantStudioTM 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.

Design the Experiment

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

This chapter covers:

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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
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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*TM 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 QuantStudioTM 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:

How do you want	t to identify this expe	riment?			
* Experiment Name:	96-Well Relative Std Curve Example		Comments:	Relative Standard Curve example	<u>^</u>
Barcode:					
User Name:	Example User				~
* Which block are	e you using to run the	experiment?			
3	84-Well	Array Card	1	96-Well (0.2mL)	Fast 96-Well (0.1mL)
Stan	kperiment do you war Idard Curve	Relative Standard Curve	Cor	nparative Cτ (ΔΔCτ)	Melt Curve
Ge	notyping	Presence/Absence			
* Which reagents	s do you want to use t	to detect the target sequence?			
🗸 TaqMa	n® Reagents	SYBR® Green Reagents		Other	
* What propertie	s do you want for the	instrument run?			
✓ s	tandard	Fast			

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:

Targets				Samples				
New Save to Library Import from Library Del	ete			New Save to Library Import from Library Delete				
Target Name	Reporter	Quencher	Color	Sample Name	Color			
FAS	FAM 🗸	NFQ-MGB 🗸 🗸	-	10				
HPRT	FAM 🗸	NFQ-MGB 🗸 🗸	- -	100		- 1		
				1000		- 1		
				10000	– •	4		
				Heart	– ~			
				Kidney	– •	· 🗸		
Biological Replicate Groups				Analysis Settings				
New Delete								
Biological Group Name Color		Comments						
				Reference Sample: Kidney Endogenous Control: FAS		*		
* Passive Reference								
ROX V								

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 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.

2

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

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

📏 Define and Set Up Standards		Plate Layout	Well Table										
Targets 🔼 🔪		U Show in Well	s 🔻 Select Wells	. 🔻 🗰 View	Lenend							+	
FAS UV		1	2	3	4	5	6	7	8	9	10	11	12
HPRT 💌	A	10000 S FAS	10000 S FAS	10000 S FAS	1000 S FAS	1000 S FAS	1000 S FAS	100 S FAS	100 S FAS	100 S FAS	10 S FAS	10 S FAS	10 S FAS
	в	1 S FAS	1 S FAS	1 S FAS	Kidney FAS	Kidney FAS	Kidney	Heart	Heart FAS	Heart			N FAS
Samples	с	10000	10000	10000	1000	1000	1000	100	100	100	10 S HPRT	10 S HPRT	10 S HPRT
1 10 100 100 1000	D	1 S HPRT	1 S HPRT	1 S HPRT	Kidney	Kidney	Kidney	Heart	Heart	Heart			N HPRT
10000 10000 ✓ Heart ✓ Kidney	E												
Biological Groups	F												
Biological Group	G												
	н												
	W	/ells: <u> </u>	30 N 2	<u>`</u>				<u> </u>		*	<u> </u>		52 Empty

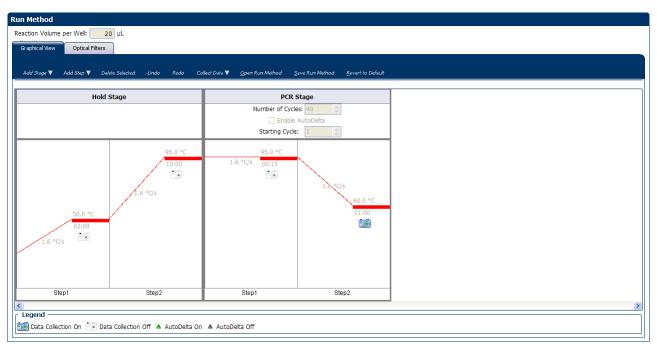
Your Assign screen should look like this:

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	Step 2	1.6°C/s	60°C	1 minute
Enable AutoDelta: Unchecked (default)				
Starting Cycle: Disabled when Enable AutoDelta is unchecked				



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 on	Refer to	Part number	
Consumables	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 12K Flex</i> System Multi-Well Plate and Array Card Experiments	4470050	
	Appendix A in Booklet 7, <i>QuantStudio™ 12K Flex System Multi-Well</i> Plate and Array Card Experiments - Appendixes		
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, <i>Getting Started with QuantStudio™ 12K Flex</i> System Multi-Well Plate and Array Card Experiments	4470050	

For more information



Chapter 2 Design the Experiment *For more information*

Prepare the Reactions

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

This chapter covers:

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Assemble required materials

3

- 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 TM kits, SuperScript [®] VILO TM 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 <i>Invitrogen VILO Kits Protocol</i> (PN 100002284) to:
	 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	1 Kidney 76	
2	Heart	76

3. Add the required volume of sample stock (100 ng/ μ L) 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

Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Relative Standard Curve and Comparative C_T Experiments

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 μ L 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 (20×)	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 (20×	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

- The reaction plate for the Relative Standard Curve example experiment contains:
 - A MicroAmp[®] Optical 96-Well Reaction Plate

components

- Reaction volume: 20 µL/well ٠
- 12 Unknown wells
- 30 Standard wells S
- 2 Negative Control wells
- 52 Empty wells

The plate layout for the example experiment looks like this:

>	Plate Layout W	ell Table										
Ĩ	🛄 Show in Wells 🔻	Select Wells 🔻 関	View Legend								№ +)	
	1	2	3	4	5	6	7	8	9	10	11	12
A	10000 S FAS	10000 S FAS	10000 S FAS	1000 S FAS	1000 S FAS	1000 S FAS	100 S FAS	100 S FAS	100 S FAS	10 S FAS	10 S FAS	10 S FAS
в	1 S FAS	1 S FAS	1 S FAS	Kidney	Kidney	Kidney FAS	Heart	Heart	Heart			N FAS
с	10000	10000	10000	1000	1000	1000	100	100	100	10	10 S HPRT	10 S HPRT
i D	1 S HPRT	1 S HPRT	1 S HPRT	Kidney	Kidney	Kidney	Heart	Heart	Heart			N HPRT
E												
F												
G												
н												
W	Wells: 11 12 30 12 52 Empty											

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 μ L 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, <i>Getting Started with QuantStudio™ 12K</i> Flex System Multi-Well Plate and Array Card Experiments	4470050
Sealing the reaction plate	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 12K</i> Flex System Multi-Well Plate and Array Card Experiments	4470050

Chapter 3 Prepare the Reactions *For more information*

3

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

This chapter covers:

- 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 QuantStudioTM 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

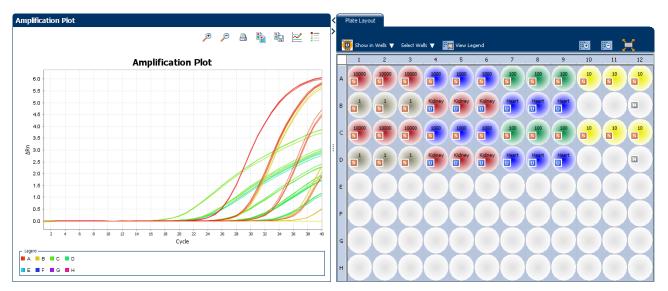
- **1.** In the Instrument Console screen, select the instrument icon.
- 2. Click Manage Instrument or double-click on the instrument icon.
- **3.** In the Manage Instrument screen, click **Monitor Running Experiment** to access the Run screen.

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.

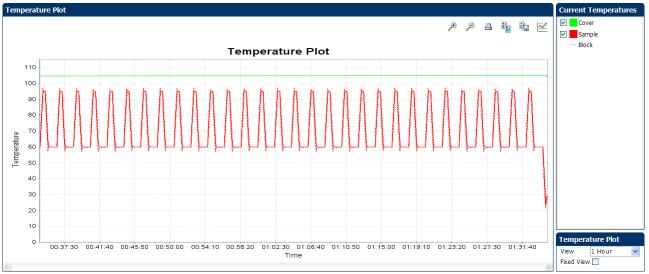
The figure below shows the Amplification Plot screen as it appears at the end of the example experiment.



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.



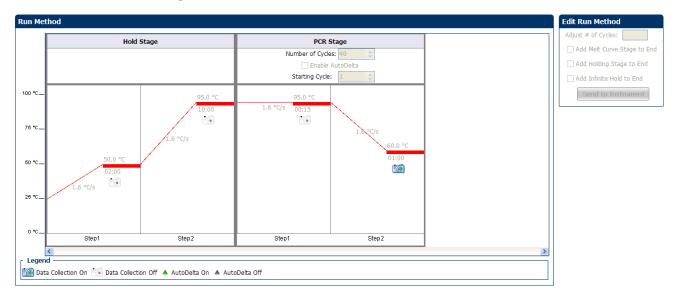
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Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

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:

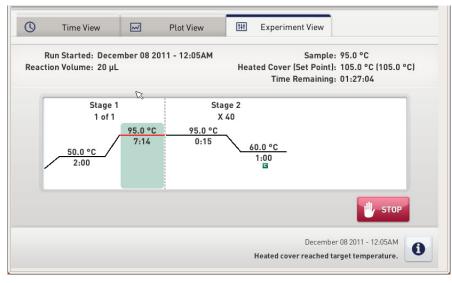
Run Data Report	
Experiment Name:	96-Well Relative Std Curve Example
Start Time:	08-09-2011 09:30:33 SGT
Stop Time:	08-09-2011 11:05:14 SGT
Run Duration:	94 minutes 40 seconds
User Name:	DEFAULT
Instrument Name:	QuantStudioDemo
Firmware Version:	0.13.1
Software Version:	QuantStudio 12K Flex Software v1.0
Instrument Serial Number:	QuantStudioDemo
Sample Volume:	20.0
Cover Temperature:	105.0
Block Type:	96-Well Block (0.2mL)
Errors Encountered:	

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

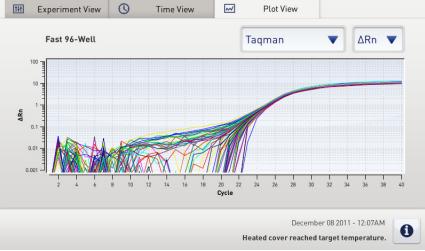


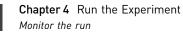
Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

Time view







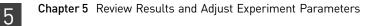


Review Results and Adjust Experiment Parameters

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:

Sec	tion 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
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Sec	tion 5.2 Adjust parameters for re-analysis of your own experiments	57
	Adjust analysis settings	57
	Improve C _T precision by omitting wells	61
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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 In the standard curve example experiment, you review the Standard Curve Plot screen for the following regression coefficient values: experiment standard curve

- Slope/amplification efficiency
- R² value (correlation coefficient)
- C_T values

To view and assess the Standard Curve plot

values

1. From the Experiment Menu pane, select **Analysis** > **Standard Curve**. Note: If no data are displayed, click Analyze.

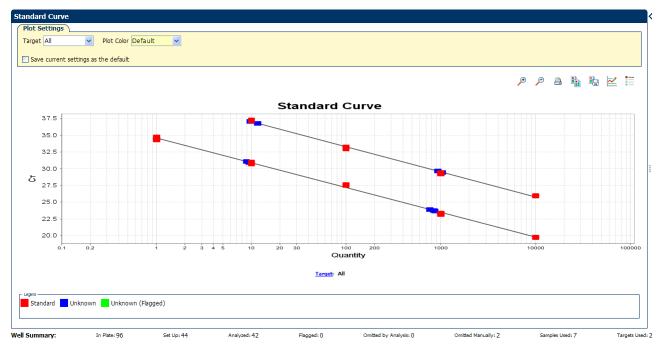
- 2. Display all 96 wells in the Standard Curve Plot screen by clicking the upper left corner of the plate layout in the Plate Layout tab.
- **3.** Enter the Plot Settings:

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).

> F	late	Layout	Well Table												
Sh	ow in	n Table 🗸	Select Wells 🔻	Group by 🔻										🗄 Expand All	😑 Collapse All
#		Well	Omit Flag	Sample N	Target Na	Task	Dyes	Ст	Ст Mean	CT SD	Quantity	Normali Norma	li Efficiency	Slope RQ	RQ I
			🗏 1 - FAS - S	FANDARD											<u>^</u>
1	14	B2		1	FAS	STANDARD	FAM-NFQ-MGB								
			🗏 1 - FAS - ST		54.0		51111150 1100				4 000		05 070	0.700	
	13 15			1	FAS FAS	STANDARD STANDARD	FAM-NFQ-MGB FAM-NFQ-MGB	Undetermi			1.000		85.379 85.379		
1	15			STANDARD - 1.0	FAS	STANDARD	FAM-NEQ-MGB	Undeterm	_		1.000		85.379	-3./30	
	27	D1		1	HPRT	STANDARD	FAM-NFQ-MGB	34.390	34.475	0.211	1.000		86.318	-3.700	
		D2		1	HPRT	STANDARD	FAM-NFQ-MGB		34.475				86.318		
		D3		1	HPRT	STANDARD	FAM-NFQ-MGB		34.475				86.318		
			🗏 10 - FAS - S	TANDARD											
	12	A12		10	FAS	STANDARD	FAM-NFQ-MGB								
1			🗏 10 - FAS - S	TANDARD - 10.0											
		A10		10	FAS	STANDARD	FAM-NFQ-MGB	37.109	37.207				85.379		
	11	A11		10	FAS	STANDARD	FAM-NFQ-MGB	37.305	37.207	0.138	10.000		85.379	-3.730	
				STANDARD - 10.											
		C10		10	HPRT	STANDARD	•	30.982	30.790				86.318		
		C11 C12		10	HPRT HPRT	STANDARD		30.706	30.790 30.790				86.318 86.318		
	30			10 STANDARD - 100		STANDARD	FAM-NFQ-MGB	30.683	30.790	0.167	10.000		86.318	-3./00	
	,	A7	100 - FAS -	100	FAS	STANDARD	FAM-NFQ-MGB	33.287	33.154	0.160	100.000		85.379	-3.730	
		A8		100	FAS	STANDARD		33.197	33.154				85.379		
		A9		100	FAS	STANDARD	FAM-NFQ-MGB		33.154				85.379		
			🗏 100 - HPRT	- STANDARD - 10	0.0										
1	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			100	HPRT	STANDARD	FAM-NFQ-MGB	27.498	27.593	0.083	100.000		86.318	-3.700	
				- STANDARD - 10											
	1	A4		1000	FAS	STANDARD	FAM-NFQ-MGB	29.403	29.296				85.379	-3.730	~
			<						· · · · ·						>
Well S	um	imary:	In F	Plate: 96	Set Up: 44	Analy	zed: 42	Flagged: ()	c	Omitted by Analy	sis: ()	Omitted Manually: 2	Samp	les Used: 7	Targets Used: 2

5

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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 (10⁵ to 10⁶ 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.
- **R² values (correlation coefficient)** The R² 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 R² 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.



Chapter 5 Review Results and Adjust Experiment Parameters Assess amplification results using the Amplification Plot

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

Plate Layout Well Table

- 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:

	1	2		3	4	5	6	7	8	9	10	11	12
I	10000	10000		0000	1000	1000	1000	100	100	100	10	10	10
l	S FAS CT: 25.9	B FAS CT:	26.01 S FAS	б Ст: 25.96	S FAS CT: 29.4	S FAS CT: 29.25	S FAS CT: 29.23	S FAS CT: 33.29	S FAS CT: 33.2	S FAS CT: 32.98	S FAS CT: 37.11	S FAS CT: 37.3	SCr: Undete
	1			1	Kidney FAS CT: 37.03	Kidney FAS CT: 36.74	Kidney	Heart FAS CT: 29.63	Heart FAS CT: 29.64	Heart FAS CT: 29.47			NCr: Undet
ŀ	SCT: Undetermin	SCr: Undete	rmined	Undetermined	U FAS CT: 37.03	U FAS CT: 36.74	I FAS CT: 36.74	U FAS CT: 29.63	I FAS CT: 29.64	U FAS CT: 29.47			
ſ	10000 S HP Ст: 19.7	10000 8 S HP CT:		.0000 Ст: 19.75	1000	1000	1000	100	100	100	10	10	10 1 S HP Ct:
	CI: 15.7		12.//3		MPRI CI: 23.2		CT: 23.14	CT: 27.65	CT: 27.65		CT: 30/30	CI: 30//	
	1	1 9 S HP CT:	34.32 S HP.	1 Ст: 34.72	Kidney	Kidney	Kidney	Heart	Heart	Heart			N CT : Undet
	CT: 34.3		24.32		U HP CI: 50.56	U HP CT: 51.06	U HPAT CI: 30/3	U HPKI CI 25.5	U HPKI CIT257	U HP CT: 25.75			
l													
l													

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.

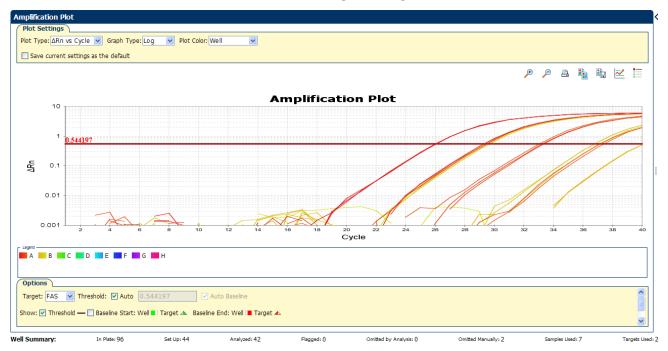


5. View the threshold values:

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:

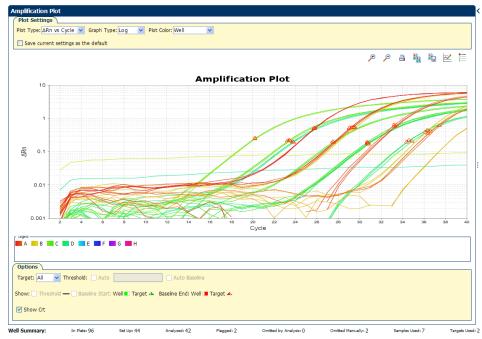
- Outliers
- A typical amplification plot The QuantStudio[™] 12K Flex Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
 - Plateau phase
 - Linear phase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

A typical amplification plot should look like this:

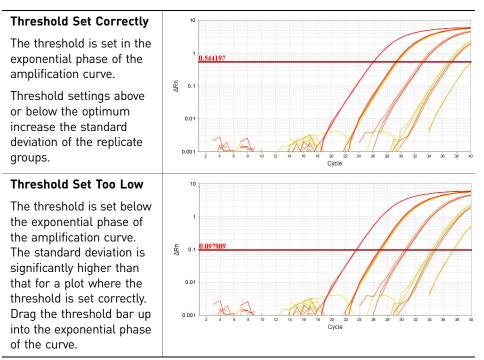


IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudio[™] 12K Flex Software. Therefore, Life Technologies recommends that you examine the Amplification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



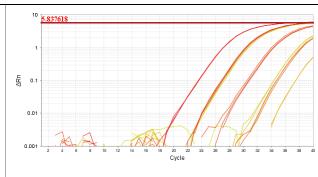
• Correct threshold values.



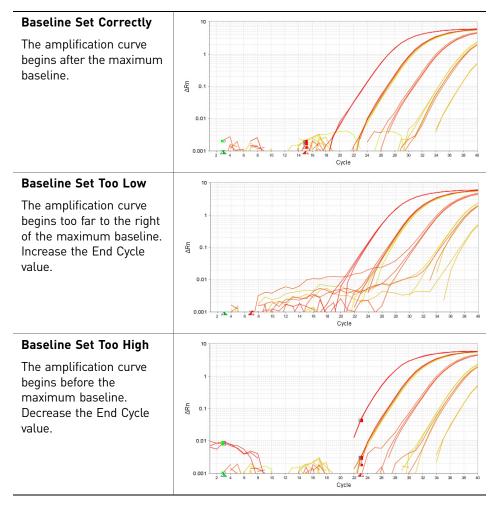
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Threshold Set Too High

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



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).



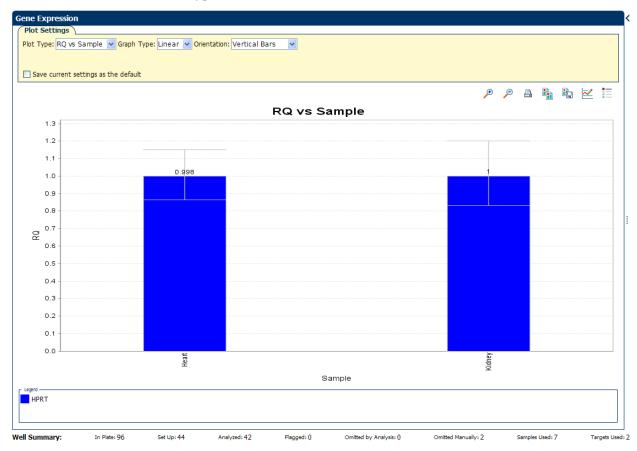
Assess the gene expression profile using the Gene Expression Plot

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:

				,₽ _}	Э д		i 🗠	
		RQ vs T	arget					
1.30			-					
1.25								
1.20								
1.15								
1.10								
1.05	0.998				1			
1.00 -	0.350							
0.95						-		
0.90						-		
0.85						-		
0.80	· • • •					-		
0.75	· · · · ·					-		
0.70	· · · · ·			· · · ·				
0.65	· • • •					-		
0.60	· · · · ·					-		
0.55				· · · ·				
0.50	· • • •					-		
0.45						-		
0.40				· · · ·				
0.35						-		
0.30						-		
0.25				· · · ·		-		
0.20						-		
0.15								
0.10								
0.05						-		
0.00			ZI SI					
			НРЯТ					
			 Target					

• **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:



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

experiment values

Example

- 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).

ne Expression						
Plot Settings						
ot Type: RQ vs Sample	🛩 Graph Type: Log10 👻 Orientation: Ve	rtical Bars 🛛 👻				
Save current settings as	the default					
				Ð	ра 🐴 🖣	5 📈 🗄
		RQ vs Sam	ple			
0.08			•			
0.07						
0.06						
0.05						
0.04						
0.03						
0.02						
9 0.01						
É 0.00				0		
2 0.01 2 0.00 2 -0.01	-0.001					
-0.02						
-0.03						
-0.04						
-0.05						
-0.06						
-0.07						
-0.08						
L	Heart			Kidney		
	_	Samp	ble	Σ		
end HPRT						
HPKI						
Summary: In Pla	ate: 96 Set Up: 44 Analyzed	: 42 Flagged: 0	Omitted by Analysis: ()	Omitted Manually: 2	Samples Used: 7	Targets

Assessing the gene expression plot in your own experiments

5

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

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

Example experiment values and flags

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.

>	Plat	e Layout	Well Table														
	Show	in Table 🏹	🗸 Select Wells 🔻	Group by 🔻											🗄 Ехра	nd All 🛛 🗖 🤇	ollapse All
	#	Well	Omit Elag	Sample N	Target Na	Task	Dves	Ст	Ст Mean	CT SD	Ouantity	Normali	Normali	Efficiency	Slana	RO	RQI
	#	wen	I - FAS - S		Target Na	Task	Dyes	CI	CT Mean	CT 3D	Quantity	NUTHAIL	Normali	Enciency	Siupe	ΝŲ	RQ I
	14	B2			FAS	STANDARD	FAM-NFQ-MGB	_				_	_	_	_	_	
	11	02	■ 1 - FAS - S	-	1713	STANDARD	174111 Q 1105	_			_			_			
	13	B1		1	FAS	STANDARD	FAM-NFO-MGB	Undetermi			1.000			85.379	-3.730		
	15	B3		1	FAS	STANDARD	FAM-NFQ-MGB	Undetermi			1.000			85.379	-3.730		E
			🗏 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		
		D2		1	HPRT	STANDARD	FAM-NFQ-MGB		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 - S														
	12	A12		10	FAS	STANDARD	FAM-NFQ-MGB										
				TANDARD - 10.0		CTANDADD	EAN NEO MOD	27.100	27.207	0.120	10.000			05 070	2,720		
		A10 A11		10 10	FAS FAS	STANDARD STANDARD	FAM-NFQ-MGB FAM-NFQ-MGB		37.207 37.207	0.138	10.000			85.379 85.379	-3.730 -3.730		
	11	AII		STANDARD - 10		STANDARD	PAM-NPQ-MOB	37.303	37.207	0.136	10.000			63.379	-3.730		
	34	C10		10	HPRT	STANDARD	FAM-NFO-MGB	30.982	30.790	0.167	10.000	_	_	86.318	-3.700	_	
		C11		10	HPRT	STANDARD	FAM-NFQ-MGB		30.790	0.167	10.000			86.318	-3,700		
	36	C12		10	HPRT	STANDARD	FAM-NFQ-MGB		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		
				- STANDARD - 1													
		C7		100	HPRT	STANDARD	FAM-NFQ-MGB		27.593	0.083	100.000			86.318	-3.700		
		C8		100	HPRT	STANDARD	FAM-NFQ-MGB		27.593	0.083	100.000			86.318	-3.700		
	33	C9	E 1000 EAC	100 - STANDARD - 10	HPRT	STANDARD	FAM-NFQ-MGB	27.498	27.593	0.083	100.000	_	_	86.318	-3.700	_	
	4	A4	1000 - PAS	1000	FAS	STANDARD	FAM-NFQ-MGB	20 403	29.296	0.093	1,000.000			85.379	-3.730		
	-			1000	FAS	STANDARD	FAM-NEQ-MOD		29.290	0.095	1,000.000			05.379	-5.750		~
			<														>
W	ell Sur	nmary:	In F	Plate: 96	Set Up: 44	Analy	/zed: 42	Flagged: ()	0	mitted by Analys	is: O	Omitted M	anually: 2	Sample	es Used: 7	Tai	gets Used: 2

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

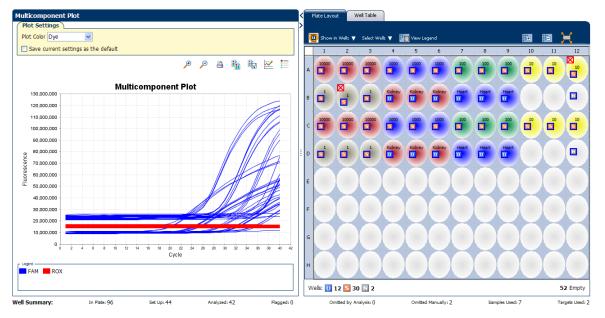
The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run. Purpose In the Relative Standard Curve example experiment, you review the Multicomponent Plot screen for: • ROXTM dye (passive reference) FAMTM dye (reporter for RNase P) Spikes, dips, and/or sudden changes Amplification in the negative control wells View the 1. From the Experiment Menu pane, select **Analysis** • **Multicomponent Plot**. Multicomponent Note: If no data are displayed, click Analyze. Plot 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).

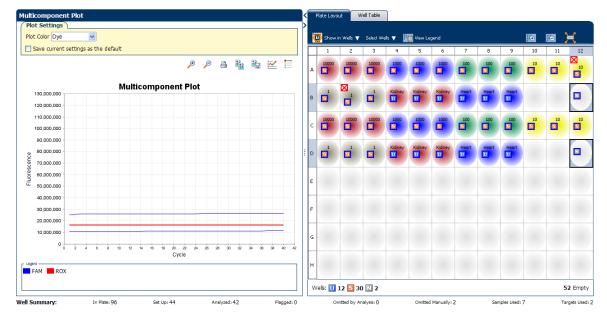
Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

5. Check the FAM dye signal. In the example experiment, the FAM dye signal increases throughout the PCR process, indicating normal amplification.



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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.

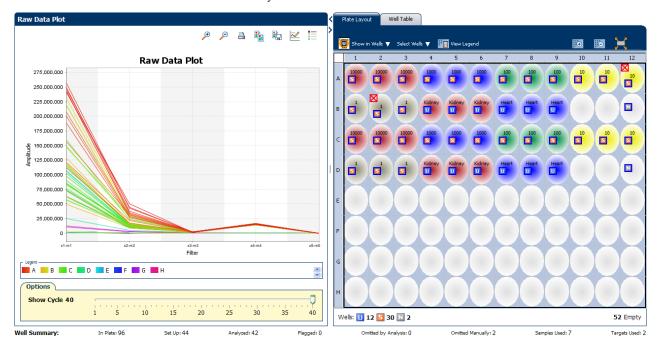
Tips for confirming dye accuracy in your own experiment When you analyze your own Relative Standard Curve experiment, look for:

- **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	 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).
- 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:

				Load Save Revertic	Defaults		
				Emission Filter			
		m1(520±15)	m2(558±11)	m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
	x1(470±15)	\checkmark					
	x2(520±10)		\checkmark				
	x3(550±11)			¥			
EXCITATION HITER	x4(580±10)						
	x5(640±10)					V	
lt C	x6(662±10) urve Filter —						
elt C				Load Save Revert to	Defaults		
elt C				Emission Filter			
elt C	urve Filter ————	m1(520±15)	m2(558±11)	Emission Filter m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
elt C		m1(520±15)	m2(558±11)	Emission Filter		m5(682±14)	
	urve Filter ————			Emission Filter m3(586±10)	m4(623±14)		m6(711±12)
	urve Filter ——— x1(470±15)			Emission Filter m3(586±10)	m4(623±14)		m6(711±12)
Excitation Filter	urve Filter			Emission Filter m3(586±10)	m4(623±14)		m6(711±12)

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

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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:

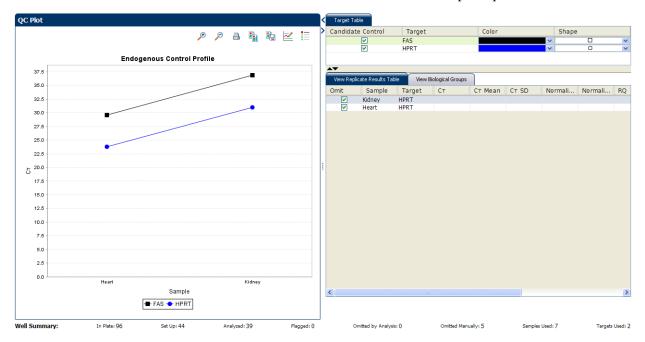
1. From the Experiment Menu pane, select Analysis > QC Plot.

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.



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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

- 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:

QC Summary									
Flag Details									
Flag:		Description			Frequency	Wells			
AMPNC		Amplification in negative	control		0			~	
BADROX		Bad passive reference si			0				
OFFSCALE		Fluorescence is offscale			0				
HIGHSD		High standard deviation	in replicate group		0				
NOAMP		No amplification			0				
NOISE		Noise higher than other	s in plate		0				
SPIKE		Noise spikes			0			8	
NOSIGNAL		No signal in well			0				
OUTLIERRG		Outlier in replicate group)		0				
EXPFAIL		Exponential algorithm fa	iled		0				
BLFAIL		Baseline algorithm failed			0				
THOLDFAIL		Thresholding algorithm f	balled		0				
CTFAIL		CT algorithm failed	aiicu		0			~	
CTITLE		er algenenn raied						<u> </u>	
Total Wells: Wells Set Up: Well Summary:	In Plate: 96	96 Processed Wells: 44 Flagged Wells: Set Up: 44	Analyzed: 39		ly Omitted Wells: s Omitted Wells: Omitted by Analysis: 0	5 Targets U 0 Samples U Omitted Manually: 5	Ised: Jsed: Samples Used: 7	2 7 Targets Us	
weii summary:	In Plate: 96	Set up: 44	Analyzed: 39	Hagged: ()	Umitted by Analysis: ()	Umitted Manually: 5	Samples Used: /	Targets U	*

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

_

Description
Pre-processing flag
Fluorescence is offscale
Primary analysis flags
Bad passive reference signal
No amplification
Noise higher than others in plate
Noise spikes
No signal in well
Exponential algorithm failed
Baseline algorithm failed
Thresholding algorithm failed
C _T algorithm failed
Amplification in the linear region is below a certain threshold, corresponding to the score set in the analysis settings
econdary analysis flags
Outlier in replicate group
Amplification in the negative control
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, <i>Getting Started with QuantStudio™ 12K Flex</i> 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**.

 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:

Analysis Settings f	or 96-Well Relative Std Curv	e Example				
Ст Settings Flag	Settings Relative Quantificat	ion Settings Advanced Settin	ngs Standard Curve Setti	ings		
Review the defa change the setti		this experiment. To use different :	settings for a target, select the	e target from the table, deselect Use Default Settings, then		
Data Step Selection Algorithm Settings Select the step and stage to use for CT analysis. Only stage/step combinations for which data suitable for CT analysis have been collected are displayed. Select the algorithm Settings Stage 2, Step 2 Image: Stage 2, Step 2 Image: Stage 2, Step 2 Image: Stage 2, Step 2 Default CT Settings Default CT Settings are used to calculate the CT for targets without custom settings. To edit the default settings, click Edit Default Settings. Threshold: AUTO Baseline Start Cycle: AUTO Baseline End Cycle: AUTO						
Select a Target		1 - I ·		CT Settings for FAS		
Target	Threshold	Baseline Start	Baseline End	CT Settings to Use: 🗹 Default Settings		
FAS				Automatic Threshold		
HPRT	AUTO	AUTO	AUTO	Threshold: 0.544197		
				✓ Automatic Baseline Baseline Start Cycle: 3 End Cycle: 15		
ave to Library	ve to Library Load from Library Revert to Default Analysis Settings Apply Analysis Settings Cancel					

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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.

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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.

🛍 Analysis Settings for 96-Well Relative Std Curve Example Flag Settings Relative Quantification Settings Advanced Settings Standard Curve Settings Ст Settings Configure the flags and filtering. In this panel you can enable, disable, and configure flags, and indicate if a well is to be rejected when a flag is raised 7 Description Attribute Condition Value Reject Well Flag Use AMPNC **~** ~ Amplification in negat. 35.000 Ст **~** BADROX Bad passive referenc.. Bad passive referenc. ~ 0.600 OFESCALE **~** Eluorescence is offscale HIGHSD High standard deviati... CT standard deviation 0.500 Amplification algorith. NOAMP No amplification ~ ✓ 0.100 NOTSE Noise higher than ot... **~** Relative noise ✓ 4.000 SPIKE Noise spikes ~ Spike algorithm result ✓ 1.000 NOSIGNAL No signal in well **~** OUTLIERRG **~** Outlier in replicate gr. EXPFAIL **~** Exponential algorithm. Baseline algorithm failed ~ BLFAIL THOLDFAIL **~** Thresholding algorith. ~ CTFAIL CT algorithm failed AMPSCORE AMP Score **~** AMP Score 1.000 Save to Library Load from Library Revert to Default Analysis Settings Apply Analysis Settings Cancel

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:

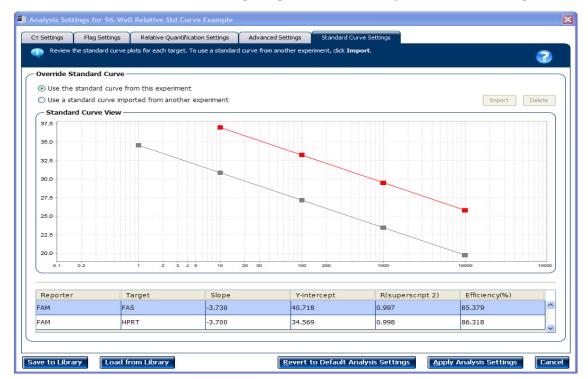
- 1. Select one or more well-target combinations in the table.
- 2. Deselect the Use C_T Settings Defined for Target check box.
- **3.** Define the custom baseline settings:
 - For automatic baseline calculations, select the **Automatic Baseline** check box.
 - To define the baseline, deselect the **Automatic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

- 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).

Applied Biosystems QuantStudioTM 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Relative Standard Curve and Comparative C_T Experiments

> Plat	te Layout	Well T	able											
Show	in Table 🔻	Select W	ells 🔻 Group by 🔻										🗄 Expand Al	🖬 Collapse All
#	Well	Omit	Flag Sample N.	Target Na	Task	Dyes	Ст	CT Mean CT SD	Quantity	Normali	Normali	Efficiency Slop	e RQ	RQ I
		🗏 1 - F/	S - STANDARD											~
14	B2		1	FAS	STANDARD	FAM-NFQ-MGB								
			S - STANDARD - 1.0											
	B1		1	FAS	STANDARD	FAM-NFQ-MGB			1.000					
15	B3	1	1 PRT - STANDARD - 1.0	FAS	STANDARD	FAM-NFQ-MGB	_		1.000)	_		_	
37	D1		1	HPRT	STANDARD	FAM-NFQ-MGB			1.000)				
	D2		1	HPRT	STANDARD	FAM-NFQ-MGB			1.000					
39	D3		1	HPRT	STANDARD	FAM-NFQ-MGB			1.000)				
		🗏 10 - i	AS - STANDARD											
12	A12	 Image: A start of the start of	10	FAS	STANDARD	FAM-NFQ-MGB								
1			AS - STANDARD - 10.											
	A10		10	FAS	STANDARD	FAM-NFQ-MGB			10.000					
11	A11		10	FAS	STANDARD	FAM-NFQ-MGB			10.000)				
			IPRT - STANDARD - 1		07410400	5444 1150 1400			10.000					
	C10 C11		10 10	HPRT HPRT	STANDARD STANDARD	FAM-NFQ-MGB FAM-NFQ-MGB			10.000					
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50	012		FAS - STANDARD - 10		STANDARD	TANHA Q-NOD			10.000	,				
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 -											
	C7		100	HPRT	STANDARD	FAM-NFQ-MGB			100.000					
	C8		100	HPRT	STANDARD	FAM-NFQ-MGB			100.000					
33	C9		100	HPRT	STANDARD	FAM-NFQ-MGB	_		100.000)	_		_	_
4	A4		- FAS - STANDARD - 1 1000	FAS	STANDARD	FAM-NFO-MGB			1,000.000					
4	A4		1000	FAS	STANDARD	FAM-INFQ-MGB			1,000.000					~
		1	1				1111							>
Well Su	mmary:		In Plate: 96	Set Up: 44	Anal	yzed: 39	Flagged: ()	Omitted by	Analysis: ()	Omitted Man	iually: 5	Samples Used	:7	Targets Used: 2

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

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

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 QuantStudioTM 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	<pre><drive>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments</drive></pre>

Your Export screen should look like this:

							_	
ort File Location: C:\Applied Bi	osystems\QuantStud	lio 12K Flex Software\U	Browse Export File	Name: 96-Well Re	elative Std Curve E	xample_Quants	6 File Type: 📋 (*.t	xt)
Sample Setup	Amplification	Multicomponent	Tech. Rep. Results	Bio. Rep. Resu	ilts 🗸 Results			
Skip Empty Wells 🔽 Skip Omit	ted Wells							
elect Content	Well	Well Position	Sample Name	Target Name	Task	Reporter	Quencher	1
All Fields	~	1 A1	10000	FAS	STANDARD	FAM	NFQ-MGB	
		2 A2	10000	FAS	STANDARD	FAM	NFO-MGB	
Well		3 A3	10000	FAS	STANDARD	EAM	NFQ-MGB	
		4 A4	1000	FAS	STANDARD	FAM	NFO-MGB	
Well Position		5 A5	1000	FAS	STANDARD	EAM	NFQ-MGB	
Sample Name		6 A6	1000	FAS	STANDARD	FAM	NFO-MGB	
		7 A7	100	FAS	STANDARD	EAM	NFQ-MGB	
Target Name		8 A8	100	FAS	STANDARD	FAM	NFO-MGB	
		9 A 9	100	FAS	STANDARD	EAM	NFQ-MGB	
Task		10 A10	10	FAS	STANDARD	FAM	NFO-MGB	
Reporter		11 A11	10	FAS	STANDARD	EAM	NFO-MGB	
Reporcer		13 B1	1	FAS	STANDARD	FAM	NFO-MGB	
Quencher	=	15 B3	1	FAS	STANDARD	FAM	NFQ-MGB	
		16 B4	Kidney	FAS	UNKNOWN	FAM	NFQ-MGB	
RQ		17 B5	Kidney	FAS	UNKNOWN	FAM	NFQ-MGB	
- no w		18 B6	Kidney	FAS	UNKNOWN	FAM	NFQ-MGB	
RQ Min		19 B7	Heart	FAS	UNKNOWN	FAM	NFQ-MGB	
RO Max		20 B8	Heart	FAS	UNKNOWN	FAM	NFQ-MGB	
		21 B9	Heart	FAS	UNKNOWN	FAM	NFQ-MGB	
СТ		24 B12		FAS	NTC	FAM	NFQ-MGB	
		25 C1	10000	HPRT	STANDARD	FAM	NFQ-MGB	
Ct Mean		26 C2	10000	HPRT	STANDARD	FAM	NFQ-MGB	
Ct SD		27 C3	10000	HPRT	STANDARD	FAM	NFQ-MGB	
CC 3D		28 C4	1000	HPRT	STANDARD	FAM	NFQ-MGB	
Quantity		29 C5	1000	HPRT	STANDARD	FAM	NFQ-MGB	
		30 C6	1000	HPRT	STANDARD	FAM	NFQ-MGB	
Quantity Mean		31 C7	100	HPRT	STANDARD	FAM	NFQ-MGB	
Oursetty CD		32 C8	100	HPRT	STANDARD	FAM	NFQ-MGB	
Quantity SD		33 C9	100	HPRT	STANDARD	FAM	NFQ-MGB	
Automatic Ct Threshold		34 C10	10	HPRT	STANDARD	FAM	NFQ-MGB	
	~ <							>

Start Export Save Export Set As Load Export Set Delete Export Set

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

96-Well Relative Std Curv		ort.txt - Notepad						
File Edit Format View Help								
<pre>* Barcode = NA * Block Type = 96-well Block (0.2mL) * Calibration Background performed on = 2011-08-08 01:15:53 AM SGT * Calibration FAM is expired = No * Calibration FAM is expired = No * Calibration ROI is expired = No * Calibration ROI is expired = No * Calibration ROI performed on = 2011-08-08 01:05:24 AM SGT * Calibration ROX is expired = No * Calibration SVBR is expired = No * Calibration SVBR performed on = 2011-08-08 01:58:11 AM SGT * Calibration SVBR performed on = 2011-08-08 01:58:11 AM SGT * Calibration TAMRA is expired = No * Calibration TAMRA performed on = 2011-08-08 01:24:47 AM SGT * Calibration Uniformity performed on = 2011-08-08 01:24:47 AM SGT * Calibration VIC is expired = No * 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 * Experiment Name = 06-well Relative Std Curve Example * Experiment Name = QuantStudioDemo * Instrument Name = QuantStudioDemo * Instrument Serial Number = QuantStudioDemo * Instrument Serial Number = QuantStudioDemo * Instrument Serial Number = QuantStudioDemo * Instrument Serial Shoothing On = true * Stage / CyCle where Analysis is performed = Stage 2, Step 2 * User Name = NA</pre>								1
[Results] Well Well Position SD Quantity Baseline End			eporter Ct Threshold	Quencher Ct Threshold	RQ F Automatic	RQ Min R Baselin	Q Max CT Ct Mean e Baseline Start	Ct
1 A1 10000	FAS STANDARD 3 19	FAM NFQ-MGB		25.990	25.985 0	0.027 "	10,000.000"	
2 A2 10000	FAS STANDARD	FAM NFQ-MGB		26.009	25.985 (0.027 "	10,000.000"	
true 0.544 true 3 A3 10000	3 19 FAS STANDARD	FAM NFQ-MGB		25.956	25.985 (0.027 "	10,000.000"	
true 0.544 true 4 A4 1000	3 19 FAS STANDARD	FAM NFQ-MGB		29.403	29.296	0.093 "	1,000.000"	
true 0.544 true 5 A5 1000	3 23 FAS STANDARD	FAM NFO-MGB			29.296 (1,000,000"	
true 0.544 true 6 A6 1000	3 22 FAS STANDARD	FAM NFQ-MGB		29.234			1,000.000"	
true 0.544 true 7 A7 100 true 0.544 true	FAS STANDARD 3 23 FAS STANDARD 3 26	FAM NFQ-MGB			33.154 (00.000	~

PART II Running Comparative C_T Experiments

About Comparative C_T Experiments

This chapter covers:

- About the example experiment 71

IMPORTANT! First-time users of the QuantStudioTM 12K Flex System, please read Booklet 1, *Getting Started with QuantStudioTM 12K Flex System Multi-Well Plate and Array Card Experiments* and Booklet 7, *QuantStudioTM 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 QuantStudioTM 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 QuantStudioTM 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: Hs9999903_m1
 - TGFB1 Assay Mix: Hs00998133_m1
 - CCKAR Assay Mix: Hs00167891_m1



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*TM 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 QuantStudioTM 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:

How do you wan	t to identify this expe	riment?			
* Experiment Name:	96-Well Fast Comparative	Ct Example.eds	Comments:	Comparative CT example	<u>^</u>
Barcode:					
User Name:	Example user				×
* Which block ar	e you using to run the	experiment?			
3	384-Well	Array Card	/	96-Well (0.2mL)	Fast 96-Well (0.1mL)
* What type of e	xperiment do you wan	it to set up?			
Star	ndard Curve	Relative Standard Curve	/ Cor	nparative Cτ (ΔΔCτ)	Melt Curve
Ge	enotyping	Presence/Absence			
* Which reagents	s do you want to use t	o detect the target sequence?			
✓ TaqMa	an® Reagents	SYBR® Green Reagents		Other	
• What propertie	es do you want for the	instrument run?			
S	Standard	✓ Fast			

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:

Targets				Samples	
New Save to Library Import from Library	Delete			New Save to Library Import from Library Delete	
Target Name	Reporter	Quencher	Color	Sample Name Co	olor
GH1	FAM 🗸	NFQ-MGB 🗸 🗸	-	Brain	*
LP1N1	FAM 🗸	NFQ-MGB 🗸 🗸	– •	Heart	~
TGFB1	FAM 🗸	NFQ-MGB 🗸 🗸	-	Liver	~
L1PC	FAM 🗸	NFQ-MGB 🗸 🗸	~	Lung	~
АСТВ	FAM 🗸	NFQ-MGB 🗸 🗸	-		
CCKAR	FAM 🗸	NFQ-MGB 🗸 🗸	-		
Biological Replicate Groups				Analysis Settings	
New Delete					
Biological Group Name Color		Comments		1	
				Reference Sample: Brain	
				Endogenous Control: ACTB	*
* Passive Reference					
ROX					

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		

Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Relative Standard Curve and Comparative C_T Experiments

Targets	<	Plate Layout	Well Table	e									
Name Task	>	-			_								5.2
GH1 U • LP1N1 •		Show in W	ells 🔻 Select	Wells 🔻 📓	View Legend	I						•	<u> </u>
TGFB1		1	2	3	4	5	6	7	8	9	10	11	12
L1PC 💌		Heart	Brain	Heart	Brain	Heart	Brain	Heart	Brain	Heart	Brain	Heart	Brain
ACTB	А	GH1	U GH1	U LP1N1	U LP1N1	U TGF	U TGF	L1PC	U L1PC	L ACTB	I ACTB	ССК	ССК
CCKAR		\sim											
		Heart	Brain	Heart	Brain	Heart	Brain	Heart	Brain	Heart	Brain	Heart	Brain
	в	GH1	U GH1	U LP1N1		TGF	TGF	L1PC	L1PC	ACTB	ACTB	CCK	ССК
Samples		Hand	Brain	Heart	Brain	Heart	Brain	Heart	Brain	Heart	Brain	Heart	Brain
Name	C	Heart GH1	GH1	L LP1N1	LP1N1	TGF	TGF	L1PC	L1PC	ACTB	ACTB	CCK	CCK
Brain Heart													
Lung	: D	Heart N GH1	Brain N GH1	Heart	Brain N LP1N1	Heart N TGF	Brain	Heart	Brain N L1PC	Heart ACTB	Brain N ACTB	Heart CCK	Brain CCK
	Е	Lung U GH1	Liver U GH1	Lung	Liver	Lung U TGF	Liver		Liver	Lung L ACTB	Liver	Lung	Liver
Biological Groups — 💽	F	Lung U GH1	Liver U GH1	Lung	Liver	Lung U TGF	Liver	Lung	Liver	Lung L ACTB	Liver Liver	Lung	Liver
Biological Group													
	G	Lung U GH1	Liver GH1	Lung	Liver	Lung	Liver		Liver	Lung LACTB	Liver ACTB	Lung	Liver
	н	Lung N GH1	Liver N GH1	Lung	Liver	Lung	Liver N TGF	Lung	Liver N L1PC	Lung N ACTB	Liver N ACTB	Lung	Liver CCK
	v	Vells: 🕕 72	N 24		1		L	1	L		L	L	0 Empty
	Ľ	/2											C chipty

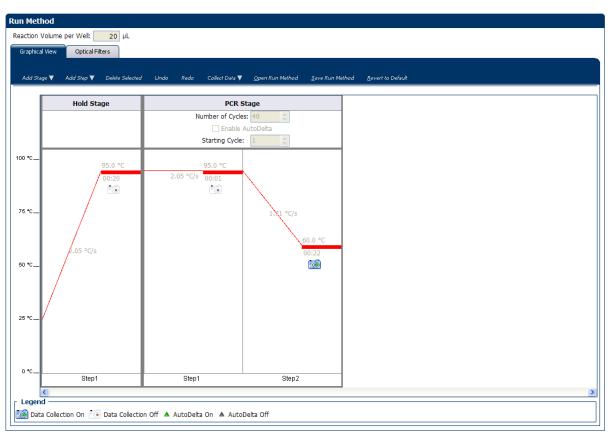
Your Assign screen should look like this:

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	Step 2	1.71°C/s	60°C	22 seconds		
Enable AutoDelta: Unchecked (default)						
Starting Cycle: Disabled when Enable AutoDelta is unchecked						



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, <i>Getting Started with</i> <i>QuantStudio™ 12K Flex System Multi-Well Plate and</i> <i>Array Card Experiments</i>	4470050
	Appendix A in Booklet 7, QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes	
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, <i>Getting Started with</i> <i>QuantStudio™ 12K Flex System Multi-Well Plate and</i> <i>Array Card Experiments</i>	4470050



Chapter 8 Design the Experiment *For more information*

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 VILOTM kits to carry out the reverse transcription.

ExampleFor the Comparative C_T example experiment, the template for the PCR reactions isexperimentcDNA reverse-transcribed from total RNA samples using one of the Invitrogen VILOTMsettingsKits, SuperScript® VILOTM cDNA Synthesis Kit (PN 4453650).

Prepare the
templateUse 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/ μ L) 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 (20×)	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

The reaction plate for the Comparative C_T example experiment contains:

- Example experiment reaction plate components
- A MicroAmp[®] Optical 96-Well Reaction Plate
- Reaction volume: 20 μ L/well
- The reaction plate contains:
 - 72 Unknown wells U
 - 24 Negative Control wells
 - 0 Empty wells

	Plate Layout	Well Table										
Ĩ	😈 Show in Wel	ls 🔻 Select Wells	View Lege	end							••	B 💥 🔤
	1	2	3	4	5	6	7	8	9	10	11	12
А	Heart GH1	Brain	Heart	Brain	Heart	Brain	Heart	Brain	Heart ACTB	Brain ACTB	Heart CCKAR	Brain
в	Heart	Brain	Heart	Brain	Heart	Brain	Heart	Brain	Heart ACTB	Brain	Heart	Brain
с	Heart Heart	Brain	Heart	Brain	Heart	Brain TGFB1	Heart	Brain	Heart ACTB	Brain ACTB	Heart CCKAR	Brain
I D	Heart	Brain	Heart	Brain	Heart TGFB1	Brain TGFB1	Heart	Brain	Heart ACTB	Brain ACTB	Heart	Brain CCKAR
E	Lung U GH1	Liver CH1	Lung	Liver	Lung TGFB1	Liver TGFB1	Lung	Liver		Liver ACTB		Liver CCKAR
F	Lung CH1	Liver GH1	Lung	Liver	Lung TGFB1	Liver TGFB1	Lung	Liver		Liver ACTB	Lung U CCKAR	Liver CCKAR
G	Lung CH1	Liver	Lung	Liver	Lung TGFB1	Liver TGFB1	Lung	Liver		Liver ACTB	Lung U CCKAR	Liver CCKAR
н	Lung R GH1	Liver N GH1	Lung LP1N1	Liver	Lung TGFB1	Liver N TGFB1	Lung	Liver		Liver N ACTB	Lung N CCKAR	Liver N CCKAR
V	Vells: 🚺 72 🛽	1 24										0 Empty

The plate layout experiment looks like this:

To prepare the reaction plate components

- 1. Add 1 μ L of each cDNA to the appropriate wells.
- **2.** Pipette 1 μ L of sterile water into the NTC wells.
- 3. Add 19 μ L 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.

\sim	

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

Tips for preparing
the reaction plateWhen you prepare your own Comparative C_T experiment, make sure the arrangement
of the PCR reactions matches the plate layout displayed in the QuantStudioTM 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, <i>Getting Started with QuantStudio™ 12K Flex System</i> Multi-Well Plate and Array Card Experiments	4470050



Chapter 9 Prepare the Reactions *For more information*

Run the Experiment

This chapter explains how to run the example experiment on the QuantStudio $^{\rm TM}$ 12K Flex Instrument.

This chapter covers:

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 QuantStudioTM 12K Flex Instrument touchscreen.

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

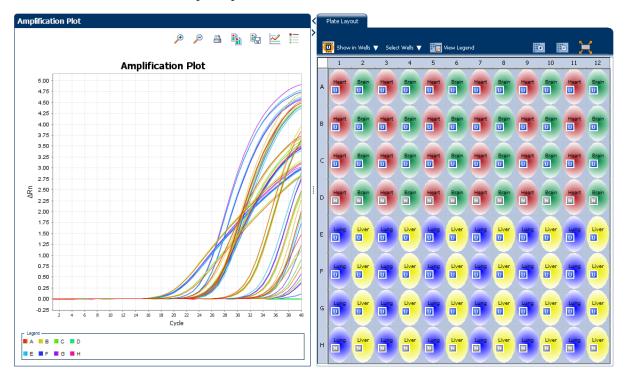
- **1.** In the Instrument Console screen, select the instrument icon.
- 2. Click **Manage Instrument** or double-click on the instrument icon.
- **3.** In the Manage Instrument screen, click **Monitor Running Experiment** to access the Run screen.

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 QuantStudioTM 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.

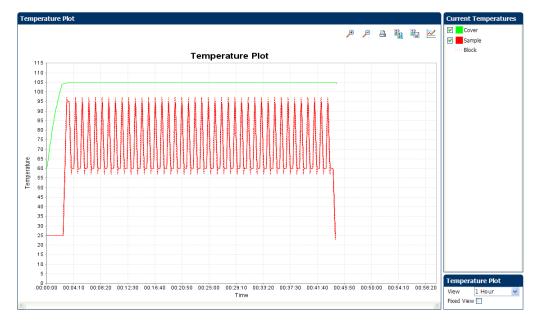
The figure below shows the Amplification Plot screen as it appears at the end of the example experiment.



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.

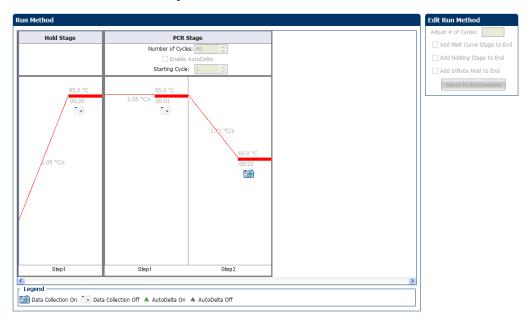


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

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.



Applied Biosystems QuantStudioTM 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Relative Standard Curve and Comparative C_T Experiments

View Run Data

Click View Run Data from the Run Experiment Menu.

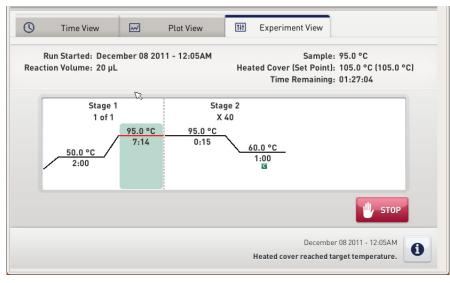
Your View Run Data screen should like this:

Run Data Report	
Experiment Name:	96-Well Fast Comparative Ct Example
Start Time:	09-28-2011 12:49:01 SGT
Stop Time:	09-28-2011 13:33:38 SGT
Run Duration:	44 minutes 36 seconds
User Name:	DEFAULT
Instrument Name:	QuantStudioDemo
Firmware Version:	0.16.1
Software Version:	QuantStudio 12K Flex Software v1.0
Instrument Serial Number:	QuantStudioDemo
Sample Volume:	20.0
Cover Temperature:	105.0
Block Type:	96-Well Block (0.2mL)
Errors Encountered:	× ×

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

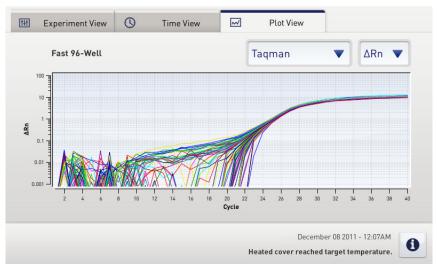


Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

Time View



Plot View





Chapter 10 Run the Experiment *Monitor the run*

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:

Sect	ion 11.1 Review Results 9)5
	Analyze the example experiment) 5
	Assess the gene expression profile using the Gene Expression Plot) 5
	Identify well problems using the Well Table	<i></i> 7
	Assess amplification results using the Amplification Plot	<i>)</i> 9
	Confirm accurate dye signal using the Multicomponent Plot 10)6
	Determine signal accuracy using the Raw Data Plot 10)8
	View the endogenous control profile using the QC Plot 11	0
	Review the flags in the QC Summary 11	1
	For more information 11	13
Sect	ion 11.2 Adjust parameters for re-analysis of your own experiments 11	15
	Adjust analysis settings 11	15
	Improve C _T precision by omitting wells 11	8



Section 11.1 Review Results

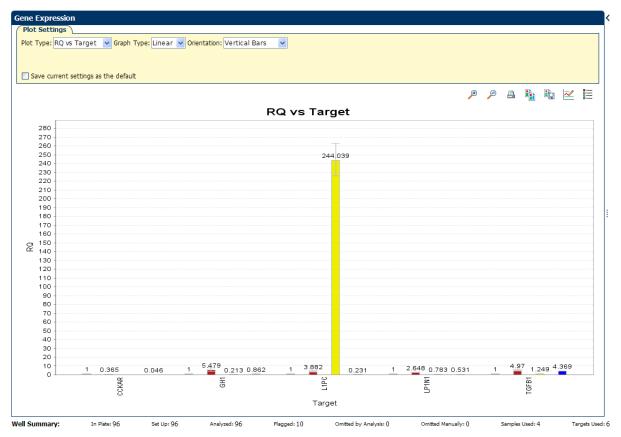
Analyze the example experiment

- 1. Open the example experiment file that you ran in Chapter 10.
- 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

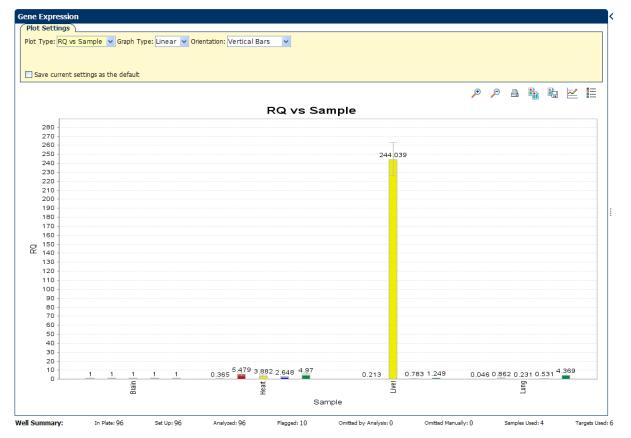
The Gene Expression Plot screen displays the results of relative quantification calculations in the gene expression profile. There are two plots available:

• **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

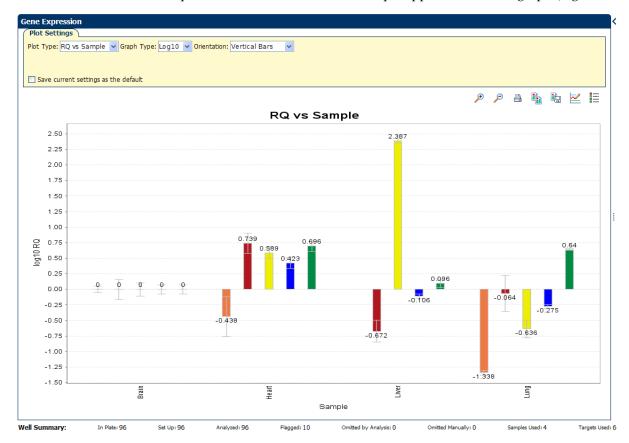
- From the Experiment Menu pane, select Analysis > Gene Expression.
 Note: If no data are displayed, click Analyze.
- 2. In the Gene Expression Plot screen, select:

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

ExampleReview the Well Table to evaluate the CT precision of the replicate groups.experiment valuesand flags



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).

> Plat	e Layout	Well Tat	ble												
Show	in Tabla 💌	Soloct Wall	s ▼ Group by										🗄 Expan	d All 🗖 Col	lapse All
Show		Select Well	s 🗸 Group by	•									U Expan		iapse Air
#	Well	Omit	Flag	Sample	Target	Task	Dyes	Ст	C⊤ Mean	CT SD	ΔCT Δ0	Ст Ме	ΔCT SE	ΔΔCτ	RQ
		🗏 Brain -	L1PC												^
8	A8			Brain	L1PC	UNKNOWN	FAM-NFQ		31.637	0.217		12.054	0.130	0.000	
20	B8			Brain	L1PC	UNKNOWN	FAM-NFQ		31.637	0.217		12.054	0.130	0.000	
32				Brain	L1PC L1PC	UNKNOWN	FAM-NFQ		31.637	0.217		12.054	0.130	0.000	
44	08	🗏 Brain -	D1N1	Brain	LIPC	NTC	FAM-NFQ	Undetermi	_						
4	A4		CF 1111	Brain	LP1N1	UNKNOWN	FAM-NFQ	24,883	24,955	0.133		5.372	0.085	0.000	
16	B4			Brain	LP1N1	UNKNOWN	FAM-NFQ		24.955	0.133		5.372		0.000	
28	C4			Brain	LP1N1	UNKNOWN	FAM-NFQ		24.955	0.133		5.372		0.000	
40				Brain	LP1N1	NTC	-	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 -													
	A11		<u> </u>	Heart	CCKAR	UNKNOWN	FAM-NFQ		37.383	0.649		15.465		1.453	
23	B11		1	Heart	CCKAR	UNKNOWN	FAM-NFQ		37.383	0.649		15.465		1.453	
35	C11 D11		1	Heart	CCKAR CCKAR	UNKNOWN	FAM-NFQ		37.383	0.649		15.465	0.387	1.453	
47	DII	📕 Heart -	CUI	Heart	CCKAR	NTC	FAM-NEQ	Undetermi					_		
1	A1		UNI	Heart	GH1	UNKNOWN	FAM-NFQ	34,655	34.427	0.292		12.509	0.194	-2.454	
13				Heart	GH1	UNKNOWN	FAM-NFQ		34.427	0.292		12.509	0.194	-2.454	
25				Heart	GH1	UNKNOWN	FAM-NFQ		34,427	0.292		12.509	0.194	-2.454	
37				Heart	GH1	NTC	-	Undetermi	011127	0.252		12.000	0.251	21101	
		E 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		32.015	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 Su	nmary:	In	Plate: 96	Set Up: 96	Analy	zed: 96	Flagged: 10	Omi	tted by Analysis: () 0	mitted Manually: ()	Sa	mples Used: 4	Targe	ts Used: (

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



>	Plate Layout	Well Table									
	🛄 Show in Wel	ls 🔻 Select Wells 🔻	View Legend							•	1
	1	2	3	4 5	6	7	8	9	10	11	12
٨	Heart	Brain	Heart U LPINI	Brain Heart	Brain	Heart	Brain	Heart	Brain ACTB	Heart CCKAR	Brain CCKAR
в	Heart	Brain	Heart	Brain LP1N1 Heart	Brain	Heart	Brain	Heart	Brain	Heart CCKAR	Brain
c	Heart	Brain	Heart U LPINI	Brain LP1NI Heart	Brain	Heart	Brain	Heart	Brain ACTB	Heart CCKAR	Brain CCKAR
:	Heart	Brain	Heart N LPINI	Brain LP1N1 Heart	Brain TGFB1	Heart	Brain	Heart ACTB	Brain ACTB	Heart CCKAR	Brain CCKAR
E	Lung U GH1	Liver		Liver Lung	Liver TGFB1	Lung LLIPC	Liver	Lung ACTB	Liver ACTB		Liver
F	Lung U GH1	Liver		Liver Lung	Liver TGFB1	Lung LLIPC	Liver	Lung ACTB	Liver ACTB	Lung L CCKAR	Liver
G	Lung Lung GH1	Liver		Liver LPINI U TGFB1	Liver	Lung U L1PC	Liver	Lung ACTB	Liver	Lung U CCKAR	Liver
F	Lung N GH1	Liver	Lung	Liver LP1NI	Liver TGFB1	Lung	Liver	Lung ACTB	Liver N ACTB	Lung CCKAR	Liver N CCKAR
,	Wells: <u> </u>	24									0 Empty
Wel	l Summary:	In Plate: 96	Set Up: 96	Analyzed: 96	Flagged: 10	Omitted	by Analysis: ()	Omitted Manu	ally: ()	Samples Used: 4	Targets Used: 6

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.

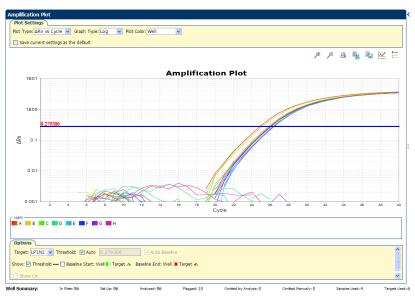
c. Verify that the baseline is set correctly: The end cycle should be set a few cycles before the cycle number where significant fluorescent signal is detected. In the example experiment, the baseline is set correctly.



5. View the threshold values:

Menu	Select
Graph Type	Log
Target	LP1N1

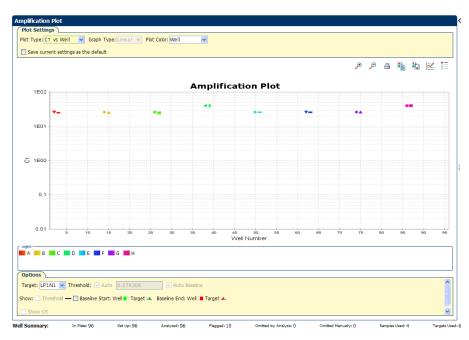
- a. Select the Threshold check box to show the threshold.
- **b.** Verify that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.



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- **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.



 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).

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

Tips for analyzing

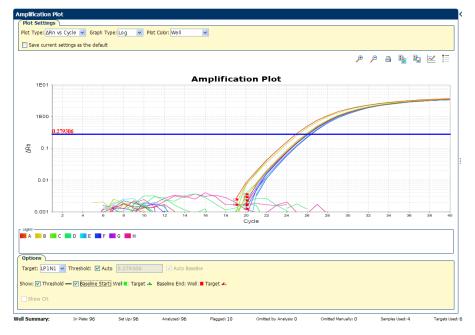
your own

experiments

- Exponential (geometric phase)

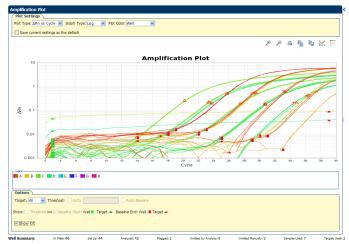
- Baseline

A typical amplification plot should look like this:

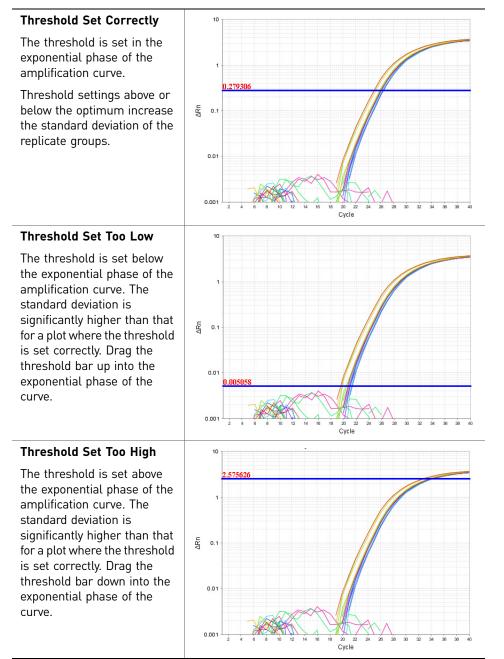


IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudioTM 12K Flex Software. Therefore, Life Technologies recommends that you examine the Amplification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

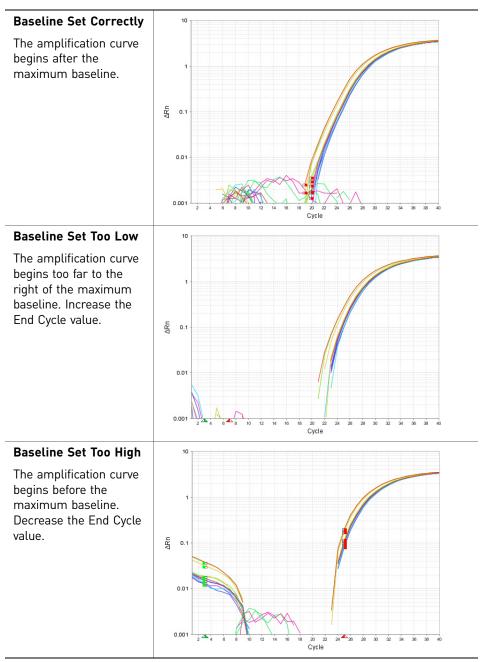
Note: If you use the Relative Threshold algorithm to analyze an experiment that includes amplification, select to view the analysis results using the Δ Rn vs Cycle, Rn vs Cycle, or C_{RT} vs Well plot type and Linear or Log graph type. Also select the **Show Crt** check box to view the derived fractional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



Correct threshold values



Correct baseline values



• 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.

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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:

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

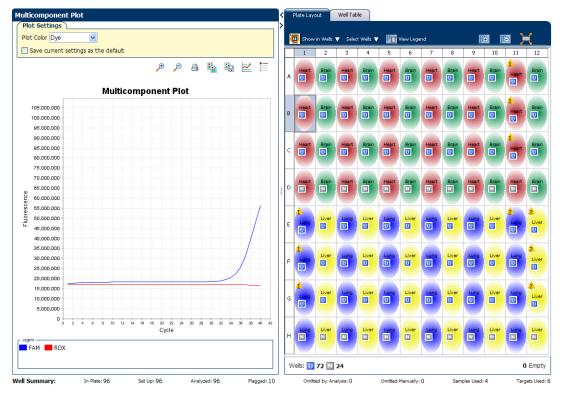
View the Multicomponent Plot

- 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.



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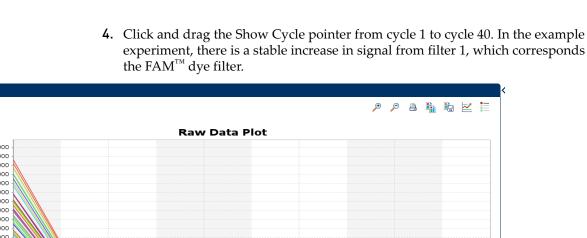
Tips for confirming dye accuracy in your own experiment When you analyze your own Comparative C_T experiment, look for:

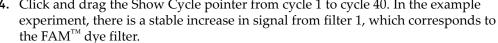
- **Passive reference** The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye** The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification 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.

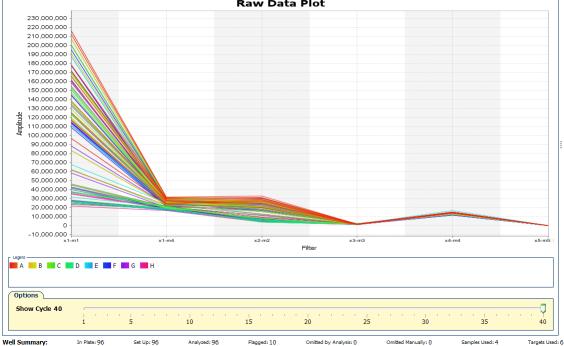
Determine signal accuracy using the Raw Data Plot

	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	 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 Example 1 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).







Raw Data Plot

				Emission Filter			
		m1(520±15)	m2(558±11)	m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
	x1(470±15)	\checkmark			\checkmark		
9	x2(520±10)		\checkmark				
	x3(550±11)						
	x4(580±10)						
	x5(640±10)						
	x6(662±10)						
lt Ci	urve Filter ———			Load Save Revert to Emission Filter	Defaults		
lt Cu	urve Filter ———	m1(520+15)	m2(550+11)	Emission Filter		m5(692+14)	m6(711+12)
lt Cu		m1(520±15)	m2(558±11)	Emission Filter m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
	x1(470±15)	m1(520±15)		Emission Filter m3(586±10)	m4(623±14)		
				Emission Filter m3(586±10)	m4(623±14)		
	x1(470±15) x2(520±10)			Emission Filter m3(586±10)	m4(623±14)		
	x1(470±15) x2(520±10) x3(550±11)			Emission Filter m3(586±10)	m4(623±14)		
	x1(470±15) x2(520±10) x3(550±11) x4(580±10)			Emission Filter m3(586±10)	m4(623±14)		
	x1(470±15) x2(520±10) x3(550±11) x4(580±10) x5(640±10)			Emission Filter m3(586±10)	m4(623±14)		

The filters used for the example experiment are:

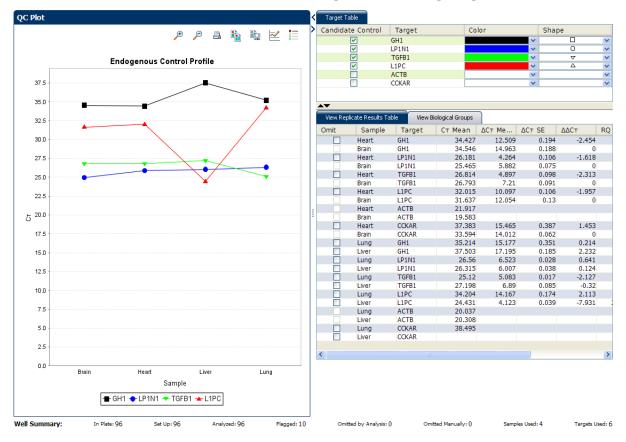


Tips for determining signal accuracy in your own experiment When you analyze your own Comparative C_T experiment, look for the following in each filter:

- 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	 From the Experiment Menu pane, select Analysis > QC Plot. Note: If no data are displayed, click Analyze. In the QC Plot screen, click Target Table. 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. Select a color for each target, from the Color drop-down menu. Select a shape for each target, from the Shape drop-down menu. Click the View Replicate Results Table. Select the check box of the samples to plot. In the example experiment, all the four samples, Brain, Heart, Liver, and Lung are selected. Click Image 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 QC1. From the Experiment Menu pane, select Analysis > QC Summary.SummaryNote: 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.

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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.

QC Summary Flag Details Flag: Wells Description Frequency AMPN Amplification in negative control Bad passive reference signal Fluorescence is offscale BADROX OFFSCALE HIGHSD A11, B11, C11, E1, F1, G1 E11, E12, F12, G12 High standard deviation in replicate group NOAMP NOISE No amplification Noise higher than others in plate SPIKE NOSIGNAL OUTLIERRG EXPFAIL Voise spikes No signal in well Outlier in replicate group Exponential algorithm failed Baseline algorithm failed E11, E12, F12, G12 BLFAIL THOLDFAIL Thresholding algorithm failed CTFAIL CT algorithm failed AMPSCORE AMP Score 96 | Processed Wells: 96 | Flagged Wells: 0 Targets Used: 0 Samples Used: Total Wells 96 Manually Omitted Wells: 6 Wells Set Up: 10 Analysis Omitted Wells: 4 Well Summary: In Plate: 96 Set Up: 96 Analyzed: 96 Flagged: 10 Omitted by Analysis: () Omitted Manually: () Samples Used: 4 Targets Used: 6

The QC Summary 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					
F	Primary analysis flags					
BADROX	Bad passive reference signal					
NOAMP	No amplification					

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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
Se	econdary 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, <i>Getting Started with QuantStudio™ 12K Flex</i> System Multi-Well Plate and Array Card Experiments	4470050



Chapter 11 Review Results and Adjust Experiment Parameters *For more information*

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

1. From the Experiment Menu pane, select Analysis.

settings

- 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:

analysis have Stage 2, S - Default C Default CT se	tep 2 r Settings	lisplayed.	targets without custor	n settings. To edit the default se	ettings, dick Edil	Select the algorithm to calculate CT. Baseline Threshold v t Default Settings.
- Select a T	arget	eshold	O Baseline End Cyc			CT Settings for ACTB
ACTB	AUTO		AUTO	AUTO	^	C⊤ Settings to Use: □ Default Settings ✓ Automatic Threshold
CCKAR	0.170	0213	AUTO	AUTO		Threshold: 0.171002
GH1	0.122	2748	AUTO	AUTO		Automatic Baseline
.1PC	AUTO)	AUTO	AUTO		Baseline Start Cycle: 3 💠 End Cycle: 15 💠
P1N1	0.279	9306	3	25		
TGFB1	AUTO	C	AUTO	AUTO		

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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 QuantStudioTM 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.

Analysis Settings for 96-Well Fast Comparative Ct Example						
Cτ Settings Flag Settings Relative Quantification Settings Advanced Settings						
Configure the flags and filtering. In this panel you can enable, disable, and configure flags, and indicate if a well is to be rejected when a flag is raised.						
Flag	Description	Use	Attribute	Condition	Value	Reject Well
AMPNC	Amplification in negat		Ст	< 🗸	35.000	
BADROX	Bad passive referenc		Bad passive referenc	> *	0.600	
OFFSCALE	Fluorescence is offscale	~				
HIGHSD	High standard deviati	~	CT standard deviation	> 🗸	0.500	
NOAMP	No amplification	✓	Amplification algorith	< 🗸	0.100	
NOISE	Noise higher than ot	✓	Relative noise	> 🗸	4.000	
SPIKE	Noise spikes	✓	Spike algorithm result	> *	1.000	
NOSIGNAL	No signal in well	~				
OUTLIERRG	Outlier in replicate gr	~				
EXPFAIL	Exponential algorithm	~				
BLFAIL	Baseline algorithm failed	~				
THOLDFAIL	Thresholding algorith	~				
CTFAIL	CT algorithm failed	~				
AMPSCORE	AMP Score	~	AMP Score	> *	1.000	
Save to Library	Load from Library		<u>Revert to Defa</u>	ault Analysis Settings	Apply A	nalysis Settings

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.

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.

- 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.

Plate	Layout	Well Table												
5how ir	n Table 🔻	Select Wells 🔻	Group by 🔻									🗉 Expan	HAII 🗖 Col	apse A
#	Well	Omit	Flag Sample	Target	Task	Dyes	Ст	Ст Mean	CT SD	ΔСт	∆Ст Ме	ΔCT SE	ΔΔCT	RQ
28	C4		Brain	LP1N1	UNKNOWN	FAM-NFQ	25.391	25.465	0.114		5.882	0.075	0.000	
40	D4		Brain	LP1N1	NTC	FAM-NFQ	Undetermi							
		🗏 Brain - TGF	B1											
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 - ACT	TB											
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 - CCK	AR											
11	A11		🚹 Heart	CCKAR	UNKNOWN	FAM-NFQ	37.170	37.383	0.649		15.465	0.387	1.453	
23	B11		🚹 Heart	CCKAR	UNKNOWN	FAM-NFQ	38.111	37.383	0.649		15.465	0.387	1.453	
35	C11		🚹 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	l											
	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	
	C1		Heart	GH1	UNKNOWN	FAM-NFQ		34.427	0.292		12.509	0.194	-2.454	
37	D1		Heart	GH1	NTC	FAM-NFQ	Undetermi							
		🗏 Heart - L1P	С											
	A7		Heart	L1PC	UNKNOWN	FAM-NFQ		32.015	0.073		10.097	0.106	-1.957	
	B7		Heart	L1PC	UNKNOWN	FAM-NFQ		32.015	0.073		10.097	0.106	-1.957	
	C7		Heart	L1PC	UNKNOWN	-		32.015	0.073		10.097	0.106	-1.957	
43	D7		Heart	L1PC	NTC	FAM-NFQ	Undetermi							
		Heart - LP1												
	A3		Heart	LP1N1	UNKNOWN	FAM-NFQ		26.181	0.073		4.264	0.106	-1.618	
	B3		Heart	LP1N1	UNKNOWN	FAM-NFQ		26.181	0.073		4.264	0.106	-1.618	
	C3		Heart	LP1N1	UNKNOWN	FAM-NFQ		26.181	0.073		4.264	0.106	-1.618	
39	D3		Heart	LP1N1	NTC	FAM-NFQ	Undetermi				_		_	
-		Heart - TGF		TOTOL	100000	5414 1150	26.020	26.014	0.000		4 007	0.000	2.242	
	A5		Heart	TGFB1		FAM-NFQ		26.814	0.026		4.897	0.098	-2.313	
17	85		Heart	TGFB1	UNKNOWN		26.784	26.814	0.026		4.897	0.098	-2.313	
		<												
	imary:	In Plate	: 96 Set Up: 9	6 Ar	nalvzed: 96	Flagged: 10	Omit	ted by Analysis: () On	nitted Manualiv: 0	Sa	mples Used: 4	Targe	ts Us

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

> Pla	ate Layout	Well Table													
Shov	w in Table 🔻	Select Wells	Group by	•									🖶 Expar	dall 🗖 G	ollapse All
#	Well	Omit	Flag	Sample	Target	Task	Dyes	Ст	Ст Mean	CT SD	ΔCτ Δ	Ст Ме	ΔCT SE	ΔΔCτ	RQ
28	8 C4			Brain	LP1N1	UNKNOWN	FAM-NFQ	25.391	25.465	0.114		5.882	0.075	0.00	0 ^
40	0 D4			Brain	LP1N1	NTC	FAM-NFQ	Undetermi							
		🗏 Brain - TG	FB1												
6				Brain	TGFB1	UNKNOWN	FAM-NFQ		26.793	0.144		7.210	0.091	0.00	
18				Brain	TGFB1	UNKNOWN	FAM-NFQ		26.793	0.144		7.210	0.091	0.00	
	0 C6			Brain	TGFB1	UNKNOWN	FAM-NFQ		26.793	0.144		7.210	0.091	0.00	0
42	2 D6		CTD	Brain	TGFB1	NTC	FAM-NFQ	Undetermi				_		_	
9	A9	Heart - A	CIB	Heart	ACTB	UNKNOWN	FAM-NFO	22.101	21.917	0.168					
21				Heart	ACTB	UNKNOWN	FAM-NEQ		21.917	0.168					
	3 C9			Heart	ACTB	UNKNOWN	FAM-NFQ		21.917	0.168					
	5 D9	H		Heart	ACTB	NTC		Undetermi	21.917	0.108					
4.	5 09	Heart - C	CKAR	Healt	ACTD	NIC	PAPI-NPQ	ondecenni				_		_	
11	1 A11			Heart	CCKAR	UNKNOWN	FAM-NFO	37,170	37.383	0.649		15.465	0.387	1.45	3
	3 B11		1	Heart	CCKAR	UNKNOWN	FAM-NFO		37,383	0.649		15.465	0.387	1.45	
	5 C11	H	1	Heart	CCKAR	UNKNOWN	FAM-NFO		37,383	0.649		15,465	0.387	1.45	
	7 D11		-	Heart	CCKAR	NTC		Undetermi	071000	01015		101100	0.007	1.1.0	
		Heart - G	H1												
1	A1			Heart	GH1	UNKNOWN	FAM-NFQ	34.655	34.427	0.292		12,509	0.194	-2.45	4
13	3 B1			Heart	GH1	UNKNOWN	FAM-NFQ		34.427	0.292		12,509	0.194	-2.45	4
25	5 C1			Heart	GH1	UNKNOWN	FAM-NFQ	34.527	34.427	0.292		12.509	0.194	-2.45	4
37	7 D1			Heart	GH1	NTC	FAM-NFQ	Undetermi							
		🗏 Heart - L1	1PC												
7	A7			Heart	L1PC	UNKNOWN	FAM-NFQ	31.978	32.015	0.073		10.097	0.106	-1.95	7
19	9 B7			Heart	L1PC	UNKNOWN	FAM-NFQ	32.099	32.015	0.073		10.097	0.106	-1.95	7
31	1 C7			Heart	L1PC	UNKNOWN	FAM-NFQ	31.967	32.015	0.073		10.097	0.106	-1.95	7
43	3 D7			Heart	L1PC	NTC	FAM-NFQ	Undetermi							
		🗏 Heart - LF	P1N1												
3				Heart	LP1N1	UNKNOWN	FAM-NFQ		26.181	0.073		4.264	0.106	-1.61	
	5 B3			Heart	LP1N1	UNKNOWN	FAM-NFQ		26.181	0.073		4.264	0.106	-1.61	
	7 C3			Heart	LP1N1	UNKNOWN	FAM-NFQ		26.181	0.073		4.264	0.106	-1.61	8
39	9 D3			Heart	LP1N1	NTC	FAM-NFQ	Undetermi							
		🗏 Heart - T	GFB1												
5				Heart	TGFB1	UNKNOWN	FAM-NFQ		26.814	0.026		4.897	0.098	-2.31	
17	7 B5			Heart	TGFB1	UNKNOWN	FAM-NFQ	26.784	26.814	0.026		4.897	0.098	-2.31	
		<													>
Well Su	ummary:	In Pla	ite: 96	Set Up: 96	Analy	zed: 96	Flagged: 10	Omit	ted by Analysis: () Or	nitted Manually: ()	Sa	mples Used: 4	Targ	gets Used: 6

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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**.

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	<pre><drive>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments</drive></pre>

Your Export screen should look like this:

								_	
ort File Location: C:\Applie	d Biosystems\Qu	iantStudi	o 12K Flex Software\Ut	Browse Export File	Name: 96-Well Fa	st Comparative C	t Example_Quar	r File Type: 📋 (*.	txt)
Sample Setup	Data 🗌 Ampl	ification	Multicomponent	Tech. Rep. Results	Bio. Rep. Resul	ts 🗸 Results			
Skip Empty Wells 🗹 Skip C	Omitted Wells								
Select Content		Well	Well Position	Sample Name	Delta Ct SD	Target Name	Task	Reporter	0
All Fields	^	wen	1 A1	Heart		GH1	UNKNOWN	FAM	N
			2 A2	Brain		GH1	UNKNOWN	FAM	NE
Well			3 A3	Heart		5LP1N1	UNKNOWN	FAM	N
I Mall Deathles			4 A4	Brain		LP1N1	UNKNOWN	FAM	N
Well Position			5 A5	Heart		TGFB1	UNKNOWN	FAM	N
Sample Name			6 A6	Brain		TGFB1	UNKNOWN	FAM	N
			7 A7	Heart	0.18	L1PC	UNKNOWN	FAM	N
Target Name			8 A8	Brain	0.22	5 L1PC	UNKNOWN	FAM	N
7 - 4			9 A 9	Heart		ACTB	UNKNOWN	FAM	N
Z Task			10 A10	Brain		ACTB	UNKNOWN	FAM	N
Reporter			11 A11	Heart	0.67	CCKAR	UNKNOWN	FAM	N
			12 A12	Brain	0.10	3 CCKAR	UNKNOWN	FAM	N
Quencher			13 B1	Heart	0.33	GH1	UNKNOWN	FAM	N
			14 B2	Brain	0.32	5 GH1	UNKNOWN	FAM	N
RQ			15 B3	Heart	0.18	5 LP1N1	UNKNOWN	FAM	N
RQ Min			16 B4	Brain		LP1N1	UNKNOWN	FAM	N
			17 B5	Heart		TGFB1	UNKNOWN	FAM	N
RO Max			18 B6	Brain		TGFB1	UNKNOWN	FAM	N
			19 B7	Heart		B L1PC	UNKNOWN	FAM	N
CT CT			20 B8	Brain	0.22	5 L1PC	UNKNOWN	FAM	N
Ct Mean			21 B9	Heart		ACTB	UNKNOWN	FAM	N
			22 B10	Brain	0.67	ACTB	UNKNOWN	FAM	N
Ct SD			23 B11	Heart		CCKAR	UNKNOWN	FAM	N
-			24 B12 25 C1	Brain		CCKAR	UNKNOWN	FAM	N
Quantity			25 C1 26 C2	Heart Brain		7 GH1 5 GH1	UNKNOWN	FAM FAM	N
Delta Ch Mann			26 C2	Brain Heart		5 LP1N1	UNKNOWN	FAM	N
Delta Ct Mean			27 C3	Brain		LPINI 7 LP1N1	UNKNOWN	FAM	N
Delta Ct SD			28 C4 29 C5	Heart		TGFB1	UNKNOWN	FAM	N
			29 00	riedit	0.17	10.01			
_			30 C6	Brain	0.15	TGFB1	UNKNOWN	FAM	N

Start Export Save Export Set As Load Export Set Delete Export Set

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

5 96-Well Fast Comparative Ct Example_QuantStudio_export.txt - Notepad		
File Edit Format View Help		
<pre>* Barcode = NA * Block Type = 96-well Block (0.2mL) * 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 ROI performed on = 2011-08-08 02:07:15 AM SGT * Calibration ROX performed on = 2011-08-08 02:07:15 AM SGT * Calibration SVBR is expired = No * Calibration SVBR is expired = No * Calibration SVBR performed on = 2011-08-08 02:07:15 AM SGT * Calibration TAMRA is expired = No * Calibration TAMRA performed on = 2011-08-08 02:16:10 AM SGT * Calibration TAMRA performed on = 2011-08-08 02:16:10 AM SGT * Calibration Uniformity performed on = 2011-08-08 01:24:47 AM SGT * Calibration Uniformity performed on = 2011-08-08 01:49:09 AM SGT * Calibration VIC is expired = No * Comparity ve C (XAMPA * Date (meet File Name = C:\Program Files\Applied Biosystems\QuantStudio12KFlex\examples\Gene Expression\Comparat * Experiment Nume = 96-well Fast Comparative Ct Example * Experiment Rune = 96-well Fast Comparative Ct (ACCT) * Instrument Serial Number = QuantStudioDemo * Instrument Name = QuantStudioDemo * Instrument Name = QuantStudioDemo * Instrument Serial Number = QuantStudioDemo * Instrument Name = QuantStudioDemo * Instrument Name = QuantStudioDemo * Instrument Syne expired = Rox * Quantification (Cycle Method = Ct * Signal Smoothing On = true * Stage(Cycle Wire Analysis is performed = Stage 2, Step 2 * User Name = NA</pre>	tive Ct∖96-well	Fast
SD Quantity Delta Ct Mean Delta Ct SD Delta Delta Ct Automatic Ct Threshold Ct Threshold A	RQ Max CT Automatic Basel	Ct Mean Ct ine
Baseline Start Baseline End Efficiency EXPFAIL HIGHS NOAMP 1 A1 Heart GH1 UNKNOWN FAM NFQ-MGB 5.479 3.769 7.965 34.655 34.427 0.292 1 false 0.123 true 3 29 1.000 N N N	12.509 0.337	-2.454
	14.963 0.326	0.000
	3.967 0.185	-1.405
	5.372 0.147	0.000
	4.897 0.170	-2.313
	7.210 0.157	0.000
	10.097 0.183	-1.957

Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Relative Standard Curve and Comparative C_T Experiments

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GETTING STARTED GUIDE



Booklet 4 - Running Genotyping Experiments

Publication Part Number 4470050 Rev. A Revision Date March 2012



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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 (Δ Rn) value per the following formula:

 Δ Rn = Rn (post-PCR read) – Rn (pre-PCR read), where Rn = normalized readings.

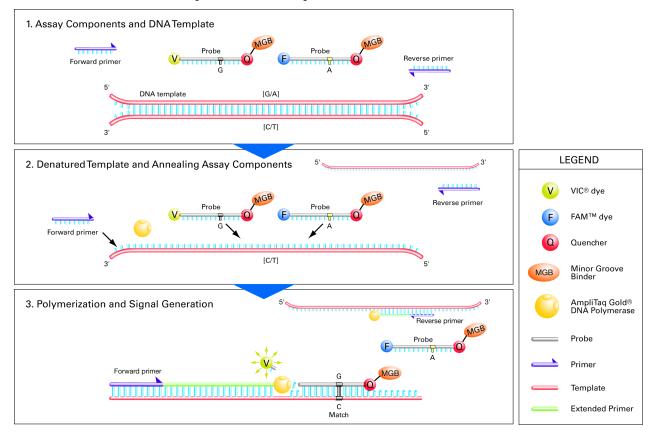
About TaqMan [®] SNP Genotyping assays	A Genotyping assay detects variants of a single nucleic acid sequence, without puantifying the target. The presence of two probes in each reaction allows Genotyping of the two possible variants at the single nucleotide polymorphism (SNP) site in a arget sequence.					
	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 [®]	Each allele-specific TaqMan [®] MGB probe has:					
MGB probes	• A reporter dye at its 5' end:					
	 VIC[®] dye is linked to the 5' end of the Allele 1 probe. 					
	- FAM TM 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 TM 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 TM 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

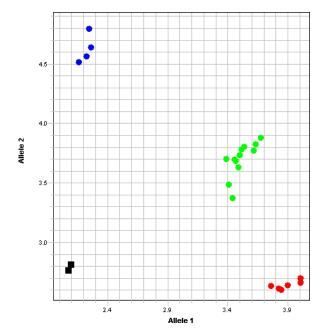
In TaqMan[®] assays, fluorescence from nonspecifically bound probes is reduced, because nucleotide mismatches between a probe and a sequence reduce the chances that the probe will be cleaved. The probe's short length means that a one-base-pair mismatch will a have a larger negative effect on the binding. 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 allelespecific probes. Finally, the QuantStudioTM 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
$VIC^{\textcircled{B}}$ 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.*

Design the Experiment

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

This chapter covers:

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Define the experiment properties.	11
Define SNPs and samples	12
Assign markers and samples	14
Set up the run method	16
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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*TM 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 QuantStudioTM 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:

How do you want to identify this experi	iment?			
Experiment Name: 96-Well Genotyping Examp Barcode: User Name: Example User	le	Comments:	Genotyping example	▲ ⊻
* Which block are you using to run the	experiment?			
384-Well	Array Card	1	96-Well (0.2mL)	Fast 96-Well (0.1mL)
What type of experiment do you want	to set up?			
Standard Curve	Relative Standard Curve	Сог	mparative Cτ (ΔΔCτ)	Melt Curve
✓ Genotyping	Presence/Absence			
* Which reagents do you want to use to	detect the target sequence?			
✓ TaqMan® Reagents	Other			
* What properties do you want for the i	instrument run?			
✓ Standard	Fast			
Include: V Pre-PCR Read V Amplification V	Post-PCR Read			

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	

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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:

SNPs									
New Edit Save to Library	Import from Library D	elete							
SNP Assay Name	NCBI SNP Refer	Context Seque	Allele 1	Reporter	Quencher	Allele 2	Reporter	Quencher	Color
NP Assay 1			Allele 1	VIC	NFQ-MGB	Allele 2	FAM	NFQ-MGB	
NP Assay 2			Allele 1	VIC	NFQ-MGB	Allele 2	FAM	NFQ-MGB	
Samples									
	t from Library Delete								
New Save to Library Impor	t from Library Delete					Color			
New Save to Library Impor Sample Name	t from Library Delete					Color			
New Save to Library Impor Sample Name ample 1	t from Library Delete					Color			
New Save to Library Impor Sample Name ample 1 ample 2	t from Library Delete					Color			`
New Save to Library Impor Sample Name ample 1 ample 2 ample 3	t from Library Delete					Color			•
New Save to Library Impor Sample Name ample 1 ample 2 ample 3 ample 4	t from Library Delete					Color			•
New Save to Library Impor Sample Name Sample 1 Sample 2 Sample 3 Sample 4 Sample 5	t from Library Delete					Color			•
New Save to Library Impor Sample Name Impor Impor Sample 1 Impor Impor Sample 2 Impor Impor Sample 3 Impor Impor Sample 4 Impor Impor Sample 5 Impor Impor Sample 6 Impor Impor	t from Library Delete					Color Color			•
New Save to Library Impor Sample Name Impor Impor Sample 1 Impor Impor Sample 2 Impor Impor Sample 3 Impor Impor Sample 4 Impor Impor Sample 5 Impor Impor Sample 6 Impor Impor	t from Library Delete					Color Color			•
New Save to Library Impor Sample Name ample 1 ample 1 ample 1 ample 3 ample 4 ample 5 ample 6 ample 7	t from Library Delete					Color Color			
New Save to Library Impor Sample Name ample 1 ample 1 ample 1 ample 2 ample 3 ample 3 ample 4 ample 5 ample 6 ample 7 ample 7 ample 8 ample 8 ample 8	t from Library Delete					Color			•
Samples New Save to Library Impor Sample Name Sample 1 Sample 2 Sample 3 Sample 4 Sample 5 Sample 6 Sample 6 Sample 8 Samnle 9 Passive Reference	t from Library Delete					Color Color			

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

Assign markers and samples

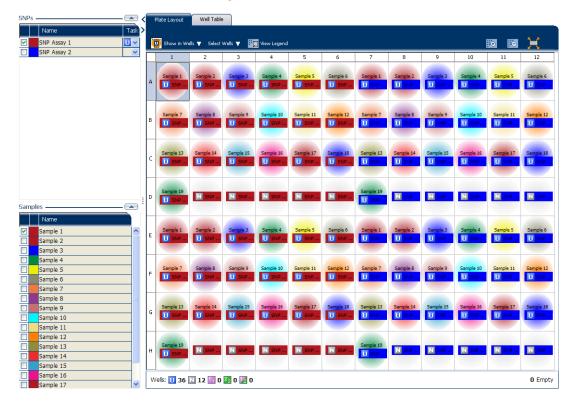
Click Assign to access the Assign screen. Enter the SNP assays and samples:

• 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	
	H2 - H6	Control	

•	SNP 2	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	
	H8 - H12	Control	



Your Assign screen should look like this:

Set up the run method

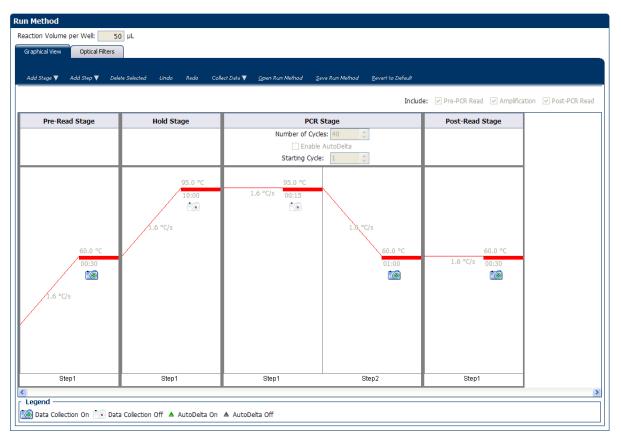
Click **Run Method** to access the Run Method screen. Set the thermal profile under the Graphical View tab. Enter:

- 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	Step 1	1.6°C/s	95°C	15 seconds
 Number of Cycles: 40 (default) 	Step 2	1.6°C/s	60°C	1 minute
 Enable AutoDelta: Unchecked (default) 				
 Starting Cycle: Disabled when Enable AutoDelta is unchecked 				
Post-Read Stage	Step 1	1.6°C/s	60°C	30 seconds

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2



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, <i>Getting Started with QuantStudio™ 12K Flex</i> System Multi-Well Plate and Array Card Experiments	4470050
	Appendix A in Booklet 7, <i>QuantStudio™ 12K Flex System Multi-Well</i> Plate and Array Card Experiments - Appendixes	
Data collection	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 12K Flex</i> 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, <i>Getting Started with QuantStudio™ 12K Flex</i> System Multi-Well Plate and Array Card Experiments	4470050



Chapter 2 Design the Experiment *For more information*

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

3

- 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 $10 \text{ ng}/\mu\text{L}$.

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 $2 \mu L$ of sample stock to each empty tube.
- 3. Add 48 μ L of sterile water (diluent) to each tube, such that each working stock tube has a final concentration of 10 ng/ μ L
- 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 volume						
Reaction component	Per we	ell (µL)	38 Reactions + 10% excess (µL)				
	Dry	Wet	Dry	Wet			
TaqMan [®] Genotyping Master Mix (2×)	10.0	10.0	420.0	420.0			
SNP Assay Mix (20X)	1.0	1.0	42.0	42.0			
H ₂ 0, 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 🛄

>	Plate Layout Well Table											
	U Show in Wells	🔻 Select Wells 🔻	View Legen	ł							٠	d 🔀
	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
в	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
c	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18
1	Sample 19		NP Assa	N SNP Assa	N SNP Assa	N SNP Assa	Sample 19	N SNP Assa				
E	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
F	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
G	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18
н	Sample 19		NP Assa	N SNP Assa	N SNP Assa	N SNP Assa	Sample 19	N SNP Assa				
١	Wells: 🚺 36 🔝 12 🔄 0 💆 0 📈 0 Empty											

The reaction plate for the example experiment looks like this:

To prepare the reaction plate: dried gDNA

1. Pipette 2.0 μ L 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, ampliconfree 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	1. Add 2 μ L of DNA to the appropriate wells.
reaction plate: wet	2. Add 2 μ L of water to wells containing the NTCs.
gDNA	3. Transfer 48 μ L 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 prepa	ring reactions for your own experiments
Tips for preparing	When you prepare the samples for your own experiment:
samples	• 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	When you prepare the reaction plate for your own experiment:
the reaction plate	• 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, <i>Getting Started with QuantStudio™ 12K</i> Flex System Multi-Well Plate and Array Card Experiments	4470050
Sealing the reaction plate	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 12K</i> Flex System Multi-Well Plate and Array Card Experiments	4470050

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

This chapter covers:

- 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 QuantStudioTM 12K Flex Instrument is in operation.

Start the run

4

- 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 is 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 QuantStudioTM 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 QuantStudioTM 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.

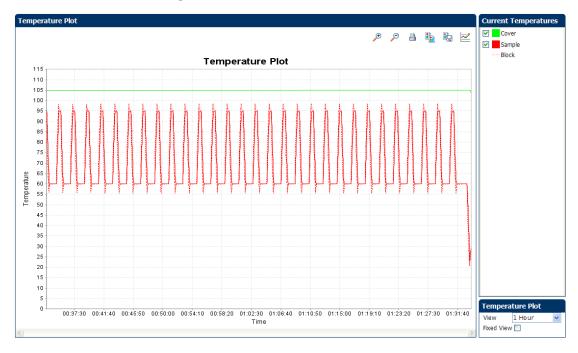
Note: The Amplification Plot is not available for experiments that do not include the PCR process.



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.

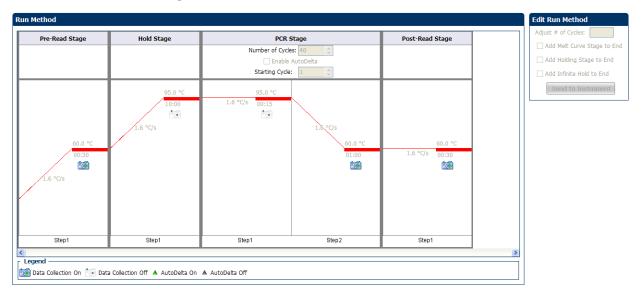


Note: The sample temperature displayed in the Current Temperatures group is a calculated value.

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.



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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.

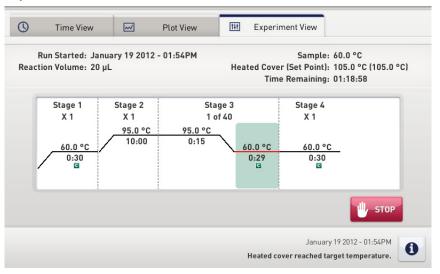
Run Data Report	
Experiment Name:	96-Well Genotyping Example
Start Time:	08-11-2011 13:39:45 5GT
Stop Time:	08-11-2011 15:13:21 SGT
Run Duration:	93 minutes 35 seconds
User Name:	DEFAULT
Instrument Name:	QuantStudioDemo
Firmware Version:	0.13.1
Software Version:	QuantStudio 12K Flex Software v1.0
Instrument Serial Number:	QuantStudioDemo
Sample Volume:	50.0
Cover Temperature:	105.0
Block Type:	96-Well Block (0.2mL)
Errors Encountered:	 X

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

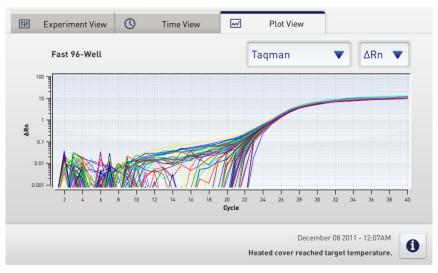


Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

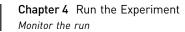
Time View



Plot View



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

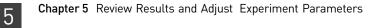


Review Results and Adjust Experiment Parameters

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:

Sect	tion 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
Sect	tion 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.
- 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.

1. From the Experiment menu pane, select **Analysis** • **Allelic Discrimination Plot**.

- To view and assess the 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.

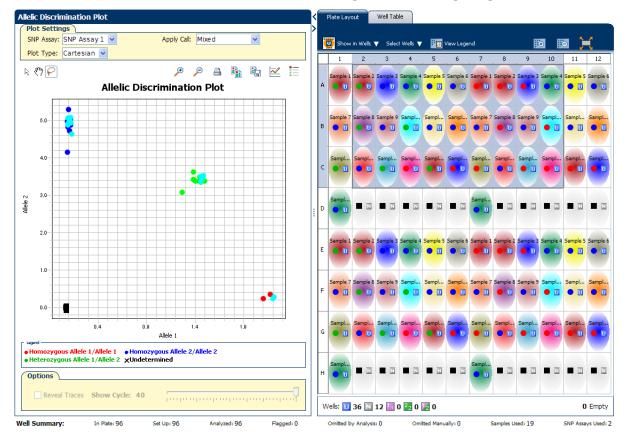
The Allelic Discrimination Plot displays allele symbols for each sample evaluated for the selected SNP. The samples are grouped on the plot as follows:

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. Source - 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.

n

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

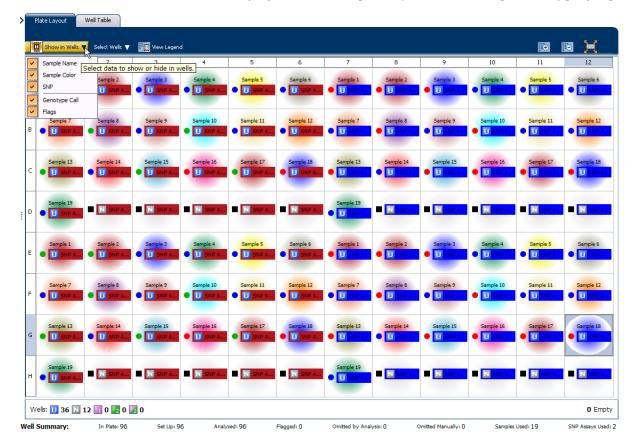
Sample Name

Review the experiment results in the Plate Layout. The plate layout displays the assayspecific 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	• 24 samples as Alle	
View the layout	experiment does not di 1. Click the ≮icon be layout	eside the Allelic Discrimination Plot to maximize the plate
	2. Click Show in wells to display. R parameters.	n Wells, then select or deselect a parameter that you want the epeat this step until the plate layout contains all of the desired

The name of the sample applied to the well.

Parameter	Description
Task	The task assigned to the well:
	🛄 – Unknown
	🔟 – No Template Control
	Interpretention - Allele 1
	22 – 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 $igslash$ 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 \triangle . 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 \leq 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:
	• Δ Rn vs. Cycle – Δ Rn is the difference in normalized fluorescence signal generated by the reporter between the pre-PCR read and the post-PCR read. This plot displays Δ R _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 Δ Rn 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).

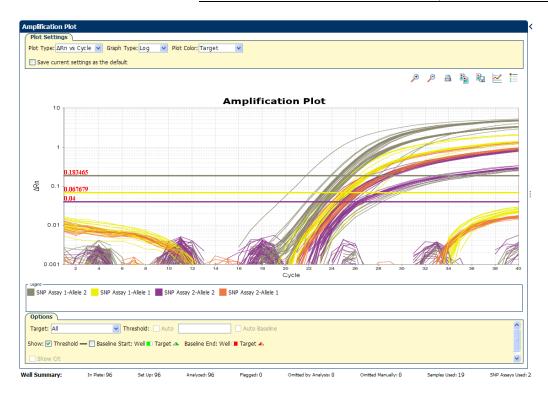
Each plot can be viewed as a linear or log10 graph type.



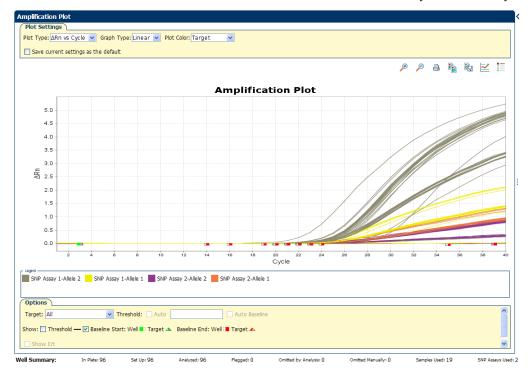
View the $\triangle Rn$ vs. Cycle plot

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

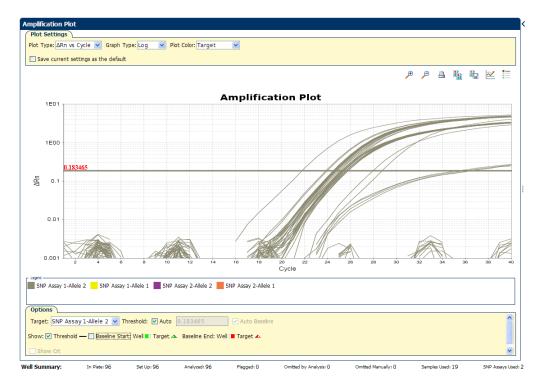


- **3.** View the baseline values:
 - **a**. From the Graph Type drop-down menu, select Linear.

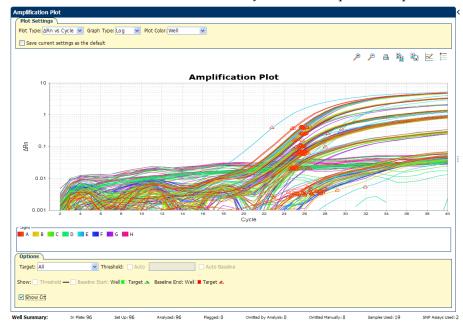


b. Select Baseline to show the start cycle and end cycle.

- **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 Δ Rn 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.

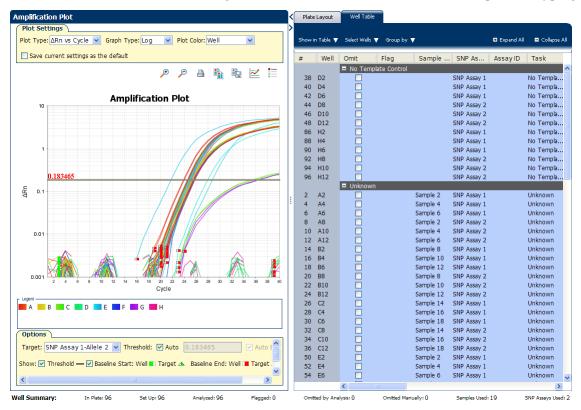


Identify well problems using the Well Table

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
View the well table
Select the Well Table tab.
Click the Flag column header to sort the data so that the wells that triggered flags appear at the top of the table.
Confirm the integrity of the controls:

From the Group By menu, select Task to organize the table rows by their function on the reaction plate.
Confirm that each of the controls do not display flags (△).
Click the I 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 (\frown) 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 X 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 (\triangle) 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 .

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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 (\triangle) 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 (\triangle) 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)
- FAMTM 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.

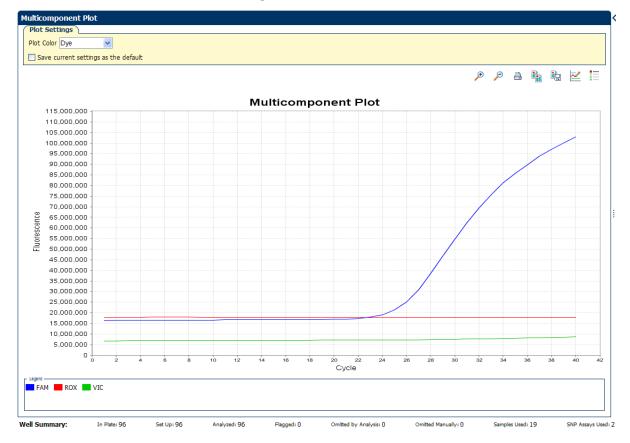
Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

- 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.



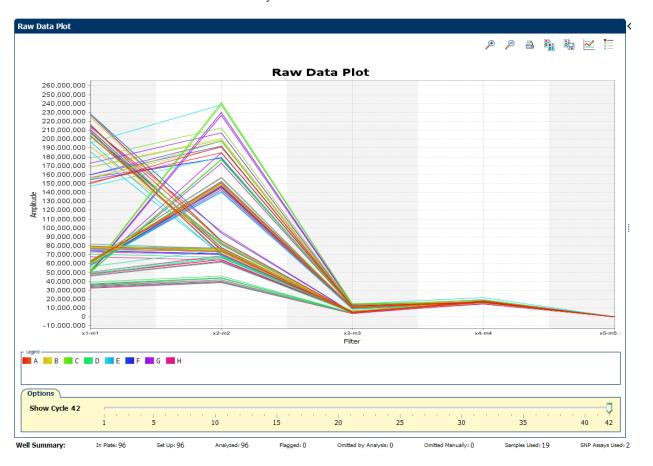
Determine signal accuracy using the Raw Data Plot

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

- 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.



			Load Save Revert to	Defaults		
			Emission Filter			
	m1(520±15)	m2(558±11)	m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
x1(470±						
x2(520± x3(550± x4(580±	.0)	V				
x3(550±	1)		×			
x4(580±	LO)			\checkmark		
x5(640±	LO)				\checkmark	
x6(662± t Curve Filter -			Lasd Save Revert to	Defaults		
x6(662±				Defaults		
	10) m1(520±15)	m2(558±11)	Load Save Revert & Emission Filter m3(586±10)	Defaults m4(623±14)	m5(682±14)	m6(711±12)
	m1(520±15)	m2(558±11)	Emission Filter		m5(682±14)	
t Curve Filter - x1(470±	m1(520±15)		Emission Filter m3(586±10)	m4(623±14)		m6(711±12)
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t Curve Filter - x1(470±	m1(520±15) 15) □ 10) □		Emission Filter m3(586±10)	m4(623±14)		m6(711±12)
t Curve Filter - x1(470± x2(520± x3(550±	m1(520±15) 15) 10) 11) 10) 10) 11) 10) 11) 11) 11) 11) 11) 11) 12) 12) 13) 14) 15) 15) 15) 15) 15) 15) 15) 15)		Emission Filter m3(586±10)	m4(623±14)		m6(711±12)

The filters are:

Tips for determining signal accuracy in your

When you analyze your own Genotyping experiment, look for the following in each filter:

Characteristic signal growth

own experiment

- 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

5

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.

g Details				
lag:	Description	Frequency	Wells	
DROX	Bad passive reference signal	0		
FSCALE	Fluorescence is offscale	0		
SIGNAL	No signal in well	0		
FAIL	Positive control failed	0		
CLUSTER	Small number of samples in cluster	0		
PNC	Amplification in negative control	0		
AMP	No amplification	0		
ISE	Noise higher than others in plate	0		
KE	Noise spikes	0		
PFAIL	Exponential algorithm failed	0		
AIL	Baseline algorithm failed	0		
OLDFAIL	Thresholding algorithm failed			
AIL	CT algorithm failed	0		
IPSCORE	AMP Score	0		
Flag Detail: Passi	OX—Bad passive reference signal /e reference signal is abnormal.			
Flag Detail: Passiv Flag Criteria: Fluore	ve reference signal is abnormal. escence < 500.0			
Flag Detail: Passiv Flag Criteria: Fluore lagged Wells: None	ve reference signal is abnormal. escence < 500.0			
Flag Detail: Passiv Flag Criteria: Fluore lagged Wells: None	ve reference signal is abnormal. escence < 500.0			
Flag Detail: Passiv Flag Criteria: Fluore lagged Wells: None	ve reference signal is abnormal. escence < 500.0			
Flag Detail: Passiv Flag Criteria: Fluore agged Wells: None	ve reference signal is abnormal. escence < 500.0			
Flag Detail: Passiv Flag Criteria: Fluore lagged Wells: None	ve reference signal is abnormal. escence < 500.0			
Flag Detail: Passiv Flag Criteria: Fluore lagged Wells: None	ve reference signal is abnormal. escence < 500.0			
Flag Detail: Passiv Flag Criteria: Fluore lagged Wells: None	ve reference signal is abnormal. escence < 500.0	96 Manually Omitted Wells: 0 Analysis Omitted Wells:	0 SNP Assays Used: 0 Samples Used:	

Possible flags	The flags listed below may be triggered by the experiment data or the assa	ıy.
----------------	--	-----

-

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					
S	econdary 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, <i>Getting Started with QuantStudio™ 12K Flex</i> 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**.

 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:

- Data Analysis () Analyze Data () Analyze Real- ⁻	from Post-PCR Read Only			Data from Pre-PCR Read a Real-Time Rn - Median(Rna	
			_	he default settings, dick Edit 95 Edit Default Set	_
- Select a SNP /	Assay				Call Settings for SNP Assay 1
SNP Assay	Analysis Type	Autocaller	Keep Manual C	Quality Value	Apply Call Settings: 🔽 Default Settings
SNP Assay 1	Default	Yes	No	95	Autocaller Enabled
SNP Assay 2	Default	Yes	No	95	Quality Value: 95.0

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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 QuantStudioTM 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 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.

Flag	Description	Use	Attribute	Condition	Value	Reject Well
ADROX	Bad passive referenc	>	Fluorescence	< •	500.000	✓
FFSCALE	Fluorescence is offscale	V				
OSIGNAL	No signal in well	~				
CFAIL	Positive control failed	V				
MCLUSTER	Small number of sam	V	Number of data poin	≤ •	2.000	
MPNC	Amplification in negat	>	Ст	< •	35.000	
DAMP	No amplification	V	Amplification algorith	< *	0.100	
OISE	Noise higher than ot	>	Relative noise	> •	4.000	
PIKE	Noise spikes	>	Spike algorithm result	> •	1.000	
KPFAIL	Exponential algorithm	V				
LFAIL	Baseline algorithm failed	~				
HOLDFAIL	Thresholding algorith					
TFAIL	CT algorithm failed	V				
MPSCORE	AMP Score	V	AMP Score	> •	1.000	
MPSCORE	AMP Score		AMP Score	>	1.000	

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



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**.

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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	<pre><drive>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments</drive></pre>

Your Export screen should look like this:

ort File Location: C:\Applied Biosystems	s\QuantStudio I	L2K Flex Software\U	Browse Export File	Name: 96-Well Ge	notyping Exampl	e_QuantStudio_e File	• Type: 📋 (*.txt)	
Sample Setup	Amplification	Multicomponent	Results					
Skip Empty Wells V Skip Omitted Wells	- Inparted to I							
Select Content	Well	Well Position	Sample Name	SNP Assay N	Task	Allele1 Delta	Allele2 Delta	
All Fields	^ Vven			1				
		1 A1 2 A2	Sample 1	SNP Assay 1	custom task	1.458	3.343	
Z Well		2 A2 3 A3	Sample 2 Sample 3	SNP Assay 1 SNP Assay 1	UNKNOWN custom task	1.474	3.455 4.941	
		4 A4	Sample 4		UNKNOWN	0.124	5.080	
Well Position		4 A4 5 A5	Sample 5	SNP Assay 1 SNP Assay 1	custom task	0.122	5.080	
Sample Name		6 A6	Sample 6	SNP Assay 1 SNP Assay 1	UNKNOWN	0.108	5.020	
Sample Name		7 A7	Sample 1	SNP Assay 2	custom task	0.979	0.371	
SNP Assay Name		8 A8	Sample 2	SNP Assay 2	UNKNOWN	0.981	0.357	
,		9 A 9	Sample 3	SNP Assay 2 SNP Assay 2	custom task	0.913	0.403	
Z Task		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	
🛛 Allele1 Delta Rn		12 A12	Sample 6	SNP Assay 2	UNKNOWN	0.002	0.885	
Allele2 Delta Rn	=	13 B1	Sample 7	SNP Assay 1	custom task	0.122	4.885	
Allelez Deita Kil	-	14 B2	Sample 8	SNP Assay 1	UNKNOWN	1.491	3.516	
Pass.Ref		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	
Quality(%)		17 B5	Sample 11	SNP Assay 1	custom task	0.098	4.933	
		18 86	Sample 12	SNP Assay 1	UNKNOWN	0.093	4.873	
🗹 Call		19 B7	Sample 7	SNP Assay 2	custom task	0.093	0.852	
Method		20 88	Sample 8	SNP Assay 2	UNKNOWN	1.011	0.280	
- Hodiod		21 89	Sample 9	SNP Assay 2	custom task	0.101	0.894	
Allele1 Automatic Ct Threshold		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	
Allele1 Ct Threshold		24 B12	Sample 12	SNP Assay 2	UNKNOWN	0.109	0.904	
Allele1 Automatic Baseline		25 C1	Sample 13	SNP Assay 1	custom task	1.501	3.380	
Allelet Automatic Baseline		26 C2	Sample 14	SNP Assay 1	UNKNOWN	0.130	4.641	
Allele1 Baseline Start		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	
Allele1 Baseline End		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	

Start Export Save Export Set As Load Export Set Delete Export Set

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

96-Well Genotyping Example_QuantStudio_export.txt - Notepad	
File Edit Format View Help	
<pre>P 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 performed on = 2011-08-08 01:39:58 AM SGT Calibration ROI performed on = 2011-08-08 01:05:24 AM SGT Calibration ROI performed on = 2011-08-08 01:05:24 AM SGT Calibration ROX performed on = 2011-08-08 01:05:24 AM SGT Calibration ROX performed on = 2011-08-08 01:05:24 AM SGT Calibration SVBR performed on = 2011-08-08 01:58:11 AM SGT Calibration SYBR performed on = 2011-08-08 01:58:11 AM SGT Calibration SYBR performed on = 2011-08-08 01:26:10 AM SGT Calibration TAMRA performed on = 2011-08-08 01:24:47 AM SGT Calibration Uniformity is expired = NO Calibration Uniformity is expired = NO State (Civic Where Analysis is performed = Stage 3, Step 2 Visen Name = NA</pre>	2. eds
[Results]well well PositionSample NameSNP Assay NameTaskAllele1Delta RnAllele2 Delta RnPass.RefCallMethodAllele1Automatic Ct ThresholdAllele1 Ct ThresholdAllele1 Automatic BaselineAllele1 Baseline StarBaselineEndAllele2Automatic Ct ThresholdAllele2Automatic BaselineAllele1 Baseline StarBaselineEndBaselineAllele2Automatic BaselineAllele1 BaselineAllele2BaselineEndSmple 1SNP Assay 1custom task1.4583.34398.416Heterozygous Allele 1/Allele 21A1Sample 2SNP Assay 1custom task1.4743.45598.416Heterozygous Allele 1/Allele 2Automatic Baseline2A2Sample 3SNP Assay 1custom task0.1244.94198.416Homozygous Allele 2/Allele 23A1Custom task0.1245.06098.416Homozygous Allele 2/Allele 2Automatic Baseline4A4Sample 3SNP Assay 1UNKNOWN 0.1225.06098.416Homozygous Allele 2/Allele 25A5Sample 5SNP Assay 1custom task0.1085.02098.416Homozygous Allele 2/Allele 26A6Sample 6SNP Assay 1UNKNOWN 0.0925.06298.416Homozygous Allele 2/Allele 26A6Sample 6SNP Assay 1UNKNOWN 0.0925.06298.416Homozygous Allele 2/Allele 26A6Sample 6SNP	t Allele2
7 A7 Sample 1 SNP Assay 2 custom task 0.979 0.371 98.416 Homozygous Allele 1/Allele 1 true 0.040 true 3 22 true 0.040 true 3 23	Auto 🗸

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www.lifetechnologies.com

GETTING STARTED GUIDE



Booklet 5 - Running Presence/Absence Experiments

Publication Part Number 4470050 Rev. A Revision Date March 2012



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About Presence/Absence Experiments

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IMPORTANT! First-time users of the QuantStudioTM 12K Flex System, please read Booklet 1, *Getting Started with QuantStudioTM 12K Flex System Multi-Well Plate and Array Card Experiments* and Booklet 7, *QuantStudioTM 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 (Δ Rn) value per the following formula:
	Δ Rn = 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 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 setup 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 QuantStudioTM 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.

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Design the Experiment

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

This chapter covers:

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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*TM 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 QuantStudioTM 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:

How do you want to identify this experin	nent?			
Experiment Name: 96-Well Presence Absence I Barcode: User Name: Example User	Example	Comments:	Presence Absence example	▲ ▼
* Which block are you using to run the e	xperiment?			
384-Well	Array Card	1	96-Well (0.2mL)	Fast 96-Well (0.1mL)
What type of experiment do you want to	to set up?			
Standard Curve	Relative Standard Curve	Сог	nparative Cτ (ΔΔCτ)	Melt Curve
Genotyping	Presence/Absence			
* Which reagents do you want to use to o	letect the target sequence?			
✓ TaqMan® Reagents	Other			
* What properties do you want for the in	strument run?			
✓ Standard	Fast			
Include: Vere-PCR Read Amplification Ver	st-PCR Read			

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

Targets				Samples		
New Save to Library Import from Library	Delete			New Save to Library Import from Library Delete		
Target Name	Reporter	Quencher	Color	Sample Name	Color	r
TGFB			~	+)		~
IPC	VIC 🗸	TAMRA	~	-)		~
				IAC		~
				ітс		~
* 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	A1 - A4 (Columns 1 - 4)	Negative	NAC
IPC		No IPC	
TGFB	A5 - A8 (Columns 5 - 8)	IPC	NTC
IPC		Negative	
TGFB	B1 - B10 (Columns 1- 10)	Unknown	(+)
IPC		IPC	
TGFB	C1 - C10 (Columns 1 - 10)	Unknown	(-)
IPC		IPC	

Your Assign screen should look like this:

Targets A	1	Plate Layout	Well Table	e										
Name Task		🛄 Show in W	ells 🔻 Select	Wells 🔻 📱	📒 View Legend	ł						÷	10	×
IPC Z		1	2	3	4	5	6	7	8	9	10	1	L	12
	A	NAC IPC		NAC IPC	NAC	NTC	NTC	NTC	NTC					
	в	(+)	(+) I IPC U TGFB	(+)	(+) I IPC I TGFB	(+) I IPC I TGFB	(+)	(+)	(+) IIPC ITGFB	(+)	(+) I IPC I TGFB			
	с	(-) TPC TGFB	(-) I IPC I TGFB	(-)	(-) I IPC I TGFB	(-) I IPC U TGFB	(-)	(-)	(-) I IPC I TGFB	(-) TPC TGFB	(-) I IPC U TGFB			
Samples	D													
Name (+) (-) NAC NTC	E													
	F													
	G													
	н													
	w	/ells: 🚺 0	1 0 <mark>U</mark> 20	N 4 🔀 4										68 Empty

Set up the run method

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

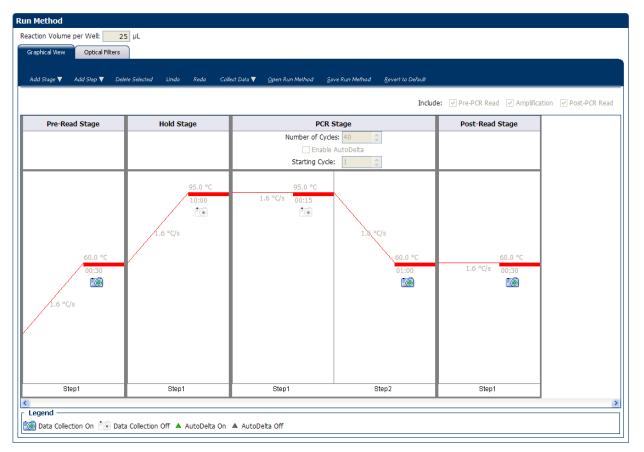
- Reaction Volume Per Well: 25 µL
- Thermal Profile

Stage	Step	Ramp rate	Temperature	Time
Pre-Read Stage	Step 1	1.6°C/s	0° C	30 seconds
Hold Stage	Step 1	1.6°C/s	95°C	10 minutes
PCR Stage	Step 1	1.6°C/s	95°C	15 seconds
 Number of Cycles: 40 (default) 	Step2	1.6°C/s	60°C	1 minute
 Enable AutoDelta: Unchecked (default) 				
 Starting Cycle: Disabled when Enable AutoDelta is unchecked 				

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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, <i>Getting Started with QuantStudio™ 12K Flex</i> System Multi-Well Plate and Array Card Experiments	4470050
	Appendix A in Booklet 7, QuantStudio™ 12K Flex System Multi-Well Plate and Array Card Experiments - Appendixes	
Data collection	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 12K Flex</i> 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, <i>Getting Started with QuantStudio™ 12K Flex</i> System Multi-Well Plate and Array Card Experiments	4470050

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Chapter 2 Design the Experiment *For more information*

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

This chapter covers:

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repare the reaction mix ("cocktail mix")	15
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1	repare the reaction plate

Assemble required materials

3

- 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 $^{\textcircled{8}}$ Universal PCR Master Mix (2.0X)	12.50	137.50
	10× IPC Mix	2.50	27.5
	50× IPC DNA	0.50	5.5
	20× 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 $^{ extsf{B}}$ Universal PCR Master Mix (2.0×)	12.50	137.50
	10× IPC Mix	2.50	27.5
	50× IPC DNA	0.50	5.5
	20× 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
	10× IPC Mix	2.50	12.5
	50× 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
		10.50	(0 F
Cocktail Mix 4 for NAC/ IPC-	TaqMan [®] Universal PCR Master Mix (2.0×)	12.50	62.5
	10× IPC Mix	2.50	12.5
	50× 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 $25 \,\mu L/well$
- 10 (+) wells U
- 10 Sample (-) wells **UI**
- 4 NTC/ IPC+ 🛛 🗹
- 4 NAC/IPC- 🛛 🚺

The plate layout looks like this:

1	Plate Layout	Well Table										_
	😈 Show in We	s 🔻 Select Wells 🐧	View Lege	nd							÷	a 🔀
Г	1	2	3	4	5	6	7	8	9	10	11	12
				NAC IPC TGFB	NTC N IPC I TGFB	NTC N IPC	NTC N IPC I TGFB	NTC N IPC I, TGFB				
	(+) I IPC U TGFB	(+) I IPC U TGFB	(+) I IPC U TGFB	(+) T TPC TGFB	(+) T IPC TGFB	(+) TIPC TGFB	(+) T IPC TGFB	(+) TIPC TGFB	(+) I IPC I TGFB	(+) TITPC TGFB		
	C (·)	(+) TPC TGFB	(-)	(-) I IPC I TGFB	(•) T IPC	(-) T IPC TGFB	(-) I IPC I TGFB	(-) T IPC TGFB	(-) T IPC TGFB	(-) T IPC TGFB		
:	D											
	E											
	F											
	G											
	н											
	Wells: 🚺 0 N	0 👖 20 📘 4	N 4									68 Empty

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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 μ L 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, <i>Getting Started with QuantStudio™ 12K</i> Flex System Multi-Well Plate and Array Card Experiments	4470050
Sealing the reaction plate	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 12K</i> Flex System Multi-Well Plate and Array Card Experiments	4470050

This chapter explains how to run the example experiment on the QuantStudio $^{\rm TM}$ 12K Flex Instrument.

This chapter covers:

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.

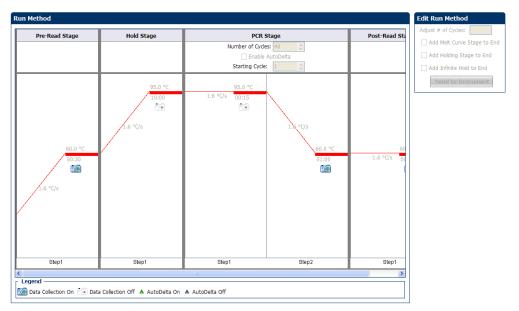


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

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.

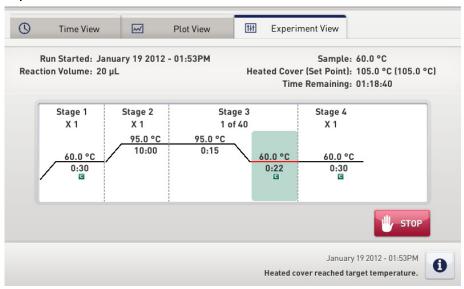
The figure below shows the View Run Data screen as it appears in the example experiment.

Run Data Report	
Experiment Name:	96-Well Presence Absence Example
Start Time:	08-11-2011 11:59:56 SGT
Stop Time:	08-11-2011 13:33:57 SGT
Run Duration:	94 minutes 1 seconds
User Name:	DEFAULT
Instrument Name:	QuantStudioDemo
Firmware Version:	0.13.1
Software Version:	QuantStudio 12K Flex Software v1.0
Instrument Serial Number:	QuantStudioDemo
Sample Volume:	25.0
Cover Temperature:	105.0
Block Type:	96-Well Block (0.2mL)
Errors Encountered:	

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

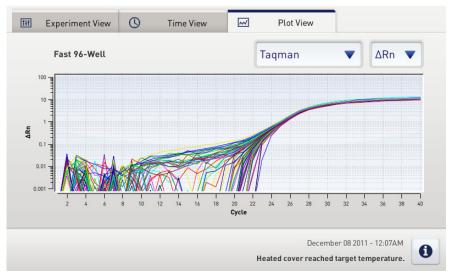


Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

Time View

D	Time View	~	Plot View	t+t	Experiment	t View		
	Run Started: Dece ion Volume: 20 µl		2011 - 12:05AM	Hea	ted Cover (Se Stage / Step	et Point):		(105.0 °C
[
	Ω	1	.1	1		7	2	
	0	1	:1	1		2	2	
	0	1	Remaining Time	-	Elapsed Time		2	

Plot View



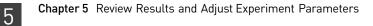
Note: You will see the Plot View only if your experiment includes the PCR process.

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:

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Section 5.1 Review Results

Analyze the example experiment

- 1. Open the Presence/Absence example experiment file that you ran in Chapter 4.
- 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 From the Experiment menu pane, select Analysis > Presence/Absence Plot.

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

- **3.** Click the **Show IPC** check box to view the fluorescence intensity of the IPC target in the Unknown-IPC wells.
- 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.
- Target Calls:
 - Presence
 - Absence
 - Unconfirmed
- IPC Calls:
 - IPC Succeeded
 - IPC Failed
- Control Well Calls:
 - negative control IPC
 - negative control Blocked IPC

Assess amplification results using the Amplification Plot

IMPORTANT! Amplification plots are not used to make Presence/Absence calls. Examine the plots to help with troubleshooting and quality control.

Amplification plots available for viewing
 The Amplification Plot displays amplification of all samples in the selected wells. There are three amplification plot views available:
 ARn 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:

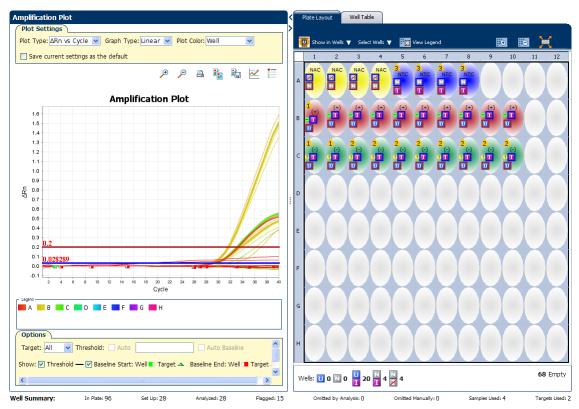
- Correct baseline and threshold values
- Irregular amplification
- Outliers

View the Amplification Plot

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

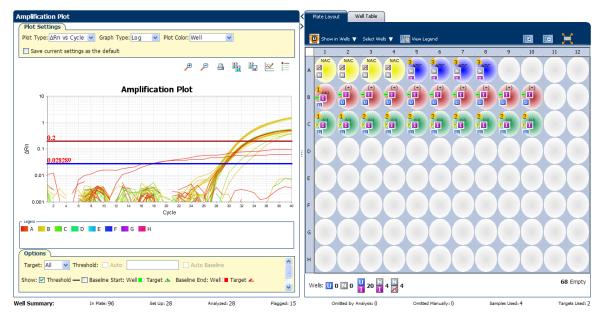
- **5.** View the baseline values:
 - a. From the Graph Type drop-down menu, select Linear.
 - b. Select the Baseline check box to show the start cycle and end cycle.
 - **c.** Verify that the baseline is set correctly: The end cycle should be set a few cycles before the cycle number where significant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

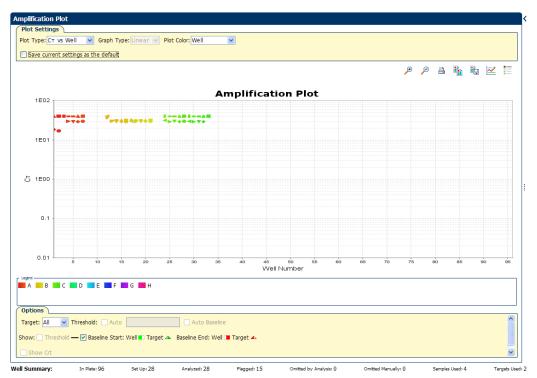
Your screen should look like this:



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- **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

5

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

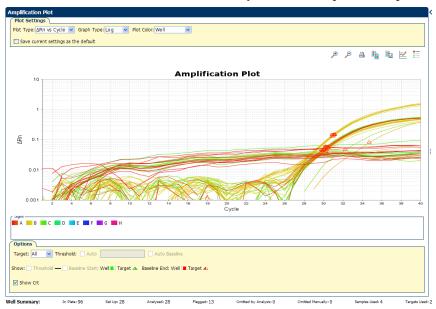
- Outliers
- A typical amplification plot The QuantStudio[™] 12K Flex Software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:
 - Plateau phase
 - Linear phase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

NC	AMPNC	Comme	Call	Thresh	ΔRn SD	∆Rn Mean	ΔRn	Dyes	Task	Target	Sample	Flag	Omit	Well	ŧ
												Vells	🗏 Flagged \		
			Negative C		0.010	0.657	0.658	VIC-TAMRA	NTC	IPC	NTC	3		A5	5
			IPC Failed		0.008	0.059	0.056	FAM-NFQ	IPC	TGFB	NTC	3		A5	5
			Negative C		0.010	0.657	0.646	VIC-TAMRA	NTC	IPC	NTC	3		A6	6
			IPC Failed		0.008	0.059	0.056	FAM-NFQ	IPC	TGFB	NTC	3		A6	6
			Negative C		0.010	0.657	0.670	VIC-TAMRA	NTC	IPC	NTC	3		A7	7
			IPC Failed		0.008	0.059	0.054	FAM-NFQ	IPC	TGFB	NTC	3		A7	7
			Negative C		0.010	0.657	0.653	VIC-TAMRA	NTC	IPC	NTC	3		A8	8
			IPC Failed		0.008	0.059	0.071	FAM-NFQ	IPC	TGFB	NTC	3		A8	8
			Absence	0.717	0.158	0.549	0.106	VIC-TAMRA	IPC	IPC	(+)	1		B1	13
			IPC Succe	0.095	0.367	1.511	0.478	FAM-NFQ	UNKNOWN	TGFB	(+)	1		B1	13
			Unconfirmed	0.717	0.177	0.622	0.532	VIC-TAMRA	IPC	IPC	(-)	1		C1	25
			IPC Failed	0.095	0.064	0.048	-0.127	FAM-NFQ	UNKNOWN	TGFB	(-)	1		C1	25
			Unconfirmed	0.717	0.177	0.622	0.681	VIC-TAMRA	IPC	IPC	(-)	1		C2	26
			IPC Failed	0.095	0.064	0.048	0.030	FAM-NFQ	UNKNOWN	TGFB	(-)	1		C2	26
			Unconfirmed	0.717	0.177	0.622	0.684	VIC-TAMRA	IPC	IPC	(-)	2		C3	27
			IPC Failed	0.095	0.064	0.048	0.056	FAM-NFQ	UNKNOWN	TGFB	(-)			C3	27
			Unconfirmed	0.717	0.177	0.622	0.700	VIC-TAMRA	IPC	IPC	(-)	2		C4	28
			IPC Failed	0.095	0.064	0.048	0.076	FAM-NFQ	UNKNOWN	TGFB	(-)			C4	28
			Unconfirmed	0.717	0.177	0.622	0.692	VIC-TAMRA	IPC	IPC	(-)	2		C5	29
				0.095	0.064	0.048	0.071	FAM-NFQ	UNKNOWN	TGFB	(-)	2		C5	29
			Unconfirmed	0.717	0.177	0.622	0.690	VIC-TAMRA	IPC	IPC	(-)	2		C6	30
				0.095	0.064	0.048	0.064	FAM-NFQ	UNKNOWN	TGFB	(-)	5		C6	30
			Unconfirmed	0.717	0.177	0.622	0.715	VIC-TAMRA	IPC	IPC	(-)	2		C7	31
				0.095	0.064	0.048	0.082	FAM-NFQ	UNKNOWN	TGFB	(-)	Ā		C7	31
			Unconfirmed		0.177	0.622	0.688	VIC-TAMRA	IPC	IPC	(-)	2		C8	32
				0.095	0.064	0.048	0.068	FAM-NFQ	UNKNOWN	TGFB	(-)	5		C8	32
			Unconfirmed		0.177	0.622	0.703	VIC-TAMRA	IPC	IPC	(-)	2	n	C9	33
				0.095	0.064	0.048	0.072	FAM-NFQ	UNKNOWN	TGFB	(-)	5		C9	33
			Unconfirmed		0.177	0.622	0.140	VIC-TAMRA	IPC	IPC	(-)	Ā		C10	34
				0.095	0.064	0.048	0.089	FAM-NFQ	UNKNOWN	TGFB	(-)	5		C10	34
_		_		0.095	0.004	0.040	0.005	174-11 Q-11	onatown	1010			🗏 Unflagge	015	34
			Blocked IP		0.013	0.098	0.096	VIC-TAMRA	BlockedIPC	IPC	NAC			A1	1
			Negative C		0.013	0.098	0.090	FAM-NFQ	NTC	TGFB	NAC			A1 A1	1
			Blocked IP		0.004	0.073	0.086	VIC-TAMRA		IPC	NAC			A2	2

- **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

how i	n Table 🔻	Select Wells	▼ Group by	v									🗄 Expand	AI 🗉 🤇	Collaps
	Well	Omit	Flag	Sample	Target	Task	Dyes	ΔRn	∆Rn Mean	ΔRn SD	Thresh	Call	Comme	AMPNC	N
		🗏 Absence													
13	B1		1	(+)	IPC	IPC	VIC-TAMRA	0.106	0.549	0.158	0.717	Absence			
14	B2			(+)	IPC	IPC	VIC-TAMRA	0.598	0.549	0.158	0.717	Absence			
15	B3			(+)	IPC	IPC	VIC-TAMRA	0.629	0.549	0.158	0.717	Absence			
16	B4			(+)	IPC	IPC	VIC-TAMRA	0.606	0.549	0.158	0.717	Absence			
17	B5			(+)	IPC	IPC	VIC-TAMRA	0.612	0.549	0.158	0.717	Absence			
18	B6			(+)	IPC	IPC	VIC-TAMRA	0.609	0.549	0.158	0.717	Absence			
19	B7			(+)	IPC	IPC	VIC-TAMRA	0.609	0.549	0.158	0.717	Absence			
20	B8			(+)	IPC	IPC	VIC-TAMRA	0.604	0.549	0.158	0.717	Absence			
21	B9			(+)	IPC	IPC	VIC-TAMRA	0.590	0.549	0.158	0.717	Absence			
22	B10			(+)	IPC	IPC	VIC-TAMRA	0.530	0.549	0.158	0.717	Absence			
		Blocked	IPC Control												
1	A1			NAC	IPC	BlockedIPC	VIC-TAMRA	0.096	0.098	0.013		Blocked IP			
2	A2			NAC	IPC	BlockedIPC	VIC-TAMRA	0.086	0.098	0.013		Blocked IP			
3	A3			NAC	IPC	BlockedIPC	VIC-TAMRA	0.094	0.098	0.013		Blocked IP			
4	A4			NAC	IPC	BlockedIPC	VIC-TAMRA	0.116	0.098	0.013		Blocked IP			
		🗏 IPC Faile	d												
5	A5		3	NTC	TGFB	IPC	FAM-NFQ	0.056	0.059	0.008		IPC Failed			
6	A6		3	NTC	TGFB	IPC	FAM-NFQ	0.056	0.059	0.008		IPC Failed			
7	A7		3	NTC	TGFB	IPC	FAM-NFQ	0.054	0.059	0.008		IPC Failed			
8	A8		3	NTC	TGFB	IPC	FAM-NFQ	0.071	0.059	0.008		IPC Failed			
25	C1		1	(-)	TGFB	UNKNOWN	FAM-NFQ	-0.127	0.048	0.064	0.095	IPC Failed			
26	C2		1	(-)	TGFB	UNKNOWN	FAM-NFQ	0.030	0.048	0.064	0.095	IPC Failed			
27	C3		2	(-)	TGFB	UNKNOWN	FAM-NFQ	0.056	0.048	0.064	0.095	IPC Failed			
28	C4		2	(-)	TGFB	UNKNOWN	FAM-NFQ	0.076	0.048	0.064	0.095	IPC Failed			
29	C5		2	(-)	TGFB	UNKNOWN	FAM-NFQ	0.071	0.048	0.064	0.095	IPC Failed			
30	C6		2	(-)	TGFB	UNKNOWN	FAM-NFQ	0.064	0.048	0.064	0.095	IPC Failed			
31	C7		2	(-)	TGFB	UNKNOWN	FAM-NFQ	0.082	0.048	0.064	0.095	IPC Failed			
32	C8		2	(-)	TGFB	UNKNOWN	FAM-NFQ	0.068	0.048	0.064	0.095	IPC Failed			
33	C9		2	(-)	TGFB	UNKNOWN	FAM-NFQ	0.072	0.048	0.064	0.095	IPC Failed			
34	C10		2	(-)	TGFB	UNKNOWN	FAM-NFQ	0.089	0.048	0.064	0.095	IPC Failed			
		IPC Suc	ceeded												
13	B1		1	(+)	TGFB	UNKNOWN	FAM-NFQ	0.478	1.511	0.367	0.095	IPC Succe			
14	B2		-	(+)	TGFB	UNKNOWN	FAM-NFQ	1.713	1.511	0.367	0.095	IPC Succe			
15	B3			(+)	TGFB	UNKNOWN	FAM-NFQ	1.628	1.511	0.367	0.095	IPC Succe			
		<													

c. From the Group By drop-down menu, select **None**. In the table, click the column heading Δ **Rn**. Wells are listed in order of increasing Δ Rn. Click the column heading again to reverse the sort order.

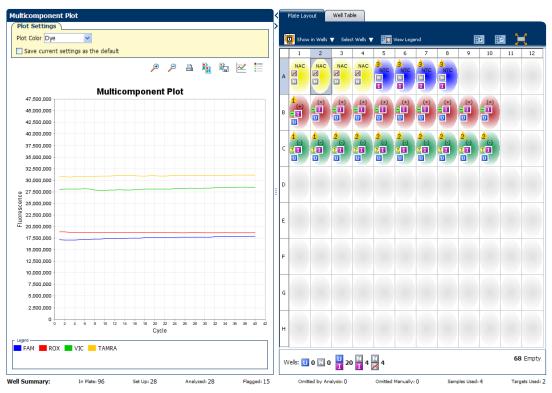
NC	AMPNC	Comme	Call	Thresh	ΔRn SD	∆Rn Mean	∆Rn ^{™1}	Dyes	Task	Target	Sample	Flag	Omit	Well	#
			IPC Succe	0.095	0.367	1.511	1.713	FAM-NFQ	UNKNOWN	TGFB	(+)			B2	14
			IPC Succe	0.095	0.367	1.511	1.656	FAM-NFQ	UNKNOWN	TGFB	(+)			B7	19
			IPC Succe	0.095	0.367	1.511	1.645	FAM-NFQ	UNKNOWN	TGFB	(+)			B8	20
			IPC Succe	0.095	0.367	1.511	1.640	FAM-NFQ	UNKNOWN	TGFB	(+)			B4	16
			IPC Succe	0.095	0.367	1.511	1.635	FAM-NFQ	UNKNOWN	TGFB	(+)			B5	17
			IPC Succe	0.095	0.367	1.511	1.628	FAM-NFQ	UNKNOWN	TGFB	(+)			B3	15
			IPC Succe	0.095	0.367	1.511	1.619	FAM-NFQ	UNKNOWN	TGFB	(+)			B6	18
			IPC Succe	0.095	0.367	1.511	1.604	FAM-NFQ	UNKNOWN	TGFB	(+)			B9	21
			IPC Succe	0.095	0.367	1.511	1.489	FAM-NFQ	UNKNOWN	TGFB	(+)			B10	22
			Unconfirmed	0.717	0.177	0.622	0.715	VIC-TAMRA	IPC	IPC	(-)	2		C7	31
			Unconfirmed	0.717	0.177	0.622	0.703	VIC-TAMRA	IPC	IPC	(-)	2		C9	33
			Unconfirmed	0.717	0.177	0.622	0.700	VIC-TAMRA	IPC	IPC	(-)	٨		C4	28
			Unconfirmed	0.717	0.177	0.622	0.692	VIC-TAMRA	IPC	IPC	(-)	2		C5	29
			Unconfirmed	0.717	0.177	0.622	0.690	VIC-TAMRA	IPC	IPC	(-)	2		C6	30
			Unconfirmed	0.717	0.177	0.622	0.688	VIC-TAMRA	IPC	IPC	(-)	2		C8	32
			Unconfirmed	0.717	0.177	0.622	0.684	VIC-TAMRA	IPC	IPC	(-)	2		C3	27
			Unconfirmed	0.717	0.177	0.622	0.681	VIC-TAMRA	IPC	IPC	(-)	1		C2	26
			Negative C		0.010	0.657	0.670	VIC-TAMRA	NTC	IPC	NTC	3		A7	7
			Negative C		0.010	0.657	0.658	VIC-TAMRA	NTC	IPC	NTC	3		A5	5
			Negative C		0.010	0.657	0.653	VIC-TAMRA	NTC	IPC	NTC	3		A8	8
			Negative C		0.010	0.657	0.646	VIC-TAMRA	NTC	IPC	NTC	4		A6	6
			Absence	0.717	0.158	0.549	0.629	VIC-TAMRA	IPC	IPC	(+)	_		B3	15
			Absence	0.717	0.158	0.549	0.612	VIC-TAMRA	IPC	IPC	(+)			B5	17
			Absence	0.717	0.158	0.549	0.609	VIC-TAMRA	IPC	IPC	(+)			B7	19
			Absence	0.717	0.158	0.549	0.609	VIC-TAMRA	IPC	IPC	(+)			B6	18
			Absence	0.717	0.158	0.549	0.606	VIC-TAMRA	IPC	IPC	(+)			B4	16
			Absence	0.717	0.158	0.549	0.604	VIC-TAMRA	IPC	IPC	(+)			B8	20
			Absence	0.717	0.158	0.549	0.598	VIC-TAMRA	IPC	IPC	(+)			B2	14
			Absence	0.717	0.158	0.549	0.590	VIC-TAMRA	IPC	IPC	(+)			B9	21
			Unconfirmed	0.717	0.177	0.622	0.532	VIC-TAMRA	IPC	IPC	(-)	4		C1	25
			Absence	0.717	0.158	0.549	0.530	VIC-TAMRA	IPC	IPC	(+)			B10	22
			IPC Succe	0.095	0.367	1.511	0.478	FAM-NFQ	UNKNOWN	TGFB	(+)	1		B1	13
			Unconfirmed	0.717	0.177	0.622	0.140	VIC-TAMRA	IPC	IPC	(-)	2		C10	34
			Blocked IP		0.013	0.098	0.116	VIC-TAMRA	BlockedIPC	IPC	NAC	-		A4	4
			Absence	0.717	0.158	0.549	0.106	VIC-TAMRA	IPC	IPC	(+)	1		B1	13
>													<		

Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Presence/Absence Experiments

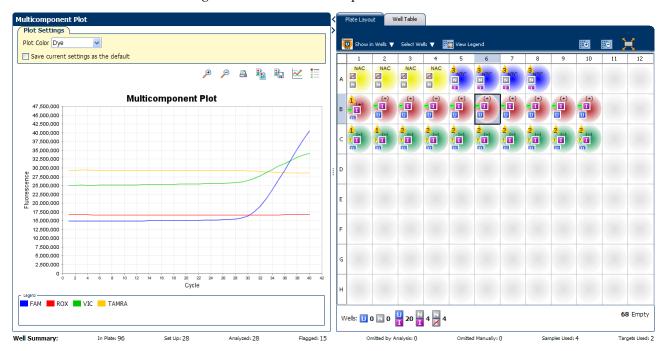
Tips for analyzing When you analyze your own Presence/Absence experiment, group the wells by: your own Flag – The software groups the flagged and unflagged wells. A flag indicates that experiments the software has found an error in the flagged well. For a description of the QuantStudio[™] 12K Flex Software 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: ROXTM dye (passive reference) • FAMTM dye (reporter) • VIC[®] dye (reporter) • TAMRA (reporter) Spikes, dips, and/or sudden changes Amplification in the negative control wells

View the 1. From the Experiment Menu pane, select Analysis > Multicomponent Plot. Multicomponent Note: If no data are displayed, click Analyze. Plot 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 **End** 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.



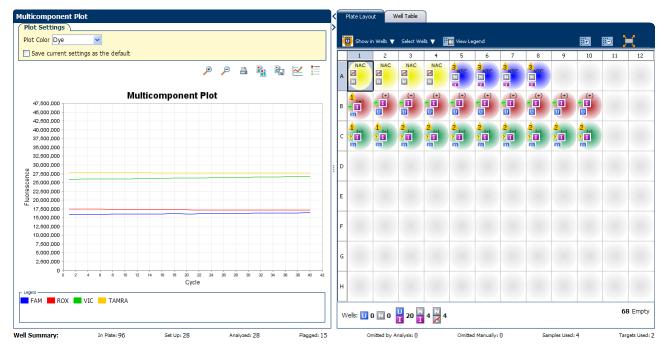
7. Check the FAM dye signal. In the example experiment, for the sample (+), the FAM dye signal increases throughout the PCR process; increase in FAM dye signal indicates normal amplification.



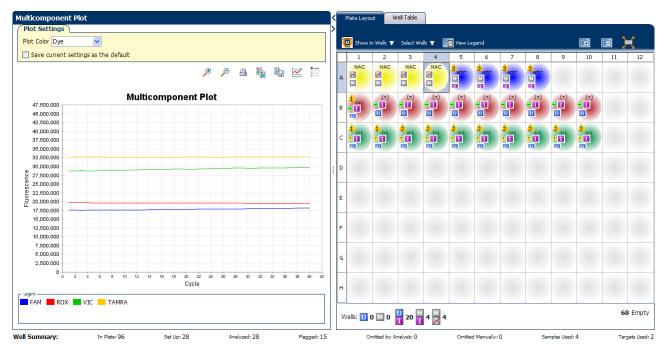
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8. Check the TAMRA dye signal. In the example experiment the TAMRA dye signal should not amplify for NAC-Blocked IPC wells or if the IPC call for the Unknown-IPC well is IPC Failed.



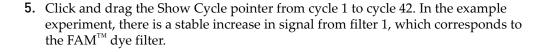
9. Select the negative control (NTC) wells one at time and check for amplification. In the example experiment, there is no amplification in the negative control wells.

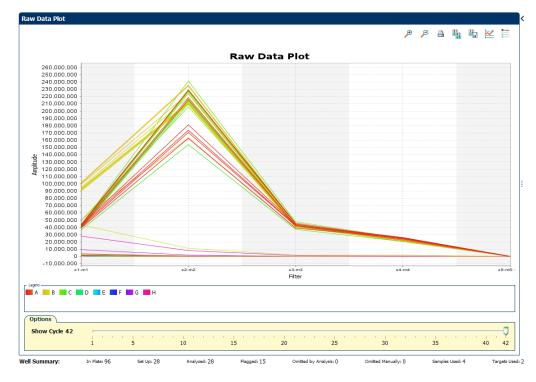


Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Presence/Absence Experiments

Tips for confirming	When you analyze your own Presence/Absence experiment, look for:
dye accuracy in your own	• Passive reference (ROX) – The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
experiment	• 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 sig	nal 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	1. From the Experiment Menu pane, select Analysis > Raw Data Plot .
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.





The filters used for the example experiment are:

				Load Save Revert k	Defaults		
				Emission Filter			
		ml(520±15)	m2(558±11)	m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
	x1(470±15)	V					
	x2(520±10)		V				
	x3(550±11)			\checkmark			
	x4(580±10)				V		
	x5(640±10)					V	
_							
t Cu	x6(662±10) urve Filter ———			Lasd Save Revert to	Defaults		
t Cu				Land Save Revert la Emission Filter	Defaults		
lt Cu		m1(520±15)	m2(558±11)		Defaults m4(623±14)	m5(682±14)	m6(711±12)
It Cu		m1(520±15)	m2(558±11)	Emission Filter		m5(682±14)	
	urve Filter ———			Emission Filter m3(586±10)	m4(623±14)		m6(711±12)
	urve Filter x1(470±15)			Emission Filter	m4(623±14)		m6(711±12)
	x1(470±15) x2(520±10)			Emission Filter m3(586±10)	m4(623±14)		m6(711±12)
	x1(470±15) x2(520±10) x3(550±11)			Emission Filter m3(586±10)	m4(623±14)		m6(711±12)

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 QC1. From the Experiment Menu pane, select Analysis > QC Summary.SummaryNote: 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.

ag Details			
Flag:	Description	Frequency	Wells
ADROX	Bad passive reference signal	0	
OSIGNAL	No signal in well	0	
FFSCALE	Fluorescence is offscale	0	
MPNC	Amplification in negative control	4	A5, A6, A7, A8
OAMP	No amplification	13	A5, A6, A7, A8, B1, C3, C4, C5, C6, C7, C8, C9, C10
OISE	Noise higher than others in plate	0	
PIKE	Noise spikes	0	
XPFAIL	Exponential algorithm failed	14	A5, A6, A7, A8, C1, C2, C3, C4, C5, C6, C7, C8, C9, C10
LFAIL	Baseline algorithm failed	0	
HOLDFAIL	Thresholding algorithm failed	0	
TFAIL	CT algorithm failed	0	
MPSCORE	AMP Score	0	
Flag Detail: The s Flag Criteria: Ampl	MP—No amplification sample did not amplify. Ilfication algorithm result < 0.1 16, A7, A8, B1, C3, C4, C5, C6, C7, C8, C9, C10		
Flag Detail: The s Flag Criteria: Ampl Flagged Wells: A5, A	sample did not amplify. lification algorithm result < 0.1		
Flag Detail: The s Flag Criteria: Ampl Flagged Wells: A5, A	sample did lifcation algorithm result < 0.1 16, A7, A8, B1, C3, C4, C5, C6, C7, C8, C9, C10	28 Manualy Omitted Wels: 15 Analysis Omitted Wels:	0 Targets Used: 0 Samples Used:

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
Se	econdary 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, <i>Getting Started with QuantStudio™ 12K Flex</i> 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:

Analysis Setting	s for 96-We	II Presence Abs	ence Example			
Call Settings	CT Settings	Flag Settings	Advanced Settings)		
Settings,th	en change the s s Settings —	settings.		e-PCR Read and Post-PCR Read		the table, deselect Use Default
Default Call S Default call settin Confidence V	ngs are used to	make presence/abs		out custom settings. To edit the defi	ault settings, click Ed	iit Default Settings.
Select a Targ	jet		in The s	Confidence		Call Settings for TGFB
Target TGFB		Default	is Type	Confidence 99%	~	Call Settings to Use: 🗹 Default Settings
IPC		Default		99%		Confidence Value: 99% 🗸
					•	
Gave to Library	Load f	rom Library		Revert to Default An	alysis Settings	Apply Analysis Settings Cano

Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Presence/Absence Experiments

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 Call Settings

settings

att 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:

Flag	Description	Use	Attribute	Condition	Value	Reject Well
ADROX	Bad passive referenc	>	Fluorescence	<	✓ 500.000	
OSIGNAL	No signal in well					
FFSCALE	Fluorescence is offscale					
MPNC	Amplification in negat		Ст	<	✓ 35.000	
OAMP	No amplification		Amplification algorith	<	✓ 0.100	
OISE	Noise higher than ot		Relative noise	>	✓ 4.000	
PIKE	Noise spikes		Spike algorithm result	>	✓ 1.000	
XPFAIL	Exponential algorithm					
LFAIL	Baseline algorithm failed					
HOLDFAIL	Thresholding algorith					
TFAIL	CT algorithm failed					
MPSCORE	AMP Score		AMP Score	>	✓ 1.000	

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



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	<pre><drive>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments</drive></pre>

Your Export screen should look like this:

ort File Location: C:\Applied Biosy	ctome\Ou	antCtudia 1		Drouten Droott File			o Evample, Quant	C File Type: M (**	
ort File Location: C:\Applied Blosy	stems\Qu	lantStudio	12K Flex Software (Us	Export File	a Name: 90-weil P	esence Absenc	e Example_Quant	S File Type:	Xt)
Sample Setup	Ampli	ification	Multicomponent	Results					
Skip Empty Wells 🔽 Skip Omitted									
	vveiis								
elect Content		Well	Well Position	Sample Name	Target Name	Task	Reporter	Ouencher	
All Fields	~	wen	1 A1	NAC	IPC	BlockedIPC	VIC	TAMRA	-
			1 A1	NAC	TGFB	NTC	FAM	NFO-MGB	
] Well			2 A2	NAC	IPC	BlockedIPC	VIC	TAMRA	
			2 A2	NAC	TGFB	NTC	FAM	NFQ-MGB	
Well Position			3 A3	NAC	IPC	BlockedIPC	VIC	TAMRA	
Sample Name			3 A3	NAC	TGFB	NTC	FAM	NFQ-MGB	
journple name			4 A4	NAC	IPC	BlockedIPC	VIC	TAMRA	
] Target Name			4 A4	NAC	TGFB	NTC	FAM	NFO-MGB	
			5 A5	NTC	IPC	NTC	VIC	TAMRA	
] Task			5 A5	NTC	TGFB	IPC	FAM	NFQ-MGB	
Reporter			6 A6	NTC	IPC	NTC	VIC	TAMRA	
Reporter			6 A6	NTC	TGFB	IPC	FAM	NFQ-MGB	
] Quencher	=		7 A7	NTC	IPC	NTC	VIC	TAMRA	
			7 A7	NTC	TGFB	IPC	FAM	NFQ-MGB	
] Delta Rn			8 A8	NTC	IPC	NTC	VIC	TAMRA	
Delta Rn Mean			8 A8	NTC	TGFB	IPC	FAM	NFQ-MGB	
J Deica Kir Mean			13 B1	(+)	IPC	IPC	VIC	TAMRA	
Delta Rn SD			13 B1	(+)	TGFB	UNKNOWN	FAM	NFQ-MGB	
			14 B2	(+)	IPC	IPC	VIC	TAMRA	
] Threshold Value			14 B2	(+)	TGFB	UNKNOWN	FAM	NFQ-MGB	
Call			15 B3	(+)	IPC	IPC	VIC	TAMRA	
			15 B3 16 B4	(+)	TGFB IPC	UNKNOWN IPC	FAM VIC	NFQ-MGB TAMRA	
Comments			16 B4	(+)	TGFB	UNKNOWN	FAM	NFO-MGB	
			17 B5	(+)	IPC	IPC	VIC	TAMRA	
Automatic Ct Threshold			17 85	(+)	TGFB	UNKNOWN	FAM	NFQ-MGB	
Ct Threshold			18 B6	(+)	IPC	IPC	VIC	TAMRA	
			18 86	(+)	TGFB	UNKNOWN	FAM	NFQ-MGB	
			19 B7	(+)	IPC	IPC	VIC	TAMRA	
Automatic Baseline									

Start Export Save Export Set As Load Export Set Delete Export Set

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

96-Well Presence Absence Exam	ple_QuantStudio_export.t	xt - Notepad							
File Edit Format View Help									
<pre>* Block Type = 96-Well Block * Calibration Background is calibration Background per * Calibration Background yel * Calibration FAM is expired * Calibration ROI is expired * Calibration ROI performed * Calibration ROX performed * Calibration ROX performed * Calibration SYBR is expire * Calibration SYBR performed * Calibration TAWRA is expire * Calibration TAWRA performed * Calibration Uniformity yel * Calibration Uniformity yel * Calibration Uniformity yel * Calibration Uniformity ser * Calibration Uniformity ser * Calibration Uniformity ser * Calibration Uniformity per * Calibration Uniformity per * Calibration VIC is expired * Chemistry = TAQMAN * Date Created = 1970-01-01 * Experiment File Name = C: Example.eds * Experiment Run End Time = * Experiment Name = 96-Well * Instrument Serial Number = * Instrument Serial Number * Instrument Serial Number * Instrument Serial Number * Instrument Serial Number * Stage / CyCle where analys* * User Name = NA</pre>	expired = No formed on = 2011-08-08 01 on = 2011-08-08 01 d = No on = 2011-08-08 01 d = No on = 2011-08-08 02 ed = No d on = 2011-08-08 02 ed on = 2011-08-08 01 or = 2011-08-08 01 07:30:00 AM SGT \Program Files\Appli Presence Absence Ex: Not Started e/Absence udioDemo = QuantStudioDemo udio 12K Flex od = Ct	:39:58 AM SGT :05:24 AM SGT :07:15 AM SGT 1:58:11 AM SGT 02:16:10 AM SG 8-08 01:24:47 :49:09 AM SGT ed Biosystems\ ample	T AM SGT	i012KFlex	:\example	s∖Presence Abs	sence∖96-well Prese	nce Abse	nce
	ple Name Target M ments Automat	Name Task ic Ct Threshol	Report d Ct Thr		Quenche Automat	er Delta ic Baseline	a Rn Delta R Baseline Start	n Mean Baselin	
1 A1 NAC IPC		VIC TAMRA	0.096	0.098	0.013	0.000 Block	ced IPC Control		false
1 A1 NAC TGFE		N NFQ-MGB 0.078	0.073	0.004	0.000	Negative Cont	rol	false	0.200
false 3 15 N 2 A2 NAC IPC	N N BlockedIPC	VIC TAMRA	0.086	0.098	0.013	0.000 вlock	ced IPC Control		false
0.028 true 3 9 2 A2 NAC TGEE	N N B NTC FAM	N NFQ-MGB 0.073	0.073	0.004	0.000	Negative Cont	rol	false	0.200
2 A2 NAC TGFE false 3 15 N 3 A3 NAC IPC	N N	VIC TAMRA		0.098	0.013	-	ced IPC Control		false
0.028 true 3 4	N N	N							
3 A3 NAC TGF8 false 3 15 N	N N	NFQ-MGB 0.070		0.004	0.000	Negative Cont		false	0.200
4 A4 NAC IPC 0.028 true 3 39	BlockedIPC N N	VIC TAMRA N	0.116	0.098	0.013	0.000 Block	ced IPC Control		false 🗸

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GETTING STARTED GUIDE



Booklet 6 - Running Melt Curve Experiments

Publication Part Number 4470050 Rev. A Revision Date March 2012



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About Melt Curve Experiments

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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.

Design the Experiment

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

This chapter covers:

2

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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*TM 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 QuantStudioTM 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:

How do you want to identify this exper	iment?				
Experiment Name: 384-Well Melt Curve Example Barcode: User Name: Example user	ole	Comments:	Melt Curve example		<
• Which block are you using to run the	experiment?				
✓ 384-Well	Array Card		96-Well (0.2mL)	Fast 96-Well (0.1mL)	
What type of experiment do you want	to set up?				
Standard Curve	Relative Standard Curve	Cor	nparative Cτ (ΔΔCτ)	✓ Melt Curve	
Genotyping	Presence/Absence				
* Which reagents do you want to use to	detect the target sequence?				
✓ SYBR® Green Reagents	Other				
What properties do you want for the i	instrument run?				
Standard	Fast				

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

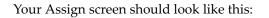
Targets					San	Samples						
New	Save to Library	Import from Library	Delete			Net	v Save to Libra	ry Import from Library	Delete			
Target Name			Reporter	Quencher	Color	Sar	nple Name				Color	
Target :	1		SYBR 🗸	None	× 📃 🕚	Sam	ole 1					~
• Dac	sive Referen	CO										
ROX	sive kereren	ue -										
NUX	~											

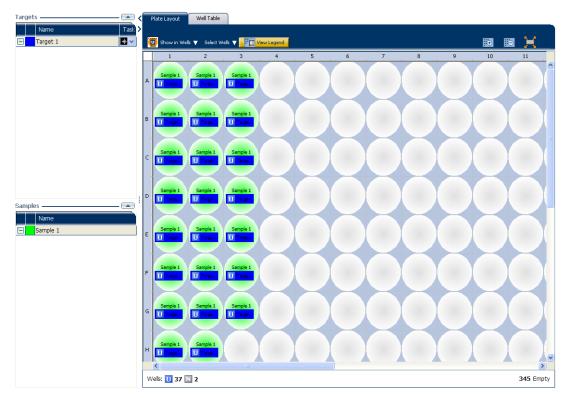
Your Define screen should look like this:

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	





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

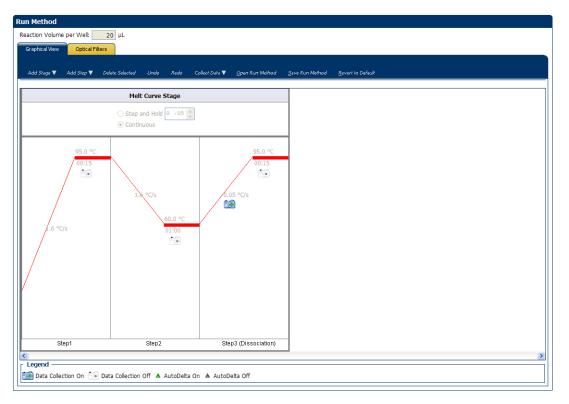
Edit the ramp increment

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

2

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

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:

Prepare the sample dilutions 1 Prepare the reaction mix ("cocktail mix")	Assemble required materials	13
Prepare the reaction plate 1	Prepare the sample dilutions	13
	Prepare the reaction mix ("cocktail mix")	14
For more information 1	Prepare the reaction plate	14
	For more information	15

Assemble required materials

3

- 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/ μ L. After you dilute the sample according to the Sample Dilutions Calculations table, the sample will have a concentration of 10 ng/ μ L. Add 2 μ L 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")

	Reaction Component	Volume for 1 reaction (µL)	Volume for 40 reactions (µL)				
	Power SYBR $^{\textcircled{8}}$ 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				
	Mix. 2. Add the required volume of each cocl	ktail mix componen	t to the tube.				
	2. Add the required volume of each cocl	ktail mix componen	t to the tube.				
	3. Mix the cocktail thoroughly by gently cap the tube.	pipetting up and d	lown several times, ti				
	4. Centrifuge the tube briefly to remove air bubbles.						
	5. Place the cocktail mix on ice until you	prepare the reaction	on plate.				
Calculations	Determine the quantity of primer to be add following calculation:	ded to the reaction r	nix by performing th				
	č						

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

Concentration (initial) C1 x Volume (primer stock) V1 = Concentration (final) C2 x Volume (final reaction) V2

 $(10\mu M) \ge (V1) = (0.05\mu M) (20\mu L)$

 $V1 = (0.05 \times 20) / 10 = 0.1$

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, <i>Getting Started with QuantStudio™ 12K Flex</i> System Multi-Well Plate and Array Card Experiments	4470050
Sealing the reaction plate	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 12K Flex</i> System Multi-Well Plate and Array Card Experiments	4470050

Chapter 3 Prepare the Reactions *For more information*

3

This chapter explains how run the example experiment on the QuantStudio $^{\rm TM}$ 12K Flex Instrument.

This chapter covers:

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

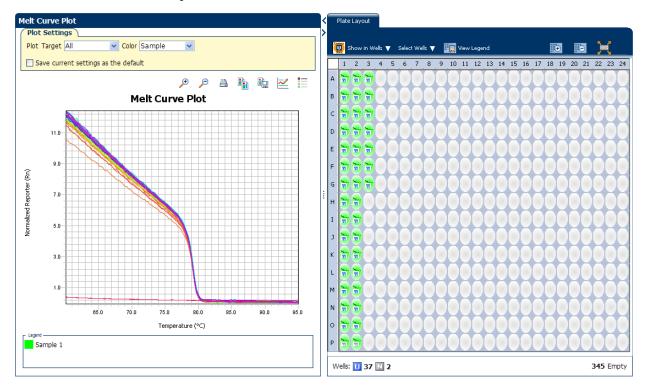
- 1. In the Instrument Console screen, select the instrument icon.
- 2. Click Manage Instrument or double-click on the instrument icon.
- **3.** On the Manage Instrument screen, click **Monitor Running Instrument** to access the Run screen.

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.

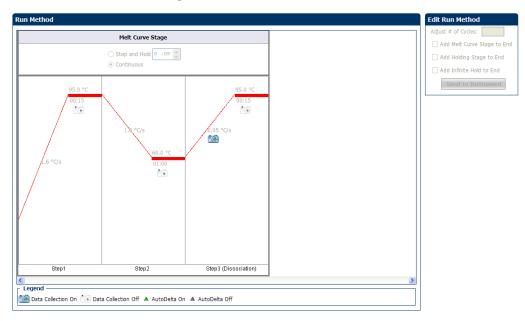


Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

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.



Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide for Melt Curve Experiments

View run data

Click View Run Data from the Run Experiment Menu.

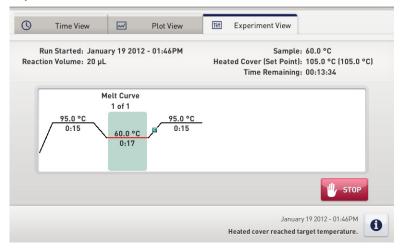
The figure below shows the View Run Data screen as it appears in the example experiment.

Run Data Report	
Experiment Name:	384-Well Melt: Curve Example
	09-20-2011 12:14:48 SGT
Stop Time:	09-20-2011 12:30:16 SGT
Run Duration:	15 minutes 28 seconds
User Name:	DEFAULT
Instrument Name:	QuantStudioDemo
Firmware Version:	0.16.1
Software Version:	QuantStudio 12K Flex Software v1.0
Instrument Serial Number:	QuantStudioDemo
Sample Volume:	20.0
Cover Temperature:	105.0
Block Type:	384-Well Block
Errors Encountered:	 A A

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

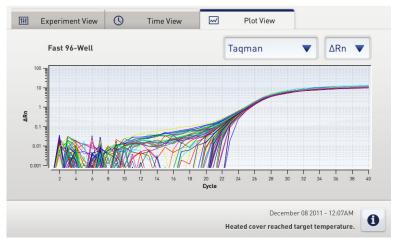


Note: The above screenshot is for visual representation only. Actual results will vary with the experiment.

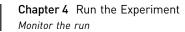
Time View

ד פ	ime View	~	Plot View	141	Experime	ent View		
	tarted: Dece olume: 20 µl		011 - 12:05AM	Heat	ed Cover l		e: 59.5 °	°Cl
icuction (orunner zo p	-		neu	Stage / St			0,
		4		1		5		
	Λ	1	-1	1		2	2	
	0	1	:1	1	•	2	2	
	0	1	:1	1	:	2	2	
	0	1		0	_		2	
	0	1	Remaining Tim	0	Elapsed Ti		2	

Plot View



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

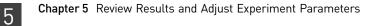


Review Results and Adjust Experiment Parameters

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:

Sect	tion 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
Sect	tion 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.
- 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:

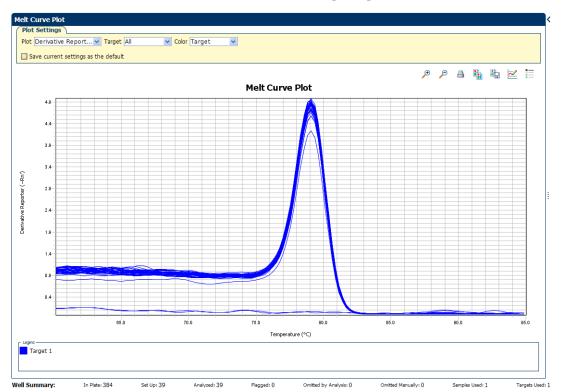
- Normalized Reporter (Rn) vs. Temperature This plot displays the fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference. You can use this plot to see the change in Rn with change in the temperature. You cannot use this plot to determine the Tm of the 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

- From the Experiment menu pane, select Analysis > Melt Curve Plot.
 Note: If no data are displayed, click Analyze.
 - Note: Il no data are displayed, click
 - **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 \land .
View the well table	 Select the Well Table tab. 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 (<u>)</u>.

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

>	Plate I	Layout	Well Table											
	Show in	Table 🔻	Select Wells 🔻	Group by 🖲	,								Expand All	Collapse All
	#	Well	Omit	Flag	Sample	Target	Task	Dyes	Tml	Tm2	Tm3	Comme		
			INTC			-		-						^
	361				Sample 1	Target 1	NTC	SYBR-None	62.140					
	362	P2			Sample 1	Target 1	NTC	SYBR-None	62.916					=
			UNKNOWN	I		-								
	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	12			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				Sample 1	Target 1	UNKNOWN	SYBR-None	79.087					
	265				Sample 1	Target 1	UNKNOWN		78.958					
	266				Sample 1	Target 1	UNKNOWN		78.958					
	289	M1			Sample 1	Target 1	UNKNOWN	SYBR-None	78.958					~
w	ell Sum	mary:	In Plate	e: 384	Set Up: 39	Ani	ilyzed: 39	Flagged: (Omitted by Analys	is: O	Omitted Manually:	0 Samples Used: 1	Targets Used: 1

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 $ ightarrow$ 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

b

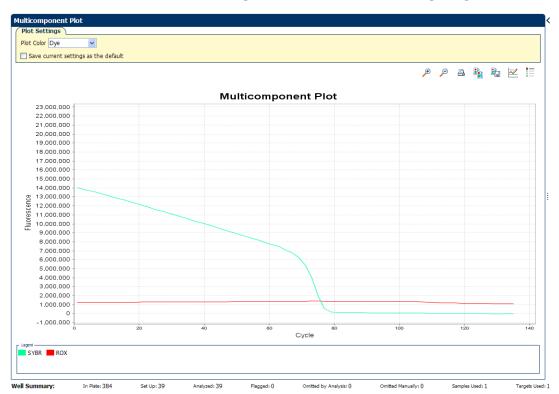
When you analyze your own experiment:

- Review the data for the Unknown samples. For each row that displays \land 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 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	 From the Experiment Menu pane, select Analysis > Multicomponent Plot. Note: If no data are displayed, click Analyze. Display the unknown wells in the plate layout to display the corresponding data in the Multicomponent Plot screen: Click the Plate Layout tab. 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. From the Plot Color drop-down menu, select Dye. Click



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

Tips for confirming dye accuracy in your own experiment

5

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.

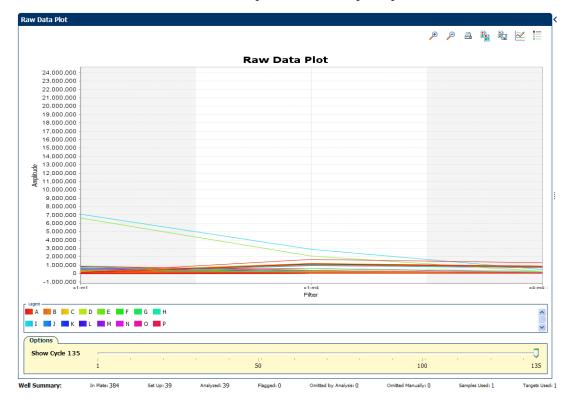
Determine signal accuracy using the Raw Data Plot

	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	 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:

Load Save Revert to Defaults							
				Emission Filter			
		ml(520±15)	m2(558±11)	m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
	x1(470±15)						
	x2(520±10)						
	x3(550±11)						
Excitation Filter	x4(580±10)						
- 6	x5(640±10)						
lt C	x6(662±10) Curve Filter ———			Load Save Revert to	refacults		
lt C	x6(662±10)			Load Save Revert to Emission Filter	nefaults		
elt C	x6(662±10)	m1(520±15)	m2(558±11)		ฟระประ m4(623±14)	m5(682±14)	m6(711±12)
elt C	x6(662±10)	m1(520±15)	m2(558±11)	Emission Filter		m5(682±14)	
	x6(662±10) Curve Filter ———			Emission Filter m3(586±10)	m4(623±14)		m6(711±12)
	x6(662±10) Curve Filter x1(470±15)			Emission Filter m3(586±10)	m4(623±14)		m6(711±12)
Excitation Filter	x6(662±10) Curve Filter x1(470±15) x2(520±10)			Emission Filter m3(586±10)	m4(623±14)		m6(711±12)
	x6(662±10) xurve Filter x1(470±15) x2(520±10) x3(550±11)			Emission Filter m3(586±10)	m4(623±14)		m6(711±12)

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

- 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.

P Multiple Tm peaks 0 SIGNAL No signal in well 0	ig Details							
P Multiple Tm peaks 0 SIGNAL No signal in well 0 FEGALE Fluorescence is offscale 0	Flag:	Des	scription	Fre	quency	v	Vells	
FSCALE Fluorescence is offscale 0 FSCALE Flag: MTP—Multiple Tm peaks Flag Detail: Melt curve analysis shows more than one Tm peak lagged Wells: None	TP	Multip	ple Tm peaks					
Flag: MTP—Multiple Tm peaks Flag Detail: Melt curve analysis shows more than one Tm peak peak Jagged Wells: None None	OSIGNAL	No si	gnal in well	0				
Flag Detail: Melt curve analysis shows more than one Tm peak lagged Wells: None	FFSCALE	Fluor	escence is offscale	0				

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, <i>Getting Started with QuantStudio™ 12K Flex</i> 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.

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:

natysis settings i	for 384-Well Melt Curve Examp	ne			
lelt Curve Settings	Cr Settings Flag Settings	Advanced Settings			
Review the def Multi-Peak Ci	ault settings for analysis of targets in the alling, then change the settings.	nis experiment. To use different se	ttings for a target, select the target from the tabk	e, deselect Enable	?
Select a Target	t		Multi-Peak Setting for Tar	get 1	
Target	Multi-Peak Calling	Peak Level (%)	Enable Multi-Peak Calling		
Farget 1	Enabled	10.00	Peak level relative to the dor	minant neak (0/) + 10.00	
			Peak level relative to the do	Timane peak (96) . 10.00	
					_
ve to Library	Load from Library	Reve	ert to Default Analysis Settings	pply Analysis Settings	Cano

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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 QuantStudioTM 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

Melt Curve Settings

Use this tab to:

• Enable or disable multi-peak calling.

You may change the following settings:

- 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.

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The Flag Settings tab looks like this:

Melt Curve Setting	gs Cr Settings Flag Set	ttings Advanced	Settings			
Configure t	he flags and filtering. In this panel y	vou can enable, disable	e, and configure flags, and	d indicate if a well is to be rej	iected when a flag is raise	ed. 📀
Flag	Description	Use	Attribute	Condition	Value	Reject Well
FFSCALE	Fluorescence is offscale	~				
ТР	Multiple Tm peaks	~				
OSIGNAL	No signal in well	~				

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

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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**.

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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	<pre><drive>:\Applied Biosystems\QuantStudio 12K Flex Software\experiments</drive></pre>

Your Export screen should look like this:

Sample Setup				Export file	Name: 384-weir r	Melt Curve Exam	ple_QuantStudio_	File Type: 📋 (*.t	xt)
	a 🚺 🔽 Amp	lification	Multicomponent	Results					
Skip Empty Wells 🛛 🗹 Skip Om		In reactory	- Harcomponence						
elect Content									
	~	Well	Well Position	Sample Name	Target Name	Task	Reporter	Quencher	C
All Fields			1 A1	Sample 1	Target 1	UNKNOWN	SYBR	None	
7 Well			2 A2	Sample 1	Target 1	UNKNOWN	SYBR	None	
-			3 A3	Sample 1	Target 1	UNKNOWN	SYBR	None	
Well Position			25 B1	Sample 1	Target 1	UNKNOWN	SYBR	None	
7			26 B2	Sample 1	Target 1	UNKNOWN	SYBR	None	
Sample Name			27 B3	Sample 1	Target 1	UNKNOWN	SYBR	None	
Target Name			49 C1 50 C2	Sample 1	Target 1	UNKNOWN	SYBR SYBR	None	
_ rarget Name			50 C2 51 C3	Sample 1	Target 1	UNKNOWN		None	
7 Task			73 D1	Sample 1 Sample 1	Target 1	UNKNOWN	SYBR SYBR	None	
			74 D2	Sample 1	Target 1 Target 1	UNKNOWN	SYBR	None	
Reporter			74 D2 75 D3	Sample 1	Target 1	UNKNOWN	SYBR	None	
Quencher	=		97 E1	Sample 1	Target 1	UNKNOWN	SYBR	None	
JQuencher			98 E2	Sample 1	Target 1	UNKNOWN	SYBR	None	
СТ			90 E2 99 E3	Sample 1	Target 1	UNKNOWN	SYBR	None	
			121 F1	Sample 1	Target 1	UNKNOWN	SYBR	None	
Ct Mean			122 F2	Sample 1	Target 1	UNKNOWN	SYBR	None	
_			123 F3	Sample 1	Target 1	UNKNOWN	SYBR	None	
Ct SD			145 G1	Sample 1	Target 1	UNKNOWN	SYBR	None	
Quantity			145 G2	Sample 1	Target 1	UNKNOWN	SYBR	None	
Qualitity			147 G3	Sample 1	Target 1	UNKNOWN	SYBR	None	
Ouantity Mean			169 H1	Sample 1	Target 1	UNKNOWN	SYBR	None	
			170 H2	Sample 1	Target 1	UNKNOWN	SYBR	None	
Quantity SD			193 I1	Sample 1	Target 1	UNKNOWN	SYBR	None	
The second se			194 12	Sample 1	Target 1	UNKNOWN	SYBR	None	
Automatic Ct Threshold			217 J1	Sample 1	Target 1	UNKNOWN	SYBR	None	
Ct Threshold			218 J2	Sample 1	Target 1	UNKNOWN	SYBR	None	
			241 K1	Sample 1	Target 1	UNKNOWN	SYBR	None	
Automatic Baseline			242 K2	Sample 1	Target 1	UNKNOWN	SYBR	None	
			265 L1	Sample 1	-	UNKNOWN	SYBR	None	
Baseline Start	~	<	265 L1	Sample 1	Target 1	UNKNOWN	SYBR	None	

Start Export Save Export Set As Load Export Set Delete Export Set

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 performed on = 2011-08-05 00 Calibration Background performed on = 2011-08-05 00 Calibration FAM is expired = NO Calibration FAM performed on = 2011-08-05 02:04:58 Calibration ROT is expired = NO Calibration ROT is expired = NO Calibration ROX is expired = NO Calibration SYBR performed on = 2011-08-05 02:23:30 Calibration SYBR performed on = 2011-09-15 05:13:51 Calibration SYBR performed on = 2011-09-15 05:13:55 Calibration SYBR 2 performed on = 2011-09-15 05:13:55 Calibration SYBR2 performed on = 2011-09-15 05:13:56 Calibration SYBR2 performed on = 2011-09-15 05:13:51 Calibration SYBR2 performed on = 2011-09-05 00 Calibration VIC sexpired = NO Calibration VIC sexpired = NO Chemistry = SYBR_GREN Comment = NA Experiment File Name = C:\Program Files\Applied Bio Experiment Run End Time = 2011-09-20 12:30:16 PM SG Experiment Run End Time = 2011-09-20 12:30:16 PM SG Experiment Run End Time = QuantStudioDemo Instrument Serial Number = QuantStudioDemo Instrument Serial Number = QuantStudioDemo Instrument Type = QuantStudioDemo Instrument Type = Reit Curve Passive Reference = ROX Quantification Cycle Method = Ct Signal Smoothing On = true User Name = NA	AM SGT AM SGT AM SGT 6 AM SGT 11 AM SGT 1:56:21 AM SGT AM SGT systems\QuantStudio12KFlex\examples\Melt Curve\384-well Melt Curve Example.eds	
[Sample Setup] well well Position Sample Name Sample Color Quencher Quantity Comments 1 Sample 1 "RGB(0,255,0)" 2 A2 Sample 1 "RGB(0,255,0)" 3 A3 Sample 1 "RGB(0,255,0)" 4 A4 Sample 1 "RGB(0,255,0)" 5 A5 Sample 1 "RGB(0,255,0)" 6 A6 A6 Sample 1 "RGB(0,255,0)" 7 A7 Sample 1 "RGB(0,255,0)" Sample 1 8 A8 Sample 1 "RGB(0,255,0)" Sample 1 9 A9 Sample 1 "RGB(0,255,0)" Sample 1 10 A10 Sample 1 "RGB(0,255,0)" Sample 1 10 A10 Sample 1 Sample 1 Sample 1 11 A11 Sample 1 Sample 1 Sample 1 12 A12 Sample 1 Sample 1 Sample 1 13 A13 Sample 1 Sample 1	Biogroup Name Biogroup Color Target Name Target Color Task Repo Target 1 "RGB(0,0,255)" UNKNOWN SYBR None Target 1 "RGB(0,0,255)" UNKNOWN SYBR None Target 1 "RGB(0,0,255)" UNKNOWN SYBR None	orter

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www.lifetechnologies.com

GETTING STARTED GUIDE



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

Publication Part Number 4470050 Rev. A Revision Date March 2012



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Ordering Information

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Consumables

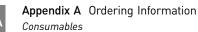
The consumables listed below are required for calibrating the QuantStudioTM 12K Flex Instrument and for performing experiments with the QuantStudioTM 12K Flex System.

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

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

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	–15 to –25°C
384-Well Spectral Calibration Plate with VIC® Dye	4432278	consumable by the expiration	
384-Well Spectral Calibration Plate with ROX [™] Dye	4432284	date mentioned	
384-Well Spectral Calibration Plate with SYBR® Green Dye	4432290	on the package	
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		



••	er der mig	
sumables		

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	–15 to –25°C
96-Well Spectral Calibration Plate with VIC® Dye	4432334	consumable by the expiration	
96-Well Spectral Calibration Plate with ROX [™] Dye	4432340	date mentioned	
96-Well Spectral Calibration Plate with $SYBR^{\circledast}$ Green Dye	4432346	on the package	
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 μ L) 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	–15 to –25°C
Fast 96-Well Spectral Calibration Plate with VIC® Dye	4432396	consumable by the expiration	
Fast 96-Well Spectral Calibration Plate with ROX [™] Dye	4432402	date mentioned	
Fast 96-Well Spectral Calibration Plate with SYBR [®] Green Dye	4432408	on the package	
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 $^{\text{TM}}/\text{ROX}^{\text{TM}}$ and $\text{VIC}^{\textcircled{B}}/\text{ROX}^{\text{TM}}$ 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	–15 to –25°C
Array Card RNase P Instrument Verification Kit	4432265	consumable by the expiration date mentioned on the package	

ExperimentThe 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	Room
	2nd Generation	4442571	consumable by the expiration	temperature
TaqMan $^{ extsf{B}}$ Array Micro Fluidic Card Sealer	ł	4331770	date mentioned	
MicroAmp [®] Optical 384-Well Reaction Plate	1000 plates	4343814	on the package	
with Barcode	500 plates	4326270		
	50 plates	4309849		
MicroAmp $^{ extsf{B}}$ Optical 384-Well Reaction Plate, 10	000 plates	4343370	_	
MicroAmp [®] Optical 96-Well Reaction Plate	500 plates	4316813		
(0.2µL)	10 plates	N8010560	_	
MicroAmp [®] Optical 96-Well Reaction Plate	500 plates	4326659	_	
with Barcode (0.2µL	20 plates	4306737	_	
MicroAmp [®] Fast Optical 96-Well Reaction Plate (0.1µL)	10 plates	4346907	_	
MicroAmp $^{\mbox{\scriptsize B}}$ Fast Optical 96-Well Reaction Plate with Barcode (0.1 μ L)	200 plates	4366932	_	
	20 plates	4346906		
Centrifuge Buckets, Array Card	1 st Generation	4337230		
	2 nd Generation	4442573		
Clip, Array Card Centrifuge Adaptor		4334682		
MicroAmp [®] Optical Adhesive Film	100 films	4311971		
	25 films	4360954		
MicroAmp $^{ extsf{B}}$ 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 $^{ extbf{B}}$ Fast Reaction Tube with cap (0.1 μ L)	1000 tubes	4358297		
MicroAmp® 96-Well Tray/Retainer Set (Blue) (for 0.2µL)	10 pairs	4381850		
MicroAmp $^{ extsf{8}}$ 96-Well Tray (Black) (for 0.1 μ L)	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	Room
Fast 96-Well Plate Adapter (0.1 µL)	1 piece	4459846	 consumable by the expiration date mentioned 	temperature
96-Well Tube Adapter (0.2 µL)	1 piece	4462077	on the package	
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 applic	ators)	4333183		

Reagents

The following table lists the reagents that can be ordered for performing experiments with the QuantStudio $^{\rm TM}$ 12K Flex System.

Recommended reagent kits				
To perform	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



Appendix A Ordering Information *General-use materials and consumables*

Documentation and Support

Related documentation

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

Document	
Allelic Discrimination Pre-Developed TaqMan $^{\circledast}$ 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 $^{ extsf{B}}$ Assay Reagents Allelic Discrimination Protocol	4312214
TaqMan [®] Drug Metabolism Genotyping Assays Protocol	4362038
TaqMan [®] SNP Genotyping Assays Protocol	4332856
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
	Allelic Discrimination Pre-Developed TaqMan® Assay Reagents Quick Reference Card Custom TaqMan® Genomic Assays Protocol Custom TaqMan® SNP Genotyping Assays Protocol Ordering TaqMan® SNP Genotyping Assays Quick Reference Card Pre-Developed TaqMan® Assay Reagents Allelic Discrimination Protocol TaqMan® Drug Metabolism Genotyping Assays Protocol TaqMan® SNP Genotyping Assays Protocol TaqMan® Drug Metabolism Genotyping Assays Protocol TaqMan® SNP Genotyping Assays Protocol Document DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol NucPrep® Chemistry: Isolation of Genomic DNA from Animal and Plant Tissue

Documents related to Relative	Document	PN
Standard Curve and Comparative C _T experiments	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	
Amplification Efficiency of TaqMan $^{\circledast}$ Gene Expression Assays Application Note	
Custom TaqMan [®] Gene Expression Assays Protocol	
Primer Express [®] Software Version 3.0 Getting Started Guide	
TaqMan [®] Gene Expression Assays Protocol	
User Bulletin #2: Relative Quantitation of Gene Expression	

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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