USER GUIDE



QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software

Getting Started Guide

for use with: QuantStudio[™]6 and 7 Flex Real-Time PCR Systems

Publication Number 4489822 Revision A





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USER GUIDE



Getting Started with QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Experiments Booklet 1

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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, refer to the instrument user guide.

IMPORTANT! Before using this product, read and understand the information in the instrument user guide.

Revision history

Revision	Date	Description
A	October 2013	New document

Purpose

The QuantStudioTM 6 and 7 Flex Real-Time PCR System Software Experiments Getting Started Guide functions as both a tutorial and as a guide for performing your own experiments on the QuantStudioTM 6 and 7 Flex Instruments.

Note: For differences between the QuantStudioTM 6 System and the QuantStudioTM 7 System, refer to the *QuantStudioTM* 6 and 7 Flex Real-Time PCR Systems Maintenance and Administration Guide (Pub. no. 4489821).

Prerequisites

This getting started 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 QuantStudioTM 6 and 7 Flex Real-Time PCR System Software, please read *Getting Started with QuantStudio*TM 6 and 7 Flex Real-Time PCR System Software 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[™] 6 and 7 Flex Real-Time PCR System Software and the example data provided on the installation CD. The following booklets are provided:

- *Getting Started with QuantStudio*[™] 6 and 7 *Flex Real-Time PCR System Software Experiments* introductory information and experiment workflow common to all experiments.
- QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Standard Curve Experiments designing, running, and analyzing a Standard Curve experiment.
- QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments – designing, running, and analyzing Relative Standard Curve and Comparative C_T experiments.

Note: This booklet also provides information on setting up, running, and analyzing a gene expression study of two Comparative C_T experiments.

- QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Genotyping Experiments designing, running, and analyzing a Genotyping experiment.
- QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Presence/Absence Experiments – designing, running, and analyzing a Presence/ Absence experiment.
- QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Melt Curve Experiments designing, running, and analyzing a Melt Curve experiment.
- QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software 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[™] 6 and 7 Flex Software Double-click \square (QuantStudioTM 6 and 7 Flex Real-Time PCR System Software shortcut) to access the Home screen, shown in the following image.



Open an example experiment

- 1. In the Home screen, select **Open** from the toolbar.
- 2. Navigate to the examples folder. The default path is: *<drive>*:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\QS6Flex or *<drive>*:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\QS7Flex.

where, $\langle drive \rangle$ is the computer hard drive on which the QuantStudioTM 6 and 7 Flex Real-Time PCR System Software is installed. The default installation drive for the software is the C: drive.

Experiment type	Example experiment file name
Standard Curve	QS6_96-Well Standard Curve Example.eds
	QS6_384-Well_Standard Curve Example.eds
	QS7_TaqMan_Array_Standard Curve Example.eds
	QS7_TaqMan_Array_RNaseP_Example.eds
	QS7_384-Well_Standard Curve Example.eds
	QS7_96-Well Standard Curve Example.eds
Relative Standard	QS6_96-Well Relative Standard Curve Example 2.eds
Curve	QS6_96-Well Relative Standard Curve Example.eds
	QS6_384-Well_Relative Standard Curve Example 2.eds
	QS6_384-Well_Relative Standard Curve Example.eds
	QS7_96-Well Relative Standard Curve Example 2.eds
	QS7_96-Well Relative Standard Curve Example.eds
	QS7_384-Well_Relative Standard Curve Example 2.eds
	QS7_384-Well_Relative Standard Curve Example.eds
Comparative C_{T}	QS6_96-Well Comparative Ct Example.eds
	QS6_384-Well_Comparative Ct_Example_1.eds
	QS6_384-Well_Comparative Ct_Example_2.eds
	QS6_384-Well_Comparative Ct_Example.eds
	QS7_96-Well Comparative Ct Example.eds
	QS7_384-Well_Comparative Ct_Example_1.eds
	QS7_384-Well_Comparative Ct_Example_2.eds
	QS7_384-Well_Comparative Ct_Example.eds
	QS7_TaqMan_Array_Comparative_Ct_Example.eds
Multiplex	QS6_96-Well Multiplex Example.eds
	QS6_384-Well_Multiplex_Example.eds
	QS7_96-Well Multiplex Example.eds
	QS7_384-Well_Multiplex_Example.eds
Genotyping	QS7_96-Well SNP Genotyping Example.eds
	QS7_384-Well_SNP_Genotyping_Example.eds
	QS6_96-Well SNP Genotyping Example.eds
	QS6_384-Well_SNP_Genotyping_Example.eds
Presence/Absence	QS6_384-Well_Presence-Absence_Example.eds
	QS6_96-Well Presence-Absence Example.eds
	QS7_96-Well Presence-Absence Example.eds
	QS7_384-Well_Presence-Absence_Example.eds

3. Select an example experiment file to open, then click **Open**.

Experiment type	Example experiment file name
Melt Curve	QS6_96-Well SYBR Green PCR w Melt Example.eds
	QS6_384-Well_SYBR_Green_PCR_with_Melt_Example.eds
	QS6_384-Well_SYBR_Green_Melt_Example.eds
	QS6_384-Well_Melt_ Example.eds
	QS7_96-Well SYBR Green PCR w Melt Example.eds
	QS7_384-Well_SYBR_Green_PCR_with_Melt_Example.eds
	QS7_384-Well_SYBR_Green_Melt_Example.eds
	QS7_384-Well_Melt_ Example.eds

Note: In addition to the example experiment files, the following user sample files are located at: C:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\User Sample Files

- Barcode template files
- Copy-paste example file
- Custom Sample Properties files
- Sample setup files
- Import custom fields file
- Export files

For more information on using the above files, see Chapter 2, "Experiment Shortcuts" on page 53.

A note on system security

The Security, Auditing, and e-Signature (SAE) feature in QuantStudioTM 6 and 7 Flex Real-Time PCR System Software enables role-based access control to enforce data integrity and authentication of users logging into the system, to strengthen system security. The feature tracks actions performed by users on experiments, templates, and studies, and it tracks changes to the SAE settings. You can enable or disable this feature to accommodate your security needs.

To enable or disable the feature, from the toolbar select **Tools > Security > Settings**.

For more information on the SAE feature, please refer to the instrument user guide.

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.

General Experiment Information and Instructions

This chapter covers:

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Note: For more information about any of the topics discussed in this guide, access the Help from within QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software by pressing F1, clicking ? in the toolbar, or selecting **Help > QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Help**.

Set up an experiment

Note: To start the QuantStudioTM 6 and 7 Flex Software, see "Start the QuantStudioTM 6 and 7 Flex Software" on page 7.

 Define experiment
 All experiments require the same general setup tasks; individual booklets supply

 properties
 Specific parameters. The following procedures outline general steps to take to set up an experiment.

Access QuantStudioTM 6 and 7 Flex Software and click \bigcirc (Experiment Setup). Click Experiment Properties to access the Experiment Properties screen.

Define experiment name and type

- 1. Enter a unique experiment name in the Experiment Name field. The default is a date and time stamp, which you can change. For example, 2010-04-12 173730.
 - Enter a name that is descriptive and easy to remember. You can enter up to 100 characters.
 - You can only use the alpha-numeric, hyphen (-), underscore (_), and spaces () characters.

Note: Ensure 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 when attempting to start 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 instrument type you are using to run the experiment
 - QuantStudio[™] 6 Flex System
 - QuantStudio[™] 7 Flex System
- 6. Select the block type you are using to run the experiment
 - 384-Well Block
 - Array Card Block (only applicable to the QuantStudio[™] 7 Flex System)

- 96-Well Block (0.2mL)
- Fast 96-Well Block (0.1mL)
- 7. 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 fluorescence data collected during post-PCR read.
 - Amplification to include real-time data.
 - **Post-PCR Read** to include data after amplification has taken place.
- **3.** For the Melt Curve experiment, select the **Include PCR** check box, to include PCR.

Review the analysis settings

Analysis Settings are different for each experiment type. The software analyzes the data using the default analysis settings. If the default analysis settings in the QuantStudio[™] 6 and 7 Flex Software are not suitable for your own experiment, you can change the settings in the Analysis Settings dialog box and save the changed analysis settings to the Analysis Settings Library

Note: For information on Analysis Settings Library, refer to Booklet 7, *QuantStudio*^{IM} 6 and 7 Flex Real-Time PCR System Software Experiments - Appendixes.

Enter the reagent information

In the Reagent Information panel you can enter detailed reagent information, including the part number, lot number, and expiration date of the reagents you will use in your experiment. This information can be entered before setting up your experiment or starting your calibration run.

- 1. In the Reagent Information panel, click **New** to add a line for reagent details, or **Delete** to remove an existing one.
- **2.** Click within the first four fields to enter your reagent **Type**, **Name**, **Part Number**, or **Lot Number**, respectively:
- **3.** Click the Expiration Date field, and click the "down arrow" to display the current month's calendar. Select the reagent's expiration date from that month, or click the "forward arrow" to select a future date.

Save the experiment

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 following is an image of the Experiment Properties screen for a Standard Curve experiment:

Experiment: QS6_QuantSI	tudio_384-Wel Type: Sta	ndard Curve		Reagents: 1	aqMan® Reagents	?
How do you want to identify	this experiment?					^
* Experiment Name: QS6_QuantStu Barcode: User Name: Example User	idio_384-Well_Standard_Curve_Exan	nple	Comments: S	tandard Curve Example		~
• Which instrument type are	you using to run the experime	nt?				
✓ QuantStudio [™] 6 Flex Syste	em QuantStudio™ 7	Flex System				
* Which block are you using t	to run the experiment?					
✓ 384-Well	96-Well (0.	2mL)	Fast	96-Well (0.1mL)		
* What type of experiment do	o you want to set up?					
✓ Standard Curve	Relative Standa	ard Curve	Compa	arative Cτ (ΔΔCτ)	Melt Curve	
Genotyping	Presence/At	osence				
* Which reagents do you wan	nt to use to detect the target s	equence?				
✓ TaqMan® Reagents	SYBR® Green F	Reagents		Other		
* What properties do you wa	nt for the instrument run?					
Standard	✓ Fast					
What is the reagent informat	ion?					
New Delete						
Туре	Name	Part Number		Lot Number	Expiration Date	
Master Mix	TaqMan Fast Universal PCR Master Mix	4984571		1206155	12-31-2013	

Define targets, samples, and biological replicate groups Use the Define screen to define targets, samples and biological replicates for your experiment.

Note: You can start a run without these definitions, but there will be no real-time data (data will not be visible) in the amplification plots (the amplification plots can be seen only after you have set up the plate).

- 1. Click **Define** to access the Define screen.
- 2. Define targets.
- **Note:** For Genotyping experiments, use this screen to specify the number of SNP assays to include in the experiment. For more information on defining SNP assays, refer to Booklet 4, *QuantStudio*[™] 6 and 7 Real-Time PCR System Software Getting Started Guide for Genotyping Experiments.
 - 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. Refer to Appendix B, Supplemental Information in Booklet 7, *QuantStudio*TM 6 and 7 Flex Real-Time PCR System Software Experiments - Appendixes for information on target libraries.

- f. (Optional) 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. Refer to Appendix B, Supplemental Information in Booklet 7, *QuantStudio*TM 6 and 7 Flex Real-Time PCR System Software Experiments - Appendixes for information on sample libraries.

- e. (*Optional*) Click **Import from Library** to add samples from the sample library.
- 4. (Optional) 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 dye from the drop-down menu.
- **6.** Define custom task name.

Note: The Custom Task Name panel is visible only when the **Hide the custom task name definition and assignment UI** check box under the Setup tab in the Preferences dialog box is unselected.

The following is an image of the Define screen for a Standard Curve experiment:

Targets				Sample	25			
New Save to Library Import from Library	Delete			New	Save to Library	Import from Library	Delete	🏣 Import from File
Target Name	Reporter	Quencher	Color	Sample	e Name	Color		
RNAse P	FAM Y	NFQ-MGB	×	5K		~		
				10K		~		
Biological Replicate Groups								
New Delete								
Biological Group Name Color		Comments						
Passive Reference				Custor	n Task Name			
ROX				New	Delete			
				Name		Color		lcon Char

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

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

- 1. Click **Assign** to access the Assign screen.
- **2.** Assign targets.
 - a. Select wells using the plate layout or the well table on the Assign screen.
 - **b.** Select a target and assign its task, in the plate, from the drop-down menu. Depending on the experiment type, options are:

Experiment type	Legend	Tasks
Standard Curve	U	Unknown
	S	Standard
	N	Negative Control
		•
Relative Standard Curve	U	Unknown
	S	Standard
	N	Negative Control
	1	-
Comparative CT	U	Unknown
	N	Negative Control
	1	-
Genotyping	U	Unknown
	1/1	Positive Control Allele 1/ Allele 1
	2/2	Positive Control Allele 2/ Allele 2
	1/2	Positive Control Allele 1/ Allele 2
	N	Negative Control
	1	-
Presence/Absence	U	Unknown
	I	Internal Positive Control
	N	Negative Control
	7	Blocked Internal Positive Control
Melt Curve	U	Unknown
	Ν	Negative Control

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

Note: You can assign only one sample to a well. If the selected wells contain mixed assignments (indicated by a _), remove existing sample assignments before you make the new sample assignment.

- 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 following is an image of the Assign screen for a Standard Curve experiment:

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

As shown below, you can also paste assignment information from an *.xls file into the plate layout of the QuantStudioTM 6 and 7 Flex Software for wells with single targets.

Note: You must select the header and the Well Number column while copying information from the *****.*x***ls** file.

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

	B1	- (*	f_{∞}	Sample					
	А	В		С	D	E	F	G	н
1	Well	Sample	Biologi	ical Group	Target	Task	Dyes	Quantity	Comments
2	1	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
3	2	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
4	3	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
5	4	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
6	5	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
7	6	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
8	7	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
9	8	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
10	9	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
11	10	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
12	11	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
13	12	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
14	13	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
15	14	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		
16	15	5K			RNaseP	UNKNOWN	FAM-NFQ-MGB		

Copy and paste

				-			, I				
🕂 Define and Set Up Standards	< Plat	e Layout	Well Table								
Targets A											
Name Tady Outers Ountity	Show	in Table 🔻	Select Wells 🔻	Group by 🔻						E Exnand All	🗏 Callanse All
Name Task Custom Quantity										- uspand / ii	
RNaseP U V	#	Well	Sample	Biological Group	Target	Task	Dyes v1	Quantity	Comments		
Target 1 💌 💌	1	A1	5K		RNaseP	UNKNOWN	FAM-NEO-MGB				^
	2	A2	5K		RNaseP	UNKNOWN	FAM-NFO-MGB				
	3	A3	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				
	4	A4	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				
	5	A5	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				_
	6	A6	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				
	7	A7	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				
	8	A8	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				
	9	A9	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				
Samples	10	A10	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				
samples	11	A11	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				
Name	12	A12	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				
🗹 🚾 5К	13	A13	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				
Sample 2	14	A14	5K		RNaseP	UNKNOWN	FAM-NFQ-MGB				
	15	A15	5K		KNaseP	UNKNOWN	FAM-NFQ-MGB				
	10	A10									
	10	A10									
	10	A10									
	20	A20									
	21	A21									
	22	A22									
	23	A23									
	24	A24									
Biological Groups	25	B1									
Biological Group	26	B2									
	27	B3									
	28	B4									
	29	B5									
	30	B6									
	31	B7									
	32	88									
	33	B9									
	34	B10									
	35	811									
	36	B12									
	37	813									~

Note: An example copy and paste file is provided with the QuantStudioTM 6 and 7 Flex Real-Time PCR System Software and is located at C:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\User Sample Files. where, *<drive>* is the computer hard drive on which the QuantStudioTM 6 and 7 Flex Software is installed. The default installation drive for the software is the C: drive.

Define the run
methodUse the Run Method screen to set up the run method for your own experiments in the
QuantStudioTM 6 and 7 Flex Software.

Note: Refer to the Booklet 7, $QuantStudio^{TM}$ 6 and 7 Real-Time PCR System Software Experiments - Appendixes for information on analysis settings.

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. Refer to Appendix B, Supplemental Information in Booklet 7, *QuantStudio*TM 6 and 7 Flex Real-Time PCR System Software Experiments - Appendixes for information on run method libraries.

- 2. Enter a number for the reaction volume per well. See "Instrument consumables" on page 17 for maximum reaction volumes for the consumables supported by the QuantStudio[™] 6 and 7 Flex Software.
- **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[™] 6 and 7 Flex Software.
 - Enable data collection by clicking 🔯.

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

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

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

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



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

4. (Optional) Complete the tasks on the Optical Filters tab:

IMPORTANT! Do not alter the optical filters for system dyes. This feature is optional when you use custom dyes, where you can select a filter set to match the profile of the dye. For more information on how to select the appropriate filter set, contact Life Technologies.

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 Default tab.

49 Preferences								
Setup	Export	Display Format	SMTP Settings	Notification S	ettings			
Defaul	lts	Startup	Experiment		Print			
Select the opening of the opening	default folders, t	he default block type, and whether	to show optical filters in the run m	ethod.				
Data Folder:	C:\Ap	plied Biosystems\QuantStu	dio 6 and 7 Flex Software\	User Files\Experim	Browse			
Import Folder:	C:\Ap	plied Biosystems\QuantStu	dio 6 and 7 Flex Software\	User Files\Import	Browse			
Instrument Typ	e: Quar	QuantStudio T Flex System						
Block Type:	384-	384-Well Block						
Decimal Places t	o Show: 3	v: 3						
Show optic	al filters for ru	method						
		Import System Pro	eferences Export Syste	m Preferences	K Cancel			

- To add a new filter set to the filter set library, click **Save**.
- To load a saved filter set, click Load.
- To go back to the original filter set combinations, click Revert to Defaults.

Getting Started with QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Experiments

Prepare reactions

Supported reagents

Life Technologies supports the following reagents for experiments performed with the QuantStudio TM 6 and 7 Flex Software.

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

Note: The QuantStudio[™] 6 and 7 Flex Software can accommodate other reagents, but performance claims have not been tested by Life Technologies.

Precautions while preparing reactions

- 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.
 - Use the appropriate consumables. The quality of pipettors and tips and the care used in measuring and mixing dilutions affect data accuracy.
 - 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.
 - Ensure that the arrangement of the PCR reactions matches the plate layout displayed in the QuantStudio[™] 6 and 7 Flex Software.

Materials required while preparing the dilutions

- DI water or DEPC water
- Microcentrifuge tubes
- Pipettors
- Pipette tips
- Vortex mixer
- Centrifuge
- Sample stock

- Standard stock
- Reaction mix components
- Plate or array card

Guidelines for preparing the dilutions, reaction mix, and plate

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

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

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

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

1. Load the reaction plate using the plate layout described in "Assign targets, samples, and biological replicate groups" on page 16.

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

2. Remove a single optical adhesive film (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). Ensure that the film completely covers all wells of the reaction plate.
- **5.** Applying firm pressure, move the applicator slowly across the film, horizontally and vertically, to ensure good contact between the film and the entire surface of the reaction plate.





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

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

7. To ensure a tight, evaporation-free seal, repeat step 5. 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 ensure that all wells are sealed. You should see an imprint of all wells on the surface of the film. The perforated tab should 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 sample block of the QuantStudio[™] 6 or 7 Instrument.

Fill and seal the array card	Fill and spin the array card				
	IMPORTANT! Wear powder-free gloves while preparing the Arrays.				
	1. Remove an array card from its box and place it on a clean, dry surface.				

- 2. Using a permanent marker, mark the side of the empty array cards.
- 3. Transfer the experiment-related chemistries and solutions into the port of the array card.

For each transfer:

- **a**. Place the array card on a lab bench, with the foil side down.
- **b**. Load 100 µL of fluid into a pipette.

c. Hold the pipette in an angled position (~45 degrees) and place the tip into the fill port. The fill port is the larger of the two holes on the left side of the fill reservoir.



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. Confirm that the labels on the buckets and clips are oriented in the same direction.

IMPORTANT! Balance the loads in opposite buckets in the centrifuge.

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



Filled array

Empty

array

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

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

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

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

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



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: Contact Life Technologies for more information on loading an array card.

Seal the array card(s)

- 1. With the carriage (roller assembly) of the Array Card Staker/ 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. Run the carriage over the array card 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 **WARNING!** Use the flat caps for the 0.2 mL tubes and 0.1 mL tubes. Rounded caps can damage the heated cover.

Note: Ensure 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 and use the following instructions:

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

Required materials:

- MicroAmp[®] Cap Installing Tool
- MicroAmp[®] Optical 8-Tube Strips or MicroAmp[®] Optical Tubes without cap
- MicroAmp[®] Optical 8-Cap Strip



MicroAmp[®] Cap Installing Tool

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

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

Remove a cap string from a plate

The MicroAmp[®] Cap Installing Tool is also used for removing the MicroAmp[®] Optical 8-Cap Strip from the 96-well optical plates and tray/ retainer assemblies. To remove the cap or cap strip:

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



Start the experiment

To start an experiment:

- 1. Prepare the instrument for use as shown below.
- 2. Load the reaction plate or array card into the instrument, as shown on page 33.
- 3. Run the experiment as shown on page 34.

Prepare the instrument for use

- Start the QuantStudio[™] 6 or 7 Instrument
 - Touch anywhere on the touchscreen to determine if the QuantStudio[™] 6 or 7 Instrument is in standby mode.

Does the touchscreen display the Standby screen after you touch it?

- Yes The instrument is ready for use. Go to step 3 below.
- No Go to step 2 to power on the instrument.
- **2.** Toggle the power button on the rear of the QuantStudio[™] 6 or 7 Instrument, then wait for it to start.



The QuantStudio $^{\text{TM}}$ 6 or 7 Instrument is ready to use when the touch screen displays the Main Menu.

- **3.** Power on the monitor.
- 4. Power on the computer:

Getting Started with QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Experiments

1

- a. Press the computer power button, then wait for it to start.
- **b.** When the Login screen appears, enter your user name and password, then click **OK**.
- **5.** Start the QuantStudio[™] 6 and 7 Flex Software:
 - **a**. From the desktop, double-click \blacksquare QuantStudioTM 6 and 7 Flex Software.
 - Note: If the shortcut is not present on the desktop, select Start ► All Programs ► Applied Biosystems ► QuantStudioTM 6 and 7 Flex Software ► QuantStudioTM 6 and 7 Flex Software to start the software.

IMPORTANT! If the QuantStudioTM 6 and 7 Flex Software will not start, confirm that no other instances of the instrument control software are open. If any instance of the software is open, close it before starting the QuantStudioTM 6 and 7 Flex Software.

b. From the Login dialog box, enter your user name and password, then click **Log In**.

Login	
Enter your user n	ame and password to log in.
User Name: Password:	
	Log In Exit Application

Note: If the QuantStudioTM 6 and 7 Flex Software displays the License Central screen after you log into the software, your license file may be corrupt. Contact Life Technologies support to obtain a replacement license file.

Add the instrument to the My Instruments group

Before you can use the QuantStudioTM 6 or 7 Instrument, you must add the instrument to the "My Instruments" group in the QuantStudioTM 6 and 7 Flex Software.

- 1. Power on the instrument and start the software as explained in "Start the QuantStudio[™] 6 or 7 Instrument" on page 30.
- From the QuantStudio[™] 6 and 7 Flex Software Home tab, click Instrument Console.
- **3.** From the Instrument Console, confirm the instrument state:
 - a. Confirm that the instrument icon appears in the My Instruments group.

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b. Confirm that a green check box appears in the upper-right corner of the instrument icon.

Refresh	Remove from My Instruments	Manage Instrument	Open Door	Close Door	Create Group	Rename Group	Delete Group	Assign to Group	
Display Gr	oup: All Groups		 Filtered by 	All States	~				3
My Inst	ruments (1)								
QS	7_0009 EEADY								
On the I	Network								

- **4.** If your instrument does not appear within the My Instruments group, add it as follows:
 - **a**. From the Instrument Console, select your QuantStudio[™] 6 or 7 Instrument from the list of instruments on the network.
 - b. Click Add to My Instruments.



Note: The details for a QuantStudioTM 6 or 7 Instrument in the My Preferred list can be exported even if the network connection has been interrupted. The exported details from the disconnected instrument would contain the data most recently downloaded from the instrument before the interruption.

Enable or change the Notification Settings

You can configure the QuantStudioTM 6 and 7 Flex Software to alert you by email when the QuantStudioTM 6 or 7 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 instrument user guide.

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1
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.

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

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

- 1. Eject the QuantStudio[™] 6 or 7 Instrument tray by doing either of the following:
 - From the QuantStudioTM 6 or 7 Instrument touchscreen, touch $\mathbf{\Delta}$.
 - From the QuantStudio[™] 6 and 7 Flex Software, select Instrument
 Instrument Console, select your instrument icon, then click Open Door.
- **2.** Load the reaction plate or array card into the plate adapter. When you load the reaction plate or array card, ensure that:
 - Well A1 is positioned at the top-left of the tray for any of the plate formats.
 - 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 in one of the following arrangements. You can load empty tubes if you do not have enough reaction volume to load the required number of tubes: 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).

- **3**. Close the QuantStudioTM 6 or 7 Instrument tray by doing either of the following:
 - From the instrument touchscreen, touch 🔼
 - From the Instrument Console screen, click Close Door.

Start the
experimentIMPORTANT! Ensure 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[™] 6 or 7 Instrument, refer to instrument user guide.

IMPORTANT! Do not attempt to open the access door during the run. The door is locked while the QuantStudioTM 6 or 7 Instrument is in operation.

Note: Ensure 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 when attempting to start the run:

Failed to	Start Run 🔀
\bigotimes	Unable to start the run. The experiment already exists. Delete the experiment before starting the run.
	ОК

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

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

- Start the experiment from the QuantStudio[™] 6 and 7 Flex Software
- Start the experiment from the QuantStudio[™] 6 or 7 Instrument touchscreen

Note: The example experiments in each of the getting started guide booklets start a run from the QuantStudioTM 6 and 7 Flex Software.

Start the experiment from the QuantStudio[™] 6 and 7 Flex Software

1. In the QuantStudio[™] 6 and 7 Flex Software, click **Q Run** in the navigation pane.

IMPORTANT! Ensure that the *.eds file you created is open before you start a run.

2. Click **START RUN**. Select the instrument for the run from the My Instruments drop-down menu.

IMPORTANT! If the preferred instrument for running the experiment 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. See "Add the instrument to the My Instruments group" on page 31 for instructions on adding an instrument to the My Instruments group.





Start the experiment from the QuantStudio[™] 6 or 7 Instrument touchscreen

1. Touch the QuantStudioTM 6 or 7 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.

Note: You can create and save new experiments from the QuantStudio[™] 6 or 7 Instrument touchscreen, or transfer experiments created and saved in the QuantStudio[™] 6 and 7 Flex Software to folders in the QuantStudio[™] 6 or 7 Instrument touchscreen via a USB flash drive.

- **5.** In the Experiments screen, select the desired experiment, then: to view or edit the experiment before starting the run.
 - Touch **View/Edit**, then go to step 6 to view or edit the experiment before starting the run.
 - Touch **F** Save & Start Run, Save and Start Run, then go to step 7 to start the run immediately.
- **6.** (*Optional*) Modify the experiment parameters as needed.
 - **a**. In the Edit Experiment screen, you can use the:
 - + Add and Delete Delete buttons to add and delete a stage or step to the thermal profile.
 - Add Melt Curve Add Melt Curve button to add a melt curve to the thermal profile.
 - Save button to save the experiment you modify.
 - **b.** In the Save Experiment screen, touch each field to edit the:
 - Experiment name
 - Folder to save the experiment
 - Reaction volume
 - Barcode Number
 - Notes

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

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

Monitor the experiment

Note: If the connection between the QuantStudio[™] 6 and 7 Flex Software and the QuantStudio[™] 6 or 7 Instrument is disrupted while running an experiment, remove and then add the instrument to the My Instruments list in the Instrument Console. You may then resume monitoring the experiment.

You can monitor an experiment run in three ways:

- From the Run screen of the QuantStudio[™] 6 and 7 Flex Software, while the experiment is in progress, as shown below.
- From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Software (to monitor an experiment started from another computer or from the QuantStudio[™] 6 or 7 Instrument touchscreen) as described in "From the QuantStudio[™] 6 and 7 Flex Software Instrument Console" on page 36.
- From the QuantStudio[™] 6 or 7 Instrument touchscreen, as described in "From the QuantStudio[™] 6 or 7 Instrument touchscreen" on page 40.

From the QuantStudio[™] 6 and 7 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[™] 6 and 7 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 37, 38, and 38 respectively) available from the QuantStudio[™] 6 and 7 Flex Software for potential problems.

То	Action				
Stop the run	 In the QuantStudio[™] 6 and 7 Flex Software, click STOP RUN. 				
	 In the Stop Run dialog, click one of the following: 				
	 Stop Immediately to stop the run immediately. 				
	 Stop after Current Cycle/Hold to stop the run after the current cycle or hold. 				
	 Cancel to continue the run. 				
View amplification data	Select Amplification Plot.				
in real time	See "To monitor the Amplification Plot" on page 37.				
View temperature data	Select Temperature Plot.				
for the run in real time	See "To monitor the Temperature Plot" on page 38.				
View progress of the run	Select Run Method.				
in the Run Method screen	See "To monitor the Run Method" on page 38.				

То	Action
Enable/disable the	Select or deselect Enable Notifications.
Notification Settings	See "Enable or change the Notification Settings" on page 32.

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 following is an image of the Run screen for a Standard Curve experiment:



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 fluorescence, refer to the instrument user guide to troubleshoot the error.

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	Select Fixed View.		
instrument run	If the entire plot does not fit in the screen, the screen is not updated as the run progresses. For example, if you select 10 minutes from the View drop-down menu, the plot will show data for 10 minutes. If the Fixed View is:		
	 Deselected, the plot updates as the run progresses even after 10 minutes. 		
	 Selected, the plot does not update as the run progresses even after 10 minutes. 		

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 temperatures, refer to the instrument user guide to troubleshoot the error.

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.

То	Action
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 6 and 7 Flex Software Help (click ?) or press **F1**).

Editing the run method during a run

You can edit the run method while an experiment run is in progress on the Run Method screen from the Setup menu.

1. Increase or decrease the number of cycles by entering the cycle number in the Adjust # of Cycles box.

Note: Ensure that you select the stage for which you want to increase or decrease the number of cycles in the graphical view of the run method. The Adjust # of Cycles box appears disabled if the corresponding stage is not selected.

- **2.** Select the appropriate check box to add a melt curve stage, holding stage, or infinite hold stage respectively, to the end of the run.
- 3. Click Send to Instrument.

Edit Run Method					
Adjust # of Cycles:					
Add Melt Curve Stage to End					
Add Holding Stage to End					
Add Infinite Hold to End					
Send to Instrument					

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 image from a Standard Curve experiment:

Run Data Report	
Experiment Name:	QS6_QuantStudio_384-Wel_Standard_Curve_Example
Start Time:	02-01-2010 22:25:14 SGT
Stop Time:	02-01-2010 23:07:07 SGT
Run Duration:	41 minutes 52 seconds
User Name:	DEFAULT
Instrument Name:	
Firmware Version:	
Software Version:	N/A
Instrument Serial Number:	278880018
Sample Volume:	10.0
Cover Temperature:	105.0
Instrument Type:	
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[™] 6 or 7 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 the time elapsed and the time remaining in the run	Touch the S 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 📵 to view the list of run events that occurred during the run. Touch 📵 again to close the event list.				

The run method on the QuantStudio[™] 6 or 7 Instrument touchscreen looks like this:

Experiment View



Time View

0	Time View	1111 Experiment Vie	2W
Reac	Run Started: July (ion Volume: 20 µL	06 2013 - 05:48PM -	Sample: 95.0 °C Heated Cover (Set Point): 105.0 °C (105.0 °C) Stage / Step / Cycle: 1 / 2 / 1
	Λ	1.0	1.52
	U	1:0	1:52
		Remaining T	ime O Elapsed Time
			July 06 2013 - 05:48PM Heated cover reached target temperature.

Unload the instrument

When your QuantStudio[™] 6 or 7 Instrument displays the Main Menu screen, unload the reaction plate from the instrument and transfer the experiment data to the computer for analysis.

Unload the reaction plate or array card

CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the temperature of the sample block(s) can exceed 100°C. Allow the consumable to cool to room temperature before removing.

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When the QuantStudioTM 6 or 7 Instrument displays the Main Menu screen, you can unload the plate or array card as follows:

- 1. After the run, touch an the QuantStudio[™] 6 or 7 Instrument touchscreen or click **Open Door** in the Instrument Console screen of the QuantStudio[™] 6 and 7 Flex Software to eject the plate or array card.
- **2.** Remove the reaction plate or array card from the instrument tray and dispose of it according to your laboratory regulations.
- Touch on the QuantStudio[™] 6 or 7 Instrument touchscreen or click Close Door to retract the plate adapter back into the instrument. If the QuantStudio[™] 6 or 7 Instrument does not eject the plate, remove the plate as follows:
 - **a**. Power off the QuantStudio[™] 6 or 7 Instrument.
 - **b.** Wait for 15 minutes, then power on the QuantStudio[™] 6 or 7 Instrument and eject the plate.
 - **c.** If the plate does not eject, power off and unplug the QuantStudio[™] 6 or 7 Instrument, then open the access door.
 - **d.** Wearing powder-free gloves, reach into the QuantStudio[™] 6 or 7 Instrument and remove the plate from the heated cover, then close the access door.

Transfer experiment results

Download the experiment from the QuantStudio $^{\mbox{\tiny TM}}$ 6 or 7 Instrument over the network

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

When the QuantStudioTM 6 or 7 Instrument completes a experiment without a connection to the QuantStudioTM 6 and 7 Flex Software, the software allows you to download the results from the instrument through the network connection.

- 1. In the QuantStudio[™] 6 and 7 Flex Software, select **Instrument** → **Instrument** Console.
- **2.** Select the instrument icon of the QuantStudio[™] 6 or 7 Instrument from the My Instruments list, then click Manage Instrument to open the Instrument Manager.

Note: If the Manage Instrument button is inactive, add your QuantStudioTM 6 or 7 Instrument to the My Instruments group as explained in "Add the instrument to the My Instruments group" on page 26.

- 3. From the Instrument Manager, click Manage Files, then click File Manager.
- 4. From the File Manager screen, download the file(s):
 - **a.** From the Folders field, select the folder that contains the files that you want to download.
 - **b.** From the Experiments field, select the files to download. To select multiple files, **Ctrl-click** or **Shift-click** files in the list.
 - **c.** From the Folders field, select the folder that contains the files that you want to download.
- **5.** From the Save dialog box, select the folder to hold the experiment results and click **Save**. The experiments folder is located at:

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C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\experiments

Transfer the experiment from the QuantStudio[™] 6 or 7 Instrument to the computer via a USB drive:

1. Plug a USB drive into the USB port below the touchscreen.



IMPORTANT! Do not use the USB ports on the rear panel of the QuantStudio[™] 6 or 7 Instrument. The rear USB ports are only for use by Life Technologies personnel to service the instrument

- 2. Touch the QuantStudio[™] 6 or 7 Instrument touchscreen, to awaken it.
- 3. If the touchscreen is not at the Main Menu screen, touch
- From the Main Menu of the QuantStudio[™] 6 or 7 Instrument touchscreen, touch
 Collect Results to save the data to the USB drive.
- 5. Select one or multiple experiments (by touching them). Then touch Save to USB to copy selected experiments to the USB drive.

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

- **6.** Touch **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: C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\experiments

Review experiment results

About analysisImmediately after a run, the QuantStudio[™] 6 and 7 Flex Software automaticallyresultsanalyzes 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.

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

To override
calibrationEach experiment file (.eds) stores the calibration data from the QuantStudio[™] 6 or 7
Instrument it was run on. The calibration data can affect the analysis results of an
experiment.

If you have run multiple experiments on different QuantStudioTM 6 or 7 Instruments and prefer the analysis results from a particular instrument, then you can choose to use the calibration data from another QuantStudioTM 6 or 7 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[™] 6 or 7 Instrument, in the QuantStudio[™] 6 and 7 Flex Software.
- 2. Go to Analysis > Override Calibration > Use Calibration From Another File....

File Edit Instrument	Analysis	Tools Help						
🛄 New Experiment 🔸	Ana Ana	lysis Settings Iyze		2	lose	🆏 Import	- 纋	Create Slide
	Ove	rride Calibration	▶		Use	Calibration From	Anothe	er File
Experiment Menu		_			Reve	ert To Original C	alibratio	n l

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 any other experiment type; however the calibration data being used must be from the same instrument type, QuantStudio[™] 6 Instrument or the QuantStudio[™] 7 Instrument. Calibration data from an experiment in the QuantStudio[™] 7 Instrument can be used to override calibration data of an experiment in the QuantStudio[™] 6 Instrument, but not vice-versa.

4. Click Open.

To revert to the original calibration data

- Open the experiment file (.eds), in which you want to import the original calibration data, in the QuantStudio[™] 6 and 7 Flex Software.
- 2. Go to Analysis > Override Calibration > Revert To Original Calibration.

File Edit Instrument	Analysis	Tools Help				
New Experiment 👻	Ana Ana	alysis Settings alyze	5	⊆lose	🎼 Import 🝷 🛷 Create Slide	
A	Ove	Override Calibration		Use Calibration From Another File		
		• –		Revert To Original Calibration		

The experiment file will display analysis results as per the calibration data of the QuantStudioTM 6 or 7 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 With 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 following is an image of the plate layout for a Standard Curve experiment:

Σ	Pl	ate Layo	out	Well Tabl	le																				
	U	Show	in Wells	 Select 	t Wells 🔻	÷.	/iew Leger	nd															÷	Ð	X
Ē		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	д	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	в	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	c 🛛	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	5	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	Е	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	F	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	з	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
1	4	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	I	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	J	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	к	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	L	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	м	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	N I	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	р	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	Р	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	We	lls: 🕕	288 💽	80 N	16																			0	Empty
Wells: 🛄 288 🛐 80 🛐 16 Summary: Wells in Plate: 384 Wells Set Up: 384 Wells Analyzed: 384 Wells Flagged: D Wells Omitted by Analysis: D Wells Omitted Manually: D Samples Used: 2 Targets											s Used: 1														

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.

ons ults



The following is an image of the Multiple Plots View screen for a Standard Curve example experiment:

To display an expanded view of a plot or wells

To edit plot properties

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

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 current settings as default

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

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

(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	Р <mark>а</mark>
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[™] 6 and 7 Flex Software allows you to export:

Data type	Description
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.

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Data type	Description
Analyzed data in different formats for further analysis	The data can be exported in the QuantStudio [™] 6 and 7 format, QuantStudio [™] Dx/ViiA [™] 7 format, the 7900 SDS format, and the RDML format.
	 The 7900 format is applicable only to Standard Curve, Relative Standard Curve, Genotyping, Presence/Absence, and Melt Curve experiments.
	 The RDML export format is applicable only to Standard Curve, Relative Standard Curve, Comparative C_T, and Melt Curve experiments. The RDML format is available only in a single file format.
	 For Standard Curve experiments, you can also export the analyzed data from the QuantStudio[™] 6 and 7 Flex Software to the external applications, TaqMan[®] Protein Expression Data Analysis Software and CopyCaller[®] Software if they are installed on your computer before the QuantStudio[™] 6 and 7 Flex Software is installed. The applications appear in the Tools menu.
Gene Expression studies	These are used to carry out a comparative analysis.

Export procedure

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

Note: If you want the data to be exported automatically after analysis, select the **Auto Export** check box during experiment setup or before running an experiment.

- **2.** Select the format for exported data:
 - QuantStudio[™] 6 and 7 Format Supports .txt, .xls, and .xlsx data.
 - **QuantStudio**TM **Dx**/ **ViiA**TM **7 Format** Supports .txt, .xls, and .xlsx data.
 - **7900 Format** Supports only .txt data, where:
 - Single experiments are exported in the SDS 2.4 detector centric export format of the 7900 Sequence Detection System.
 - Studies are exported in the SDS 2.3 RQ manager detector centric export format of the 7900 Sequence Detection System
 - **RDML Format** RDML (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				×				
ſ	Defaults		Startup	Experiment	Print				
	Setup	Export	Display Format	SMTP Settings	Notification Settings				
Select the default export location.									
 Use Last File Location Use Default Folder C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\Export Browse Restore Defaults Apply 									
			Import System Pre	ferences Export System	m Preferences 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

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Select	To export
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
Clipped Data	Information that is unique to the 7900 format. Data from the last three raw data points per step (clipped from the rest). The three data points are averaged to give you the final fluorescence data value for each step.
Reagent Information	Information about the reagent selected for the experiment

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

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

7. (*Optional*) For Standard Curve experiments, select the external application, TaqMan[®] Protein Expression Data Analysis Software or CopyCaller[®] Software if either Software is installed on your computer.

Note: For more information on the TaqMan[®] Protein Expression Data Analysis Software or CopyCaller[®] Software, contact Life Technologies.

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**.

Note: It is advisable to keep the default order of the table headings if you are using the external Life Technologies applications, **TaqMan[®] Protein Expression Data Analysis Software** or **CopyCaller[®] Software** for further analysis.

9. Click Start Export.

The following is an image of the Export screen for a Standard Curve experiment:

Auto Export F	ormat : QuantStud	lio™ 6 and 7	*	Export Data	To: 💿 One File 🔘	Separate Files	🗹 Open fil	e(s) when export is	complete
Export File Location: C:\Appli	ed Biosystems\Qu	antStudio 6	and 7 Flex Softwar	Browse Export File	Name: QS6_Quar	ntStudio_384-We	ell_Standard_Curv	e File Type: 📳 (*.:	txt) 🗸
Sample Setup	w Data 🛛 🔽 Ampli	fication [Multicomponent	Results					
🗹 Skip Empty Wells 🛛 🗹 Skip	Omitted Wells								
Select Content		Well	Well Position	Sample Name	Target Name	Task	Reporter	Ouencher	С
✓ All Fields	~		1 A1		RNAse P	NTC	FAM	NFO-MGB	~
			2 A2		RNAse P	STANDARD	FAM	NFQ-MGB	29
Vel Wel			3 A3		RNAse P	STANDARD	FAM	NFQ-MGB	28
Well Position			4 A4		RNAse P	STANDARD	FAM	NFQ-MGB	27
Vien Posicion			5 A5		RNAse P	STANDARD	FAM	NFQ-MGB	26
Sample Name			6 A6		RNAse P	STANDARD	FAM	NFQ-MGB	25
			7 A7	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27
Target Name			8 A 8	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27
E Tools			9 A 9	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27
✓ Task			10 A10	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27
Reporter			11 A11	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27
			12 A12	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27
Quencher			13 A13	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27
			14 A14	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27
CT CT			15 A15	5K	RNAse P	UNKNOWN	FAM	NFQ-MGB	27
Ch Mann			16 A16	10K	RNAse P	UNKNOWN	FAM	NFQ-MGB	26
Ct Mean			17 A17	10K	RNAse P	UNKNOWN	FAM	NFQ-MGB	26
Ct SD			18 A18	10K	RNAse P	UNKNOWN	FAM	NFQ-MGB	26 🗸
	~	<							>

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

The following is an image of the exported file when opened in Notepad:



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Getting Started with QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Experiments

Experiment Shortcuts

This chapter provides you with shortcuts to use in the QuantStudio[™] 6 and 7 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.

The chapter covers:

Using experiment templates	53
Run an experiment with QuickStart	57
Import plate setup for an experiment	58
Import sample information	59
Create an experiment using ReadiApp	62

Using experiment templates

You can use a template (.edt) 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 QuantStudioTM 6 and 7 Flex Software and from the QuantStudioTM 6 or 7 Instrument touchscreen.

Note: To access the QuantStudioTM 6 and 7 Flex Software example templates, navigate to the templates folder located at *drive*:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\User Sample Files. where, *drive*> is the computer hard drive on which the QuantStudioTM 6 and 7 Flex Software is installed. The default installation drive for the software is the C: drive.

To create a
template1. Log in to the QuantStudio[™] 6 and 7 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 12.

2. Select File > Save As Template.

Note: The information saved in a template includes plate setup information (defined targets and samples, plate assignment of targets and samples), reagent information, thermal protocol, and analysis settings such as quantification cycling method.

3. Enter a file name, select a location for the template, then click **Save** and **Solution** Close. You can use that experiment as a template for similar experiments.

To create a new experiment using a template

- 1. From the Home screen, click **From Template**.
- Locate and select the template file, then click Open.
 A new experiment is created using the setup information from the template.
- **3.** Confirm that the following are correct before you prepare the reactions and run the experiment:
 - Experiment properties (experiment name, experiment type, block type, reagent, run properties)
 - Plate definitions (targets, samples, and biological replicates)
 - Plate assignments (targets, samples, and biological replicates)
 - Run method (thermal protocol)
- **4.** Proceed to preparing reactions, running the experiment, and analyzing the data.

To create an
experiment using a
template on the
QuantStudio™ 6 orYou can run experi
touchscreen by imp
instrument console
the templates as pe7 Instrument
touchscreenTo edit a template

You can run experiments using templates from the QuantStudioTM 6 or 7 Instrument touchscreen by importing the templates from the QuantStudioTM 6 and 7 Flex Software instrument console or a USB drive. You can also modify the experiment parameters in the templates as per your requirement.

To edit a template before running the experiment

1. Touch **Hew** 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 Save & Start Run Save

To run a pre-existing template

- 1. Touch **View Templates** View Templates on the Home screen of the QuantStudioTM 6 or 7 Instrument touchscreen.
- **2.** Select a pre-existing template from the templates list on the View Templates screen.
- **3.** Touch **View** to see the run profile before you start a run.
- **4.** After confirming the template setup is correct, touch **s** to go back to View Templates screen. Touch **Start Run**.

2

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 following is an image of the Batch Experiment Setup Utility dialog box:

Batch Experiment Setup Utility		×							
Provide input files, select the file naming convention, and export location, then click Create Experiments .									
1 Januard Files									
* Experiment Template File: lio 6 and 7 F	Flex Software\examples\User Sample Files\OS6_OuantStudio_384-Well_Standard_Curve_Example.e	dt Browse							
Assay Information File:		Browse							
Plate Setup File:		Browse							
2. Barcode(s) and Naming Convention									
Create Experiment Files Using: 🔿 Barcode:		Browse							
 Specify Num 	nber of Files: 25								
File Name Format: Attribute	Include Custom Name Field:								
Custom Name Field									
Plate Barcode	Move Up File Name Preview:								
ID									
	Move Down								
3. Export Location									
* Export Setup Files to:		Browse							
	Create Experiments	Cancel							

- 2. Select the file(s) to use to create the new experiments:
 - a. Click Browse in the Experiment Template File field.

Note: To use one of the example setup files, browse to <drive>:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\User Sample Files

- b. Locate an *.edt file to import, then click Select.
- c. (*Optional*) Repeat **steps 2a** and **2b** for the remaining setup file types to import (Assay Information File (*.txt or *.xml), Plate Setup File (*.txt)).

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

Note: A Barcode File contains one barcode per line. An example Barcode File looks like the following image:

- **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** 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 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**.
- **6.** Click **Create Experiments**. A confirmation message appears when the batch of experiments has been created.

2

Run an experiment with QuickStart

You can use a template to run an experiment with the QuantStudio[™] 6 and 7 Flex Software QuickStart feature:

QuickStart from the QuantStudio[™]6 and 7 Flex Software

- **1.** Prepare the reactions.
- Log in to the QuantStudio[™] 6 and 7 Flex Software and, from the Home screen, click **QuickStart** to access the QuickStart 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 template file.
 - d. (Optional) Barcode and User Name for the experiment.
- **4.** (*Optional*) To review the experiment or to make changes to any of the experiment parameters, click **Experiment Setup**.

The following is an image of the QuickStart dialog box:

😃 QuickStart							
2 Select an instrument and an experiment template file to run a new experiment. I	Load the reaction plate into the instrument, then click Start Run.						
Select Instrument							
QS7_0009 READY							
Enter Experiment Name and Location							
* Experiment Name: 2013-07-04 160106	Location: e\User Files\Experiments\2013-07-04 160106.eds						
Barcode (Optional):	Comments (Optional):						
User Name (Optional):	<u> </u>						
Select Experiment Template							
* Experiment Template File:	✓ Browse						
Samples							
Sample	Provide						
	DIOMSE						
	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						

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

QuickStart from the QuantStudio[™] 6 or 7 Instrument touchscreen

You can QuickStart an experiment from the QuantStudio[™] 6 or 7 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[™] 6 or 7 Instrument touchscreen displays up to 18 shortcut buttons to templates or folders that contain experiments to be run. The shortcut buttons are present under MY SHORTCUTS on the Home screen. To start a run, touch any of the pre-defined experiment or folder buttons.

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

- 1. Touch Ettings 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 experiment setup

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
- *.eds EDS file format
- *.edt EDS template files format
- *.sdt Sequence Detection System (SDS) template files format
- *.sds 7900 v2.4 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 47.

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.

Note: To use one of the example setup files, browse to C:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\User Sample Files

Import Plate Setup	×
Select the plate setup file to import, then click Start Import .	۷
Select File:	Browse
	Start Import Cancel

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

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

- **5.** After importing plate setup information, use Experiment Setup to set up your experiment, and then run the experiment.
- **Note:** You can import plate setup information from a 96-well plate into a 384-well plate, provided that the file you are importing the information from is a .txt file.

Import sampleYou 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 32 user-defined custom fields (for example, **Custom 1**, **Custom 2**, etc.)

- **3.** For each subsequent row, enter the well number, press the **Tab** key or enter a comma, then enter the sample name. Optionally, press the **Tab** key, then enter the custom properties for the sample.
- **4.** Save the file with the .txt or .csv file extension.

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

	A	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	25	red	No
13	12	Sample 12	12	34	Male	26	grey	No
14	13	Sample 13	13	35	Female	50	black	Yes
15	14	Sample 14	14	35	Male	33	black	No
16	15	Sample 15	15	36	Female	46	black	Yes
17	16	Sample 16	16	36	Male	35	brown	Yes
18	17	Sample 17	17	37	Female	33	blonde	No
19	18	Sample 18	18	37	Male	67	red	No
20	19	Sample 19	19	38	Female	55	grey	Yes
21	20	Sample 20	20	38	Male	44	black	No

Import sample information from a sample definition file

- 1. Create a new experiment or open the experiment to receive the setup data (select **File → Open**, select the file to open, then click **Open**).
- 2. From the open experiment, select **File > Import Plate Setup**.
- **3.** Click **Browse** to browse your computer for a sample definition text file (*.csv). After you locate the file and select it, click **Select**.

Note: To use one of the example setup files, browse to C:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\User Sample Files

4. Click Start Import.

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

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

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

The following is an image of the Assign screen with information from the above sample definition file:

🚬 🐂 Define and Set Up Standards		Plate Layout	Well Table							
Targets A		10012								
Name Task Quantity		Show in Wells	▼ Select Wells ▼	View Legend						. ,
Target 1		1	2	3	4	5	6	7	8	9
	A			٠						
Samples	в		Sample: Custom Proper ID: Age: Sex:	Sample 1 ty: 1 22 Female						
Name Sample 1 Sample 2 Sample 2 Sample 3 Comple 4	с		Weight: HairColor: Smoker:	25 black Yes						
Sample 5 Sample 5 Sample 6 Sample 7 Sample 8 Sample 8 Sample 9	D									
Biological Groups	E									
	F									
		4								V F
		'ells: 🔟 0 0	0		_					364 Empty

The following is an image of the Well Table in the Analysis section:

Z	Plate	e Layout	Well Table													
	Show i	in Table 🔻	Select Wells 🔻	Group By 🔻										Expand Al	Collapse	All
																_
Γ	#	Well	Ст	Ст Mean	CT SD	Quantity	Quantity	Quantity	Comments	ID	Age	Sex	Weight	HairColor	Smoker	Г
ľ	1	A1							1	2	2	Female	25	black	Yes	
	2	A2							2	2	5	Male	26	brown	No	
	з	AЗ							3	4	5	Female	50	blonde	Yes	
	4	A4							4	3	1	Male	33	red	Yes	
	5	A5							5	2	9	Female	46	grey	No	_
	6	A6							6	2	6	Male	35	black	No	
	7	A7							7	3	1	Female	33	black	Yes	
	8	AB							8	3	2	Male	67	black	No	
	9	A9							9	3	2	Female	55	brown	Yes	
	10	A10							10	3	3	Male	44	blonde	Yes	
	11	A11							11	3-	4	Female	25	red	No	
	12	A12							12	3-	4	Male	26	grey	No	
	13	A13							13	3	5	Female	50	black	Yes	
	14	A14							14	3	5	Male	33	black	No	
	15	A15							15	3	6	Female	46	black	Yes	
	16	A16							16	3	6	Male	35	brown	Yes	
	17	A17							17	3	7	Female	33	blonde	No	
	18	A18							18	3	7	Male	67	red	No	
	19	A19							19	3	8	Female	55	grey	Yes	
	20	A20							20	3	8	Male	44	black	No	
	21	A21														
	22	A22														
	23	A23														
	24	A24														
	25	B1														
	26	82														
	2/	83	-													
	28	84														
	29	BD DC														
	30	80														
	31	B/														
	32	88														
	33	B9														
	25	B10														
	33	010														-
			1												•	
We	ell Sun	nmary:	In Plate: 3	384 Set U	p: 384	Analyzed: 3	84 1	Flagged: ()	Omitted by Anal	lysis: 🛛	Omitted	Manually: 🛛	Samples	Jsed: 384	Targets Use	d: 0

2

Create an experiment using ReadiApp

You can use the ReadiApp feature to set up an experiment in the QuantStudioTM 6 and 7 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 $^{\text{TM}}$ 6 and 7 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[™] 6 and 7 Flex Software and, from the Set Up 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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USER GUIDE



QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Standard Curve Experiments

Booklet 2

Publication Number 4489822 Revision A



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About Standard Curve Experiments

- About the example experiment 6

IMPORTANT! First-time users of the QuantStudioTM 6 and 7 Flex Real-Time PCR System Software please read Booklet 1, *Getting Started with QuantStudioTM 6 and 7 Flex Real-Time PCR System Software Experiments* and Booklet 7, *QuantStudioTM 6 and 7 Flex Real-Time PCR System Software 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[™] 6 and 7 Flex Real-Time PCR System Software by pressing F1, clicking ② in the toolbar, or selecting Help → QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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. components • Standard - A sample that contains known quantities of the target; used in quantification experiments to generate standard curves. Standard dilution series – A set of standards containing a range of known • quantities. The standard dilution series is prepared by serially diluting standards. **Replicates** – The total number of identical reactions containing identical samples, components, and volumes. Negative controls – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells. PCR Options When performing real-time PCR, choose between: Singleplex and multiplex PCR (below) 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[™] 6 and 7 Flex Real-Time PCR System Software.

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

6

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.

- Three replicates of each sample and each dilution point in the standard curve are performed to ensure statistical significance.
- The experiment is designed for singleplex PCR, where every well contains a primer/probe set for a single target.
- Reactions are set up for 2-step RT-PCR.
- Primer/probe sets are from Life Technologies RNase P assay.

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

Design the Experiment

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

This chapter covers:

Define the experiment properties.	. 9
Define targets, samples, and biological replicates	10
Assign targets, samples, and biological groups	11
Set up the run method	13
For more information	14

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 2 in Booklet 1, Getting Started with QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Experiments.

Define the experiment properties

2

Click Experiment Setup • Experiment Properties to create a new experiment in the QuantStudio[™] 6 and 7 Flex Software. Enter:

Field or selection	Entry
Experiment Name	QS6_QuantStudio_384-Well_Standard_Curve_Example
Barcode	Leave field empty
User Name	Example User
Comments	Standard Curve example
Instrument type	QuantStudio [™] 6 Flex System
Block	384-Well Block
Experiment Type	Standard Curve
Reagents	TaqMan [®] Reagents
Ramp speed	Fast
Reagent Information	NA

Save the experiment.

Your Experiment Properties screen should look like this:

Experiment: QS6_QuantStudio_3	384-Wel Type: Sta	ndard Curve		Reagents: T	aqMan® Reagents	2
How do you want to identify this ex	periment?					^
* Experiment Name: QS6_QuantStudio_384 Barcode: User Name: Example User	-Well_Standard_Curve_Exam	ple	Comments: 9	Standard Curve Example		>
Which instrument type are you usi	ng to run the experime	nt?				
✓ QuantStudio™ 6 Flex System	QuantStudio™ 7 F	lex System				
Which block are you using to run t	he experiment?					
✓ 384-Well	96-Well (0.2	2mL)	Fast	: 96-Well (0.1mL)		
* What type of experiment do you w	ant to set up?					
✓ Standard Curve	Relative Standa	rd Curve	Comp	parative CT (ΔΔCT)	Melt Curve	
Genotyping	Presence/Ab	sence				=
Which reagents do you want to use	e to detect the target se	quence?				
 TaqMan® Reagents 	SYBR® Green R	eagents		Other		
What properties do you want for the second secon	he instrument run?					
Standard	✓ Fast					
What is the reagent information?						
Type Name		Part Number		Lot Number	Expiration Date	
<u>k</u>						

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
5K	
10K	

- **3.** Dye to be used as a Passive Reference ROX
- 4. Custom Task Name Not applicable

Targets				Sampl	es			
New Save to Library Import from Library	Delete			New	Save to Library	Import from Library	Delete	🏣 Import from File
Target Name	Reporter	Quencher	Color	Sampl	e Name	Color		
RNAse P	FAM 🗸	NFQ-MGB	~ ~	5К				
				10K		~		
							I	
Biological Replicate Groups								
New Delete								
Biological Group Name Color		Comments						
Passive Reference				Custo	n Task Name			
ROX				New	Delete			
				Name		Color		Icon Char

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:

Uptine and Set Up Standards	
Select a target from the list of targets in the reaction plate. Define the standard curve, select wells for the standards, then dick Apply. Repeat for each standard curve in the reaction plate, then dick	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
* # of Points: 5 (5 Recommended)	
* # of Replicates: 3 (3 Recommended)	
* Starting Quantity: 1.0 (Enter the highest or lowest standard quantity for the standard curve.)	4E-2
* Serial Factor: 1:5	8E-3 1.6E-3
5 Points X 3 Replicates = 15 Required Wells	
Select and arrange wells for the standards	
Arrange standards h: Olumns O Rows Use Welk: Automatically Select Wells for Me O 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 23 24 15 Required Wells / 80 Selected Wells A	als ,E3,E4,E5,E6,F2,F3,F4,F5,F6,G2, (S,K6,L2,L3,L4,L5,L6,M2,M3,M4,
	Apply Reset Close

2. Assign targets and samples.

Target name	Well number	Task	Quantity	Sample name
RNaseP	A1 - P1 (column 1)	Negative	None	None
RNaseP	A2 - P2 (column 2)	Standard	1250	None
RNaseP	A3 - P3 (column 3)	Standard	2500	None
RNaseP	A4 - P4 (column 4)	Standard	5000	None
RNaseP	A5 - P5 (column 5)	Standard	10000	None

Target name	Well number	Task	Quantity	Sample name
RNaseP	A6 - P6 (column 6)	Standard	20000	None
RNaseP	A7 - P15 (columns 7 -15)	Unknown	Determined by run	5K
RNaseP	A16 - P24 (columns1 6 - 24)	Unknown	Determined by run	10K

Your Assign screen should look like this:

C Define and Set Up Standards		Plate La	ayout	W	ell Tabl	e																			
Targets A Name A Task Quantity		U) She	ow in W	'ells ▼	Selec	t Wells	•	🔛 Vie	w Leger	nd												E	÷	Ð	X
RNAse P		1	2	З	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	A		S	<mark>S</mark>	<mark>s</mark>	<mark>S</mark>	<mark>s</mark>	U	U	U	U	U	U	U	U	U	U	U	<mark>U</mark>	U	<mark>U</mark>	<mark>U</mark>	U	U	U
	в	N	<u>s</u>	<mark>s</mark>	S	<mark>S</mark>	S .	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
	c	N	S	S	S	S	S .	U	U	U	υ.	υ.	U	U	U	υ.	Ū.,	U	U	U	U	U	U	U	U
		N	S	S	S	S	S.	Π.	Π.	Π.		Π.	Π.		Π.		Π.		п.	п.	п.	п.		Π.	
	F																								
Samples	E	N	S	<u>s</u> .	<u>s</u>	<u>s</u>	<u>s</u>	<u> </u>	<u>U</u>	<u>U</u>	<u>U</u>	<u> </u>	<u>U</u>	<u>U</u>	<u>U</u>	<u>U</u> .	<u>U</u>	<u>U</u>	<u>U</u>	<u>U</u>	<u>U</u>	<u>U</u>	<u>U</u>	<u>U</u>	<u>U</u>
Name 1	F	N	<mark>S</mark>	<mark>S</mark>	<mark>S</mark>	<mark>S</mark>	<mark>S</mark>	U	U	U	U	U	U	U	U	U .	<mark>U</mark>	U	<mark>U</mark>	<mark>U</mark>	<mark>U</mark>	<mark>U</mark>	U	<mark>U</mark>	U
	G	N .	<mark>S</mark>	<mark>S</mark>	S	S	S .	U	U	U	U	U .	U	U	U	U .	U	U	U	U	U	U	U	U	U
I	н	N .	<mark>s</mark>	<mark>s</mark>	S	S	S .	U	U	U	U	U -	U	U	U	U	U	U	U	U	U	U	U	U	U
	I	N	<u>s</u>	<mark>S</mark>	S	S	S .	U	U	U	U	U .	U	U	U	U	U	U	U	U	U	U	U	U	U
	5	N	<u>s</u>	<u>s</u>	S .	S	S .	U	U	Π.	U	Π.	U	<u>.</u>	U	υ.	Ū.	U	U	U	Π.	U	U	U	U
	F	N	3	3	R	3	3	_				_	_	_						_					
Biological Groups	F			· · ·																					
Biological Group	Ŀ	Ν.	<u>s</u>	<u>s</u>	<u>s</u>	<u>s</u>	<u>s</u> .	U	U	Ш.	U	Ш.	U	Ш.,	Ш.,	U	U	U	Ш.,	U	U	U	U	U	U
	м	N .	<mark>s</mark>	<mark>S</mark>	S	<mark>S</mark>	<mark>S</mark> .	U	<mark>U</mark>	U .	U	U .	U	U	U	U	U	U	U	U	U	U	<mark>U</mark>	U	U
	N	N	<mark>S</mark>	<mark>S</mark> .	S	<mark>S</mark>	S .	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
	0	N .	<mark>s</mark>	<mark>s</mark>	S	<mark>S</mark>	S	U	<mark>U</mark>	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
	P	N .	<mark>s</mark>	<mark>s</mark>	S	S	S	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
	v	- Vells: [288	8 <mark>15</mark> 8	:0 🔃	16																		0	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: 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:



For more information

For more information on	Refer to	Publication number
Consumables	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7</i> Flex Real-Time PCR System Software Experiments	4489822
	Appendix A in Booklet 7, <i>QuantStudio[™] 6 and 7 Flex Real-Time PCR</i> System Software - Appendixes	

2

For more information on	Refer to	Publication number
Using other quantification methods	Booklet 3, Running Relative Standard Curve and Comparative C _T Experiments.	Chapter 1 in Booklet 1, Getting Started with QuantStudio [™] 6 and 7 Flex Real- Time PCR System Software Experiments
		Appendix A in Booklet 7, QuantStudio [™] 6 and 7 Flex Real- Time PCR System Software - Appendixes
Amplification efficiency	Amplification Efficiency of TaqMan [®] Gene Expression Assays Application Note	127AP05-03
Using Alternative Setup	Chapter 3 in Booklet 1, <i>Getting Started with QuantStudio[™] 6 and 7</i> Flex Real-Time PCR System Software Experiments	4489822



Chapter 2 Design the Experiment *For more information*

Prepare the Reactions

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

Assemble required materials

- Items listed in Booklet 1, *Getting Started with QuantStudio*[™] 6 and 7 Flex Real-Time PCR System Software 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×) (Part no. 4316831)

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

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

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

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

1. Prepare five standard dilutions:

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

For each dilution:

- a. Use a new pipette tip to add 18 μL of source to the tube containing the standard.
- **b.** Vortex the tube for 3 to 5 seconds, then centrifuge the tube briefly.

З

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	2115
RNase P Assay (20X)	0.5	211.5
Water	3.5	1480.5
Total reaction mix volume	9	3807

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

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

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

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

Prepare the reaction plate

The reaction plate for the Standard Curve example experiment contains:

- A MicroAmp[®] Optical 384-Well Reaction Plate
- Reaction volume: 20 µL/well
- 288 Unknown wells
- 80 Standard wells S
- 16 Negative control wells

 $QuantStudio^{\text{TM}}$ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Standard Curve Experiments

Example experiment reaction plate components З

The following is an image of the plate layout:

>	Pla	ate Layc	ut	Well Tabl	e																				
	U	Show	in Wells '	🗸 Selec	t Wells 🔻		View Leger	nd															e is	a):	ť,
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	۹ (N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	3	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	= [N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	> [N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	: [N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	- 1	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	5	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
:	+ [N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	r [N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	, [N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	< [N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	- [N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	1	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	4 [N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
-	> [N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	•	N R	S R	S R	S R	S R	S R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R	U R
	We	lls: 🕕	288 S	80 N	16																			a) 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)	Water volume (µL)
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 μ L 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)	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 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	RNase P pop1	RNase P reaction mix	1422	pop1	158
2	RNase P pop2	RNase P reaction mix	1422	pop2	158

- **b.** Mix the reactions by gently pipetting up and down, then cap the tubes.
- **c.** Centrifuge the tubes briefly to remove air bubbles.
- d. Add 10 μL of the unknown (sample) reaction to the appropriate wells in the reaction plate.
- 4. Seal the reaction plate with optical adhesive film.
- 5. Centrifuge the reaction plate briefly to remove air bubbles.

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

For more information

For more information on	Refer to	Publication number
Assigning the reaction plate components	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and</i> 7 Flex Real-Time PCR System Software Experiments	Chapter 1 in Booklet 1, Getting Started with QuantStudio [™] 6 and 7 Flex Real- Time PCR System Software Experiments
		Appendix A in Booklet 7, QuantStudio [™] 6 and 7 Flex Real- Time PCR System Software - Appendixes
Sealing the reaction plate	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and</i> 7 <i>Flex Real-Time PCR System Software Experiments</i>	Chapter 1 in Booklet 1, Getting Started with QuantStudio [™] 6 and 7 Flex Real- Time PCR System Software Experiments
		Appendix A in Booklet 7, QuantStudio [™] 6 and 7 Flex Real- Time PCR System Software - Appendixes

This chapter explains how to run the example experiment on the QuantStudio $^{\rm TM}\,$ 6 or 7 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 6 or 7 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[™] 6 and 7 Flex Real-Time PCR System Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software (to monitor an experiment started from another computer or from the QuantStudio[™] 6 or 7 Instrument touchscreen).
- From the QuantStudioTM 6 or 7 Instrument touchscreen.

From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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 QuantStudioTM 6 and 7 Flex Real-Time PCR System Software for potential problems.

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

The following is an image of 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 following is an image of 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 following is an image of 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 following is an image of the View Run Data screen as it appears in the example experiment.

Run Data Report	
Experiment Name:	QuantStudio_384-Well_Standard_Curve_Example
Start Time:	02-01-2010 22:25:14 SGT
Stop Time:	02-01-2010 23:07:07 SGT
Run Duration:	41 minutes 52 seconds
User Name:	DEFAULT
Instrument Name:	
Firmware Version:	
Software Version:	N/A
Instrument Serial Number:	278880018
Sample Volume:	10.0
Cover Temperature:	105.0
Block Type:	384-Well Block
Errors Encountered:	A

From the QuantStudio[™] 6 or 7 Instrument touchscreen You can also view the progress of the run from the QuantStudio $^{\rm TM}\,$ 6 or 7 Instrument touchscreen.

The following are images of the Run Method screen on the QuantStudio $^{^{\rm TM}}$ 6 or 7 Instrument touchscreen:

Experiment View



Time View

S Time View Experiment View					
Run Started: July 06 2013 - 05:48PM Reaction Volume: 20 µL	Sample: 95.0 °C Heated Cover (Set Point): 105.0 °C (105.0 °C) Stage / Step / Cycle: 1 / 2 / 1				
01.2	1.52				
01:3	1:52				
Remaining Time	C Elapsed Time				
	July 06 2013 - 05:48PM Heated cover reached target temperature.				



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	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	39
	Confirm accurate dye signal using the Multicomponent Plot	42
	Determine signal accuracy using the Raw Data Plot	44
	Review the flags in the QC Summary	46
	For more information	47
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	52
	For more information	53



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 QuantStudioTM 6 and 7 Flex Real-Time PCR System 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

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

Menu	Selection
Target	All
Plot Color	Target
	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	-3.289
R ²	0.997
Amplification efficiency	101.414%
Error	0

5. Check that all samples are within the standard curve. In the example experiment, as shown in the following image, 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).

۶	Plate	Layout	Well Table													
	Show in	n Table 🔻	Select Wells 🔻	Group By	v									🛨 Expand	All 📔 🖂 Collaj	pse All
				Torgo	t Nomo									_		
[#	Well	Omit	Flag	Sample Na	Target Na	Task	Dyes	Ст	Ст Mean	CT SD	Quantity	Quantity	Quantity SD	Comments	
	17	A17	Ξ													-
	18	A18			10K	RNAse P	UNKNOWN	FAM-NFQ	27.638	27.678	0.09	9,662.697	9,414.698	572.472		
	19	A19			10K	RNAse P	UNKNOWN	FAM-NFQ	27.627	27.678	0.09	9,738.641	9,414.698	572.472		_
	20	A20			10K	RNAse P	UNKNOWN	FAM-NFQ	27.684	27.678	0.09	9,352.924	9,414.698	572.472		
	21	A21			10K	RNAse P	UNKNOWN	FAM-NFQ	27.601	27.678	0.09	9,913.167	9,414.698	572.472		
	22	A22			10K	RNAse P	UNKNOWN	FAM-NFQ	27.778	27.678	0.09	8,758.838	9,414.698	572.472		
	23	A23			10K	RNAse P	UNKNOWN	FAM-NFQ	27.758	27.678	0.09	8,884.947	9,414.698	572.472		
	24	A24			10K	RNAse P	UNKNOWN	FAM-NFQ	27.78	27.678	0.09	8,744.812	9,414.698	572.472		
	40	B16			10K	RNAse P	UNKNOWN	FAM-NFQ	27.731	27.678	0.09	9,051.594	9,414.698	572.472		
	41	B17			10K	RNAse P	UNKNOWN	FAM-NFQ	27.653	27.678	0.09	9,559.844	9,414.698	572.472		
	42	B18			10K	RNAse P	UNKNOWN	FAM-NFQ	27.664	27.678	0.09	9,485.763	9,414.698	572.472		
	43	B19			10K	RNAse P	UNKNOWN	FAM-NFQ	27.576	27.678	0.09	10,087.83	9,414.698	572.472		
	44	B20			10K	RNAse P	UNKNOWN	FAM-NFQ	27.674	27.678	0.09	9,420.996	9,414.698	572.472		
	45	B21			10K	RNAse P	UNKNOWN	FAM-NFQ	27.713	27.678	0.09	9,164.339	9,414.698	572.472		
	46	B22			10K	RNAse P	UNKNOWN	FAM-NFQ	27.688	27.678	0.09	9,331.501	9,414.698	572.472		
	47	B23			10K	RNAse P	UNKNOWN	FAM-NFQ	27.731	27.678	0.09	9,054.931	9,414.698	572.472		
	48	B24			10K	RNAse P	UNKNOWN	FAM-NFQ	27.733	27.678	0.09	9,041.301	9,414.698	572.472		
	64	C16			10K	RNAse P	UNKNOWN	FAM-NFQ	27.75	27.678	0.09	8,929.64	9,414.698	572.472		
	65	C17			10K	RNAse P	UNKNOWN	FAM-NFQ	27.651	27.678	0.09	9,575.573	9,414.698	572.472		
	66	C18			10K	RNAse P	UNKNOWN	FAM-NFQ	27.636	27.678	0.09	9,677.329	9,414.698	572.472		
	67	C19			10K	RNAse P	UNKNOWN	FAM-NFQ	27.562	27.678	0.09	10,188.251	9,414.698	572.472		
	68	C20			10K	RNAse P	UNKNOWN	FAM-NFQ	27.62	27.678	0.09	9,780.859	9,414.698	572.472		
	69	C21			10K	RNAse P	UNKNOWN	FAM-NFQ	27.654	27.678	0.09	9,554.062	9,414.698	572.472		
	70	C22			10K	RNAse P	UNKNOWN	FAM-NFQ	27.606	27.678	0.09	9,883.055	9,414.698	572.472		
	71	C23			10K	RNAse P	UNKNOWN	FAM-NFQ	27.631	27.678	0.09	9,710.744	9,414.698	572.472		-
				0.4	102	DNIA D	- IN1261-004	EAM NEO	77224	072 77		0.400.004	n 414 600 		T 1	
¥¥6	en sum	mary:	in Plate: E	384	Set Up: 384	An	alyzed: 384	⊢lagged: (J	Omitted by Analysis: I	U On	hitted Manually: (J Sa	mples Used: 2	Targets	Used: 1

5

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 52). 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 Table

U) Shov	w in Wells	▼ Sele	ct Wells 🔻		View Leg	end															•	•	-(
	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							lin.	- I				lin.		lin.										- I
в				R .					- TT-		- TT -						- 11 -	.	.	.	- 11 -		.	
c İ		Rn						- III -				. III n		. III n				III.			III n	III n		
b İ		.				R •													m.					
: İ		.		.		.											III							
-		.		R n		Rn	111.									111.								
G		Rn				R n				m.						III.	m.		m.		m.		III.	
, 1		R1 •	Rn	R .	Rn	R .																		
		.		Rn	Rn	R .																		
		R .	-	R1	-	R .																		.
		N .	Rn	R .		.							-											.
		.																						
																								
																								-
				Ra ,											-									
							-						-		—									
	-																							
Wells: 1 288 5 80 1 16 0 Empty																								

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

3. In the Amplification Plot screen, enter:

Menu	Select
Plot Type	Δ Rn vs Cycle
Plot Color	Well (default)
E com a	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.
- **b.** Verify that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.



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



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

Outliers

- A typical amplification plot The QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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



The following is an image of a typical amplification plot:

IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software. Life Technologies recommends that you examine the Amplification Plot screen and review the assigned baseline and threshold values for each well after analysis.

• Correct threshold values:



Threshold Set Too Low

The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the exponential phase of the curve.

Threshold Set Too High

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





• Correct baseline values:




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

- Omit wells (see "Improve C_T precision by omitting wells" on page 52). *Or*
- Manually adjust the baseline and/or threshold (see "Adjust analysis settings" on page 49).

Identify well problems using the Well Table

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

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

Purpose The purpose of viewing the well table is to identify:

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

View the well 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.

У	Plate Layout	Well Table										
	Show in Table 🔻	Select Wells 🔻	Group By 🔻							Expand All	Collapse Al	
Ī	# Well	Omit	Target Name	ntity Mean	Quantity SD Sample Name	Target Name	Task	Dyes	Ст	Ct Mean	CT SD CC	-
	CC PCC	-	Sample Name	2,121020	072.172 20K	1010-001	ONIGIOTH	homming	27.70	27.070	0.07	
	23 A23		Task	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ	27.731	27.678	0.09	
	24 A24		Renlicate	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NFQ	27.653	27.678	0.09	
	40 816		- W	9,414.698	572.472 1UK	RNAse P	UNKNOWN	FAM-NEQ	27.664	27.678	0.09	
	41 81/		5,0	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ	27.576	27.678	0.09	
	42 818		Hag	9,414.698	572.472 IUK	RNASE P	UNKNOWN	FAMENEQ	27.674	27.678	0.09	
	43 B19		Ст	9,414.698	572.472 IUK	RNASE P	UNKNOWN	FAMINEQ	27.713	27.678	0.09	
	44 B2U		Comments	9,414.698	572.472 10K	RNASE P	UNKNOWN	FAMI-NEQ	27.688	27.678	0.09	
	45 B21		Well Position (Row)	9,414.698	572.472 1UK	RNASE P	UNKNOWN	FAM-NEQ	27.731	27.678	0.09	
	46 B22		Wall Pasitian (Caluma)	9,414.698	572.472 1UK	RNAse P	UNKNOWN	FAM-NEQ	27.733	27.678	0.09	
	47 B23		Wei Posidon (Coldnin)	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ	27.75	27.678	0.09	
	48 824		None	9,414.698	572.472 IUK	RNASE P	UNKNOWN	FAMINEQ	27.651	27.678	0.09	
	64 C16		9,677.329	9,414.698	572.472 IUK	RNASE P	UNKNOWN	FAMENEQ	27.636	27.678	0.09	
	65 C1/		10,188.251	9,414.698	572.472 10K	RNASE P	UNKNOWN	FAMINEQ	27.562	27.678	0.09	
	66 C18		9,780.859	9,414.698	572.472 10K	RNASE P	UNKNOWN	FAMINEQ	27.62	27.678	0.09	
	67 C19		9,554.062	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ	27.654	27.678	0.09	
	68 (20		9,883.055	9,414.698	572.472 IUK	RNASE P	UNKNOWN	FAMINEQ	27.000	27.678	0.09	
	69 (21		9,710.744	9,414.698	572.472 IUK	RNASE P	UNKINOWN	FAMINEQ	27.031	27.678	0.09	
	70 (22		9,489.336	9,414.698	572.472 IUK	RNASE P	UNKNOWN	FAMENEQ	27.664	27.678	0.09	
	/1 (23		9,705.506	9,414.698	572.472 IUK	RNASE P	UNKNOWN	FAMENEQ	27.631	27.678	0.09	
	72 (24		9,349.664	9,414.698	572.472 10K	RNASE P	UNKNOWN	FAMI-NEQ	27.685	27.678	0.09	
	88 D16		10,197.494	9,414.698	572.472 10K	RNASE P	UNKNOWN	FAM-NEQ	27.561	27.678	0.09	
	89 D17		10,077.892	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ	27.578	27.678	0.09	
	90 D18		9,934.107	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ	27.598	27.678	0.09	
	91 D19		9,700.193	9,414.698	572.472 IUK	RNASE P	UNKNOWN	FAMINEQ	27.032	27.678	0.09	
	92 D20		9,638.235	9,414.698	572.472 IUK	RNASE P	UNKNOWN	FAMENEQ	27.641	27.678	0.09	
	93 D21		10,246.75	9,414.698	572.472 10K	RNASE P	UNKNOWN	FAMINEQ	27.554	27.678	0.09	
	94 D22		8,886.359	9,414.698	572.472 1UK	RNASE P	UNKNOWN	FAMI-NEQ	27.757	27.678	0.09	
	95 D23		8,459.933	9,414.698	572.472 1UK	RNASE P	UNKNOWN	FAM-NEQ	27.828	27.678	0.09	
	96 D24		/,/64.318	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ	27.95	27.678	0.09	
	112 E16		10,065.235	9,414.698	572.472 10K	KNASE P	UNKNOWN	FAMINEQ	27.58	27.678	0.09	
	113 E1/		9,138.209	9,414.698	5/2.4/2 10K	RNASE P	UNKNOWN	FAMINEQ	27.718	27.678	0.09	
	114 E18		9,806.181	9,414.698	572.47210K	KNASE P	UNKNOWN	HAMHNEQ	27.617	27.678	0.09	
	115 E19		9,142.86	9,414.698	572.47210K	RNASE P	UNKNOWN	FAMI-NEQ	27.717	27.678	0.09	
	116 E20		9,424.23	9,414.698	572.47210K	RNASE P	UNKNOWN	FAM-NEQ	27.673	27.678	0.09	
	11/ E21	-1	8,763.623	9,414.698	572.47210K	RNASE P	UNKNOWN	FAM-NEQ	27.777	27.678	0.09	Ŧ
		4									•	

Flagged: ()

Omitted by Analysis: []

Samples Used: 2

Targets Used: 1

Omitted Manually: ()

The well table looks like this:

Set Up: 384

Analyzed: 384

In Plate: 384

Well Summary:

>(Plate	Layout	Well Table	2											
	Show i	n Table 🔻	Select Wells	▼ Group by ▼									🐮 Expan	d All 🔳 Co	lapse All
	#	Well	Omit	Flag San	nple Target	Task	Dyes	Ст	CT Mean	CT SD	Quantity	Quantit	Quantit	Comme	
			🗏 10K - RN	Ase P - UNKNOWN											~
	16	A16		10K	RNAse P	UNKNOWN	FAM-NEO	26,902	26,907	0.080	9.550.178	9.533.951	524,797		
	17	A17	i i	10K	RNAse P	UNKNOWN	FAM-NEO	26,907	26,907	0.080	9.523.232	9,533,951	524,797		=
	18	A18	i i	10K	RNAse P	UNKNOWN	FAM-NFO	26.955	26.907	0.080	9,206,665	9.533.951	524.797		
	19	A19	Ē	10K	RNAse P	UNKNOWN	FAM-NFO	26.849	26.907	0.080	9,912.618	9,533,951	524,797		_
	20	A20	Ē	10K	RNAse P	UNKNOWN	FAM-NEQ	26.982	26.907	0.080	9,028.357	9,533.951	524.797		
	21	A21	Ē	10K	RNAse P	UNKNOWN	FAM-NEQ	26.991	26.907	0.080	8,977.218	9,533.951	524.797		
	22	A22	Ē	10K	RNAse P	UNKNOWN	FAM-NEQ	26.974	26.907	0.080	9,080.458	9,533.951	524.797		
	23	A23	Ē	10K	RNAse P	UNKNOWN	FAM-NEQ	26.975	26.907	0.080	9,074.082	9,533.951	524.797		
	24	A24	Ē	10K	RNAse P	UNKNOWN	FAM-NEO	26.915	26.907	0.080	9,464.632	9,533.951	524.797		
	40	B16	Ē	10K	RNAse P	UNKNOWN	FAM-NFO	26.916	26.907	0.080	9,459.534	9,533.951	524.797		
	41	B17		10K	RNAse P	UNKNOWN	FAM-NFQ	26.799	26.907	0.080	10,267.638	9,533.951	524.797		
	42	B18		10K	RNAse P	UNKNOWN	FAM-NFQ	26.924	26.907	0.080	9,407.755	9,533.951	524.797		
	43	B19		10K	RNAse P	UNKNOWN	FAM-NFQ	26.939	26.907	0.080	9,308.513	9,533.951	524.797		
	44	B20		10K	RNAse P	UNKNOWN	FAM-NFQ	26.923	26.907	0.080	9,410.605	9,533.951	524.797		
1	45	B21		10K	RNAse P	UNKNOWN	FAM-NFQ	26.953	26.907	0.080	9,219.866	9,533.951	524.797		
	46	B22		10K	RNAse P	UNKNOWN	FAM-NFQ	26.949	26.907	0.080	9,240.837	9,533.951	524.797		
	47	B23		10K	RNAse P	UNKNOWN	FAM-NFQ	26.972	26.907	0.080	9,092.796	9,533.951	524.797		
	48	B24		10K	RNAse P	UNKNOWN	FAM-NFQ	26.871	26.907	0.080	9,763.808	9,533.951	524.797		
	64	C16		10K	RNAse P	UNKNOWN	FAM-NFQ	26.876	26.907	0.080	9,728.589	9,533.951	524.797		
	65	C17		10K	RNAse P	UNKNOWN	FAM-NFQ	26.822	26.907	0.080	10,107.170	9,533.951	524.797		
	66	C18		10K	RNAse P	UNKNOWN	FAM-NFQ	26.902	26.907	0.080	9,554.992	9,533.951	524.797		
	67	C19		10K	RNAse P	UNKNOWN	FAM-NFQ	26.922	26.907	0.080	9,422.722	9,533.951	524.797		
	68	C20		10K	RNAse P	UNKNOWN	FAM-NFQ	26.863	26.907	0.080	9,820.851	9,533.951	524.797		
	69	C21		10K	RNAse P	UNKNOWN	FAM-NFQ	26.821	26.907	0.080	10,112.306	9,533.951	524.797		
	70	C22		10K	RNAse P	UNKNOWN	FAM-NFQ	26.922	26.907	0.080	9,423.139	9,533.951	524.797		
	71	C23		10K	RNAse P	UNKNOWN	FAM-NFQ	26.880	26.907	0.080	9,704.001	9,533.951	524.797		
	72	C24		10K	RNAse P	UNKNOWN	FAM-NFQ	26.949	26.907	0.080	9,244.182	9,533.951	524.797		
	88	D16		10K	RNAse P	UNKNOWN	FAM-NFQ	26.801	26.907	0.080	10,254.792	9,533.951	524.797		
	89	D17		10K	RNAse P	UNKNOWN	FAM-NFQ	26.819	26.907	0.080	10,130.240	9,533.951	524.797		
	90	D18		10K	RNAse P	UNKNOWN	FAM-NFQ	26.851	26.907	0.080	9,898.796	9,533.951	524.797		
	91	D19		10K	RNAse P	UNKNOWN	FAM-NFQ	26.879	26.907	0.080	9,712.068	9,533.951	524.797		
	92	D20		10K	RNAse P	UNKNOWN	FAM-NFQ	26.909	26.907	0.080	9,507.545	9,533.951	524.797		
	93	D21		10K	RNAse P	UNKNOWN	FAM-NFQ	26.792	26.907	0.080	10,321.120	9,533.951	524.797		
	94	D22		10K	RNAse P	UNKNOWN	FAM-NFQ	26.964	26.907	0.080	9,143.233	9,533.951	524.797		
	95	D23		10K	RNAse P	UNKNOWN	FAM-NFQ	27.017	26.907	0.080	8,809.055	9,533.951	524.797		~
w		mane	In D		10.294	and and 204	Elacordu	0	Omitted by Analysis	0 0	mitted Manualiu	0 c-	and an United 2		de theod. 1

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Standard Curve Experiments

To group by flag

Х	Plate	e Layout	Well Table												
	Show i	in Table 🔻	Select Wells 🔻	Gro	upBy ∀								Expand All	🗖 Collap	ise All
- i	*	1 Mol	Omit		Target Name	otity Mooro	Outpotity SD	Comple Marge	Target Name	Tack	Dyoc	CT	Crittoon	CT SD	60
		MO	Onin		Sample Name	4,994.994	222.402	JK III III III III III III III III III I	NIVASE P	OTAKINO WIN	FRIMING	28.349	20,302	0.003	
	9	A9			Task	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.651	28.582	0.063	-
	10	A10			Peolicate	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.62	28.582	0.063	
	11	A11			-	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.682	28.582	0.063	
	12	A12		_	Dye	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NEQ	28.475	28.582	0.063	
	13	A13		~	Flag	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.575	28.582	0.063	
	14	A14			c7	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.624	28.582	0.063	
	15	A15			Comments	4,994.994	222.4525	ōΚ	RNAse P	UNKNOWN	FAM-NFQ	28.649	28.582	0.063	
	16	A16			Well Decition (Dow)	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NFQ	27.638	27.678	0.09	
	17	A17			well rusiculi (Ruw)	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NFQ	27.627	27.678	0.09	
	18	A18			Well Position (Column)	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NFQ	27.684	27.678	0.09	
	19	A19			None	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NFQ	27.601	27.678	0.09	
	20	A20			8,758.838	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NFQ	27.778	27.678	0.09	
	21	A21			8,884.947	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NFQ	27.758	27.678	0.09	
	22	A22			8,744.812	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NEQ	27.78	27.678	0.09	
	23	A23			9,051.594	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NFQ	27.731	27.678	0.09	
	24	A24			9,559.844	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NFQ	27.653	27.678	0.09	
	25	B1							RNAse P	NTC	FAM-NFQ	Undetermi			
	26	B2			1,250	1,250			RNAse P	STANDARD	FAM-NFQ	30.401	30.568	0.087	
	27	B3			2,500	2,500			RNAse P	STANDARD	FAM-NFQ	29.452	29.515	0.075	
	28	B4			5,000	5,000			RNAse P	STANDARD	FAM-NFQ	28.569	28.611	0.054	
	29	B5			10,000	10,000			RNAse P	STANDARD	FAM-NFQ	27.584	27.646	0.057	
	30	B6			20,000	20,000			RNAse P	STANDARD	FAM-NFQ	26.467	26.553	0.049	
	31	B7			5,160.045	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.534	28.582	0.063	
	32	B8			5,114.345	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.546	28.582	0.063	
	33	B9			4,843.689	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.624	28.582	0.063	
	34	B10			4,609.773	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.695	28.582	0.063	
	35	B11			4,678.537	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.674	28.582	0.063	
	36	B12			4,990.247	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.582	28.582	0.063	
	37	B13			4,778.531	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.643	28.582	0.063	
	38	B14			4,736.198	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.656	28.582	0.063	
	39	B15			5,170.109	4,994.994	222.4525	5K	RNAse P	UNKNOWN	FAM-NFQ	28.531	28.582	0.063	
	40	B16			9,485.763	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NFQ	27.664	27.678	0.09	
	41	B17			10,087.83	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NEQ	27.576	27.678	0.09	
	42	818			9,420.996	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NFQ	27.674	27.678	0.09	
	43	B19			9,164.339	9,414.698	572.4721	LOK	RNAse P	UNKNOWN	FAM-NEQ	27.713	27.678	0.09	-
			•												•
w	ell Sun	nmary:	In Plate: 3	384	Set Up: 384	Analyzed: 384	4 Flag	ged: ()	Omitted by Analysis	0 0	mitted Manually:	0 Sa	mples Used: 2	Targets '	Used: 1

From the Group By drop-down menu, select **Flag**. The software groups the flagged and unflagged wells. In the example experiment, there are no flagged wells.

To group by C_T value

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

У	Plate Layout	Well Table									
	Show in Table 🔻	Select Wells 🔻	Group By 🔻							Expand All	Collapse All
Ī	# Well	Omit	Target Name	htity Mean	Quantity SD Sample Nam	e Tarnet Name	Task	Dives	(T	CT Mean	CT SD CC
H	92 020		Sample Name	9,414.090	J72.472 10K	KINASE P	OLAKIAOAALA	FARINEQ 2	27.041	27.078	0.09
- 1	93 D21		Task	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.554	27.678	0.09 🚽
- 1	94 D22		Replicate	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.757	27.678	0.09
- 1	95 D23		-	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.828	27.678	0.09
- 1	96 D24		Dye	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.95	27.678	0.09
- 1	99 E3		Flag	2,500		RNAse P	STANDARD	FAM-NEQ 2	29.429	29.515	0.075
- 1	100 E4		🗸 СТ 🚺	5,000		RNAse P	STANDARD	FAM-NFQ 2	28.649	28.611	0.054
- 1	101 E5		Comments	10,000		RNAse P	STANDARD	FAM-NEQ 2	27.662	27.646	0.057
- 1	102 E6		Well Position (Row)	20,000		RNAse P	STANDARD	FAM-NFQ 2	26.533	26.553	0.049
- 1	103 E7		Weil Position (Kow)	4,994.994	222.452.5K	RNAse P	UNKNOWN	FAM-NEQ 2	28.584	28.582	0.063
- 1	104 E8		Well Position (Column)	4,994.994	222.4525K	RNAse P	UNKNOWN	FAM-NEQ 2	28.543	28.582	0.063
- 1	105 E9		None	4,994.994	222.452.5K	RNAse P	UNKNOWN	FAM-NEQ 2	28.524	28.582	0.063
- 1	106 E10		4,860.265	4,994.994	222.4525K	RNAse P	UNKNOWN	FAM-NFQ 2	28.619	28.582	0.063
- 1	107 E11		4,745.296	4,994.994	222.452.5K	RNAse P	UNKNOWN	FAM-NEQ 2	28.653	28.582	0.063
- 1	108 E12		4,869.692	4,994.994	222.4525K	RNAse P	UNKNOWN	FAM-NFQ 2	28.616	28.582	0.063
	109 E13		4,783.282	4,994.994	222.4525K	RNAse P	UNKNOWN	FAM-NFQ 2	28.642	28.582	0.063
1	110 E14		5,098.292	4,994.994	222.4525K	RNAse P	UNKNOWN	FAM-NEQ 2	28.551	28.582	0.063
- 1	111 E15		5,181.023	4,994.994	222.4525K	RNAse P	UNKNOWN	FAM-NFQ 2	28.528	28.582	0.063
- 1	112 E16		10,065.235	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.58	27.678	0.09
- 1	113 E17		9,138.209	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.718	27.678	0.09
- 1	114 E18		9,806.181	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.617	27.678	0.09
- 1	115 E19		9,142.86	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.717	27.678	0.09
- 1	116 E20		9,424.23	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.673	27.678	0.09
- 1	117 E21		8,763.623	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.777	27.678	0.09
- 1	118 E22		9,553.755	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.654	27.678	0.09
- 1	119 E23		8,161.914	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	27.879	27.678	0.09
- 1	120 E24		7,265.062	9,414.698	572.472 10K	RNAse P	UNKNOWN	FAM-NEQ 2	28.045	27.678	0.09
- 1	123 F3		2,500	2,500		RNAse P	STANDARD	FAM-NEQ 2	29.485	29.515	0.075
- 1	124 F4		5,000	5,000		RNAse P	STANDARD	FAM-NEQ 2	28.625	28.611	0.054
- 1	125 F5		10,000	10,000		RNAse P	STANDARD	FAM-NEQ 2	27.612	27.646	0.057
- 1	126 F6		20,000	20,000		RNAse P	STANDARD	FAM-NEQ 2	26.543	26.553	0.049
- 1	127 F7		4,672.368	4,994.994	222.4525K	RNAse P	UNKNOWN	FAM-NEQ 2	28.676	28.582	0.063
- 1	128 F8		5,241.754	4,994.994	222.4525K	RNAse P	UNKNOWN	FAM-NEQ 2	28.511	28.582	0.063
- 1	129 F9		4,766.071	4,994.994	222.4525K	RNAse P	UNKNOWN	FAM-NEQ 2	28.647	28.582	0.063
- 1	130 F10		4,806.276	4,994.994	222.4525K	RNAse P	UNKNOWN	FAM-NEQ 2	28.635	28.582	0.063
	131 F11		5,342.352	4,994.994	222.4525K	RNAse P	UNKNOWN	FAM-NEQ 2	28.484	28.582	0.063 💌
- [•									Þ
We	ell Summary:	In Plate: (384 Set Up: 384	Analyzed: 38	4 Flagged: 0	Omitted by Analysis	:0 c	mitted Manually: ()	Sar	nples Used: 2	Targets Used: 1

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Standard Curve Experiments

Tips for analyzing

your own experiments	standards, and samples. Look in the Quantity columns to make sure the quantity values for each replicate group are similar indicating tight C_T precision.
	• Flag – The software groups the flagged and unflagged wells. A flag indicates that the software has found a potential error in the flagged well. For a description of the QuantStudio [™] 6 and 7 Flex Real-Time PCR System Software flags, see "Flag Settings" on page 50.
	• 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 accura	ate dye signal using the Multicomponent Plot
	The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.
Purpose	In the standard curve example experiment, you review the Multicomponent Plot screen for:
	 ROX[™] dye (passive reference)
	• FAM [™] dye (reporter)
	Spikes, dips, and/or sudden changes
	Amplification in the negative control wells
View the	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.

• Replicate – The software groups the wells by replicate: negative controls,

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.

5

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.









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.

code for each row of the reaction plate (see the legend in the Raw Data Plot shown

• **Negative control wells** – There should not be any amplification in the negative control wells.

Determine signal accuracy using the Raw Data Plot

below).

	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

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Standard Curve Experiments

5

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:

PCR I	ilter —						
				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)	\checkmark					
Filter	x2(520±10)		1				
ation	x3(550±11)			V			
Excita	x4(580±10)				V		
	x5(640±10)					\checkmark	
	x6(662±10)						
				·		·	
Melt	Curve Filter ———						
				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)						
Filter	x2(520±10)						
ation	x3(550±11)						
Excita	x4(580±10)						
	x5(640±10)						
	x6(662±10)						
				•	•		



Tips for determining signal accuracy in your own experiments When you analyze your own standard curve experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

Review the flags in the QC Summary

The QC Summary screen displays a list of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software flags, including the flag frequency and location for the open experiment.

View the QC Summary

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

Note: If no data are displayed, click Analyze.

2. Review the Flags Summary.

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

In the example experiment, there are no flagged wells.

- **3.** In the Flag Details table, click each flag with a frequency >0 to display detailed information about the flag. In the example experiment, the Frequency column displays 0 for all flags.
- **4.** (*Optional*) For those flags with frequency >0, click the troubleshooting link to view information on correcting the flag.

otal Wells:	384	Processed Wells:	384 Manually Or	nitted Wells:	0	Targets Used:	
/ells Set Up:	384	Flagged Wells:	0 Analysis Om	itted Wells:	ō	Samples Used:	1
ag Details							
Flag:		Name		Frequency		Wells	
MPNC	Amp	ification in negative control	()			
ADROX	Bad (assive reterence signal		J			
IGHED	Hidb	standard deviation in renlicate group		<u>ן</u>			
DAMP	No a	nolification		1			
OISE	Noise	higher than others in plate	()			
PIKE	Noise	spikes	()			
IOSIGNAL	No si	gnal in well	()			
UTLIERRG	Outli	er in replicate group	()			
XPFAIL	Expo	nential algorithm failed	0)			
LFAIL	Base	ne algorithm tailed	l]			
HULDFAIL	Inte	molung agontrim taleu		J			
TFAIL	CT al	jorithm failed	į]			
TFAIL	ICT al	jorithm failed					
TFAIL Flag: Flag Detail:	CT al AMPNC—Amplifica A sequence amplifi reaction.	jonthm failed jon in negative control ed in a negative control					
TFAIL Flag: Flag Detail: Flag Criteria:	CT al AMPNC—Amplifica A sequence amplifi reaction. CT < 35.0	jonthm failed jon in negative control ed in a negative control					
FFAIL Flag: Flag Detail: Flag Criteria: Flagged Wells:	CT al AMPNC—Amplifica A sequence amplifi reaction. CT < 35.0 None	jonthm failed jon in negative control ed in a negative control					
FFAIL Flag: Flag Detail: Flag Criteria: Flagged Wells:	CT al AMPNC—Amplifica A sequence amplifi reaction. CT < 35.0 None View AMPNC Trout	ion in negative control ed in a negative control ed in a negative control					
FFAIL Flag: Flag Detail: Flag Criteria: Flagged Wells:	CT al AMPNC—Amplifica A sequence amplifi reaction. CT < 35.0 None View AMPNC Trout	ion in negative control ed in a negative control ed in a negative control ieshooting Information					
FFAIL Flagg Flag Detail: Flag Criteria: Flagged Wells:	[Cr al AMPNC—Amplifica A sequence amplific reaction. : CT < 35.0 : None View AMPNC Trout	ion in negative control ed in a negative control ed in a negative control leshooting Information					
FFAIL Flag: Flag Detail: Flag Criteria: Flagged Wells:	[Cr al AMPNC—Amplifica A sequence amplifi reaction. C T < 35.0 : CT < 35.0 : None Yiew AMPNC Trout	ion in negative control ed in a negative control eleshooting Information					
FFAIL Flag: Flag Detail: Flag Criteria: Flagged Wells:	Cr al AMPNC—Amplifica A sequence amplifi reaction. CT < 35.0 None <u>View AMPNC Trout</u>	ion in negative control ed in a negative control ed in a negative control					
FFAIL Flagg Flag Detail: Flag Criteria: Flagged Wells:	[Cr al AMPIXC—Amplifica A sequence amplifi reacton. : CT < 35.0 None View AMPIXC Trout	ion in negative control ed in a negative control ed in a negative control keshooting Information					
FFAIL Flag: Flag Detail: Flag Criteria: Flagged Wells:	[Cr al AMPNC—Amplifica A sequence amplifi reaction. : CT < 35.0 : None View AMPNC Trout	ion in negative control ed in a negative control eleshooting Information					
Flag: Flag Detail: Flag Criteria: Flagged Wells:	[Cr al AMPNC—Amplifica A sequence amplifi reaction. CT < 35.0 None View AMPNC Trout	ion in negative control ed in a negative control ed in a negative control					

Possible flagsThe flags listed below may be triggered by the experiment data.Note: To change the flag settings, refer to "Flag Settings" on page 50.

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
Se	econdary analysis flags
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group

For more information

For more information on	Refer to	Publication number
Publishing data	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7</i> Flex Real-Time PCR System Software Experiments	4489822



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[™] 6 and 7 Flex Real-Time PCR System Software are not suitable for your own experiment, you can change the settings in the Analysis Settings dialog box, then reanalyze your experiment.

View the analysis settings

1. From the Experiment Menu pane, select Analysis.

2. Click **Analysis** → **Analysis Settings** to open the Analysis Settings dialog box. In the example experiment, the default analysis settings are used for each tab:

- C_T Settings
- Flag Settings
- Advanced Settings
- Standard Curve Settings

The following is an image of the Analysis Settings dialog box for a Standard Curve experiment:

Analysis Settings	for QS6_QuantStudio_384-	Well_Standard_Curve_	_Example				
CT Settings Eli	a Settings Advanced Setting	s Standard Curve Sett	ings				
Review the det Settings, the	ault settings for analysis of targets n change the settings.	in this experiment. To use di	ifferent settings for a target,	select the target from the table, deselect Use Default			
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. Baseline Threshold ▼ Stage 2, Step 2 ▼ Default CT Settings							
Default CT setting Threshold: AU	s are used to calculate the CT for t	argets without custom setting Baseline End Cycle: AU	ps. To edit the default settings	is, dick Edit Default Settings. tings			
Select a Targe	t			CT Settings for RNAse P			
Target	Threshold	Baseline Start	Baseline End	CT Settings to Use: 🗹 Default Settings			
RNAse P	AUTO	AUTO	AUTO	Automatic Threshold			
				Threshold: 0.134184			
				✓ Automatic Baseline			
				Baseline Start Cycle: 3 🗇 End Cycle: 15 🗇			

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*[™] 6 and 7 Flex Real-Time PCR System Software 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

Use the Baseline Threshold algorithm to determine the C_T values.

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

• 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[™] 6 and 7 Flex Real-Time PCR System 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 following is an image of the Flag Settings tab:

CT Settings Fl	ag Settings Advanced Setl	tings Standard C	urve Settings			
Configure the	flags and filtering. In this panel y	you can enable, disable	, and configure flags, and indica	ate if a well is to be rejected	l when a flag is raised.	2
Flag	Description	Use	Attribute	Condition	Value	Reject Well
AMPNC	Amplification in negat		Ст	< 🗸	35.000	
BADROX	Bad passive referenc	v	Bad passive referenc	> 🗸	0.600	
OFFSCALE	Fluorescence is offscale	~				
HIGHSD	High standard deviati	~	CT standard deviation	> 🗸	0.500	
NOAMP	No amplification	v	Amplification algorith	< *	0.100	
VOISE	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	v				
EXPFAIL	Exponential algorithm	~				
BLFAIL	Baseline algorithm failed	~				
THOLDFAIL	Thresholding algorith	v				
CTFAIL	CT algorithm failed	✓				
Save to Library	Load from Library		Revert to Def	ault Analysis Settings	Annly Analysis	Settings Cand

Advanced Settings

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

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.

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

Standard Curve Settings

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

Note: The run method must be the same. Life Technologies recommends using the standard curve from the current experiment.

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



Improve C_T precision by omitting wells

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

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

Note: If no data are displayed, click Analyze.

- **2.** In the Amplification Plot screen, select C_T vs Well from the Plot Type drop-down menu.
- **3.** Select the **Well Table** tab.
- 4. In the Well Table, view outliers:
 - a. From the Group By drop-down menu, select Replicate.
 - **b.** Look for any outliers in the replicate group (make sure they are flagged).
 - **c.** Select the **Omit** check box next to outlying well(s), as shown in the following image.

2	Plate	Layout	Well Table												
	Show i	n Table 🔻	Select Wells	🔻 🛛 Group By	V									🗉 Expand All	😑 Collapse All
	#	Well	Omit	Flag	Sample Na	Target Na	Task	Dyes	Ст	Ct Mean	CT SD	Quantity	Quantity	Quantity SD Co	mments
	1	A1				RNAse P	NTC	FAM-NFQ	Undetermi.						<u> </u>
- 1	2	A2				RNAse P	STANDARD	FAM-NFQ	29.846	29.801	0.084	1,250			
- 1	3	AЗ				RNAse P	STANDARD	FAM-NFQ	28.856	28.762	0.071	2,500			
- 1	4	A4				RNAse P	STANDARD	FAM-NFQ	27.837	27.858	0.055	5,000			
- 1	5	A5				RNAse P	STANDARD	FAM-NFQ	26.825	26.891	0.053	10,000			
- 1	6	A6				RNAse P	STANDARD	FAM-NFQ	25.831	25.804	0.047	20,000			
- 1	7	A7			5K	RNAse P	UNKNOWN	FAM-NFQ	27.88	27.828	0.065	4,803.257	4,989.372	229.799	
- 1	8	A8			5K	RNAse P	UNKNOWN	FAM-NFQ	27.806	27.828	0.065	5,059.929	4,989.372	229.799	
- 1	9	A9			5K	RNAse P	UNKNOWN	FAM-NFQ	27.909	27.828	0.065	4,708.362	4,989.372	229.799	
- 1	10	A10			5K	RNAse P	UNKNOWN	FAM-NFQ	27.875	27.828	0.065	4,823.056	4,989.372	229.799	
- 1	11	A11			5K	RNAse P	UNKNOWN	FAM-NFQ	27.931	27.828	0.065	4,634.604	4,989.372	229.799	
- 1	12	A12			5K	RNAse P	UNKNOWN	FAM-NFQ	27.705	27.828	0.065	5,433.725	4,989.372	229.799	
- 1	13	A13			5K	RNAse P	UNKNOWN	FAM-NFQ	27.823	27.828	0.065	4,999.525	4,989.372	229.799	
- 1	14	A14			5K	RNAse P	UNKNOWN	FAM-NFQ	27.909	27.828	0.065	4,709.555	4,989.372	229.799	
	15	A15			5K	RNAse P	UNKNOWN	FAM-NFQ	27.894	27.828	0.065	4,757.323	4,989.372	229.799	
	16	A16			10K	RNAse P	UNKNOWN	FAM-NFQ	26.902	26.907	0.08	9,550.178	9,533.951	524.797	
- 1	17	A17			10K	RNAse P	UNKNOWN	FAM-NFQ	26.907	26.907	0.08	9,523.245	9,533.951	524.797	
- 1	18	A18			10K	RNAse P	UNKNOWN	FAM-NFQ	26.955	26.907	0.08	9,206.665	9,533.951	524.797	
- 1	19	A19			10K	RNAse P	UNKNOWN	FAM-NFQ	26.849	26.907	0.08	9,912.618	9,533.951	524.797	
- 1	20	A20			10K	RNAse P	UNKNOWN	FAM-NFQ	26.982	26.907	0.08	9,028.357	9,533.951	524.797	
- 1	21	A21			10K	RNAse P	UNKNOWN	FAM-NFQ	26.991	26.907	0.08	8,977.218	9,533.951	524.797	
- 1	22	A22			10K	RNAse P	UNKNOWN	FAM-NFQ	26.974	26.907	0.08	9,080.47	9,533.951	524.797	
- 1	23	A23			10K	RNAse P	UNKNOWN	FAM-NFQ	26.975	26.907	0.08	9,074.082	9,533.951	524.797	
- 1	24	A24			10K	RNAse P	UNKNOWN	FAM-NFQ	26.915	26.907	0.08	9,464.632	9,533.951	524.797	
- 1	25	B1				RNAse P	NTC	FAM-NFQ	Undetermi.						
- 1	26	B2				RNAse P	STANDARD	FAM-NFQ	29.654	29.801	0.084	1,250			
- 1	27	B3				RNAse P	STANDARD	FAM-NFQ	28.696	28.762	0.071	2,500			
- 1	28	B4				RNAse P	STANDARD	FAM-NFQ	27.829	27.858	0.055	5,000			
- 1	29	85				RNAse P	STANDARD	FAM-NFQ	26.85	26.891	0.053	10,000			
- 1	30	B6				RNAse P	STANDARD	FAM-NFQ	25.731	25.804	0.047	20,000			
- 1	31	B7			5K	RNAse P	UNKNOWN	FAM-NFQ	27.774	27.828	0.065	5,175.613	4,989.372	229.799	
	32	B8			5K	RNAse P	UNKNOWN	FAM-NFQ	27.796	27.828	0.065	5,097.28	4,989.372	229.799	
	33	B9			5K	RNAse P	UNKNOWN	FAM-NFQ	27.871	27.828	0.065	4,834.493	4,989.372	229.799	
	34	B10			5K	RNAse P	UNKNOWN	FAM-NFQ	27.94	27.828	0.065	4,606.265	4,989.372	229.799	
	35	B11			5K	RNAse P	UNKNOWN	FAM-NFQ	27.935	27.828	0.065	4,623.702	4,989.372	229.799	
	36	B12			5K	RNAse P	UNKNOWN	FAM-NFQ	27.859	27.828	0.065	4,876.215	4,989.372	229.799	T
We		mary:	In Pla	te: 384	Set Up: 384	Anal	vzed: 3R4	Flagged: (1 0	Omitted by Analysis: () On	nitted Manually: F	1	mples Used: 2	Targets Used: 1

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	Publication number
Amplification efficiency	Amplification Efficiency of TaqMan [®] Gene Expression Assays Application Note.	127AP05-03

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Standard Curve Experiments



Export Experiment 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**[™] **6 and 7** 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	QS6_QuantStudio_384-Well_Standard_Curve_Example_data
File Type	*.txt
Export File Location	C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\Export

6

Your Export screen should look like this:

Auto Export Forr	nat : QuantStuc	dio™ 6 and	7 💌	Export Data	To: 💿 One File 🔘 Se	eparate Files	✓ Open file(s)	when export is co	mplete
Export File Location: C:\Applied	Biosystems\Qu	iantStudio	6 and 7 Flex Softwar	Browse Export File	Name: QS6_Quant	Studio_384-Well_S	tandard_Curve F	le Type: 📋 (*.tx	t) 🗸
Sample Setup	ata 🛛 🔽 Ampl	lification	Multicomponent	Results					
⊂Select Content		346 11						- · · ·	
	~	well	Well Position	Sample Name	Sample Color	Biogroup Name	Biogroup Color	Target Name	1.
			1 A 1					RNASE P	RG A
Vel Vel			2 A2					RNASE P	PG
			4 44					RNAse P	RG
Well Position			5 A5					RNAse P	RG
Sample Name			6 46					RNAse P	RG
C Sample Name			7 47	5K	RGB(132,193,241)			RNAse P	RG
Sample Color			8 48	5K	RGB(132,193,241)			RNAse P	RG
			9 A 9	5K	RGB(132,193,241)			RNAse P	RG
Biogroup Name			10 A10	5K	RGB(132,193,241)			RNAse P	RG
Ringroup Color			11 A11	5K	RGB(132,193,241)			RNAse P	RG
			12 A12	5K	RGB(132,193,241)			RNAse P	RG
Target Name			13 A13	5K	RGB(132,193,241)			RNAse P	RG
			14 A14	5K	RGB(132,193,241)			RNAse P	RG
Target Color			15 A15	5K	RGB(132,193,241)			RNAse P	RG
			16 A16	10K	RGB(168,255,222)			RNAse P	RG
I dSK			17 A17	10K	RGB(168,255,222)			RNAse P	RG
Reporter			18 A18	10K	RGB(168,255,222)			RNAse P	RG 🗸
	~								

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

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

SG_QuantStudio_384-Well_Standard_Curve_Example_data.txt - Notepad	
File Edit Format View Help	
The Edit Format Vew HeD Fle Edit Format Vew HeD Calibration Background is expired = Yes Calibration Background is expired = Yes Calibration HRM MELTOOCTOR performed on = 01-29-2010 Calibration Normalization FAM-ROX is expired = Yes Calibration Normalization FAM-ROX performed on = 01-29-2010 Calibration Normalization VIC-OX performed on = 01-29-2010 Calibration Normalization VIC-OX performed on = 01-29-2010 Calibration Normalization VIC-OX performed on = 01-29-2010 Calibration Pure Dye FAM performed on = 01-29-2010 Calibration Pure Dye FAM performed on = 01-14-2010 Calibration Pure Dye FAM performed on = 01-14-2010 Calibration Pure Dye MELTDOCTOR is expired = Yes Calibration Pure Dye NED performed on = 01-29-2010 Calibration Pure Dye TAWAA is expired = Yes Calibration Pure Dye TAWAA performed on = 01-29-2010 Calibration Pure Dye TAWAA performed on = 01-29-2010 Calibration Pure Dye VIC is expired = Yes Calibration Pure Dye VIC Signifed = Yes Calibration Pure Dye VIC Signifed = Yes Calibration NoI is expired = Yes Calibration NoI is expired = Yes Calibration Uniformity Is expired = Yes Calibration Uniformity Is expired = Yes Calibration Uniformity Signifed = Yes Calibratio	
* Stage/ Cycle where Analysis is performed = Stage 2, Step 2	
User Name Name Sample Color Biogroup Name Biogroup Color Target Name Target Color Target Color Reporter Quantity Comments Comments RGB (139,189,249)" NTC FAM NFQ-MGB 1 A1 RNASE P "RGB (139,189,249)" STANDARD FAM NFQ-MGB 2 A2 RNASE P "RGB (139,189,249)" STANDARD FAM NFQ-MGB 3 A3 RNASE P "RGB (139,189,249)" STANDARD FAM NFQ-MGB 4 A4 RNASE P "RGB (139,189,249)" STANDARD FAM NFQ-MGB 5 A5 RNASE P "RGB (139,189,249)" STANDARD FAM NFQ-MGB	
0 A0 FAM NFQ-MGB 20,000.000 7 A7 5K "RGB(132,193,241)" RNASE P "RGB(139,189,249)" UNKNOWN FAM NFQ-MGB 8 A8 5K "RGB(132,193,241)" RNASE P "RGB(139,189,249)" UNKNOWN FAM NFQ-MGB	~

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Standard Curve Experiments

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USER GUIDE



QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments

Booklet 3

Publication Number 4489822 Revision A



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PART 1 Running Relative Standard Curve Experiments

About Relative Standard Curve Experiments

This chapter covers:

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- About the example experiment 13

IMPORTANT! First-time users of the QuantStudioTM 6 and 7 Flex Real-Time PCR System Software, please read Booklet 1, *Getting Started with QuantStudioTM 6 and 7 Flex Real-Time PCR System Software Experiments* and Booklet 7, *QuantStudioTM 6 and 7 Flex Real-Time PCR System Software 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[™] 6 and 7 Flex Real-Time PCR System Software by pressing F1, clicking ② in the toolbar, or selecting Help > QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Help.

About Relative Standard Curve experiments

The Relative Standard Curve method is used to determine relative target quantity in samples. The QuantStudioTM 6 and 7 Flex Real-Time PCR System 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 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.

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 6 and 7 Flex Real-Time PCR System Software measures amplification of the target and of the endogenous control in samples and in a reference sample. For more information on Comparative CT $(\Delta\Delta C_T)$ experiments, refer to Part II, Running Comparative CT $(\Delta\Delta C_T)$ Experiments of this booklet.

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 negative control wells.
PCR options	When performing real-time PCR, choose between:
	• Singleplex and multiplex PCR (below) <i>and</i>
	• 1-step and 2-step RT-PCR (page 13)
	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. <i>Or</i>
	• Multiplex PCR – In multiplex PCR, two or more primer and probe sets are present in the reaction tube or well. Each set amplifies a specific target or endogenous control. Typically, a probe labeled with FAM [™] dye detects the target and a probe labeled with VIC [®] dye detects the endogenous control.
	IMPORTANT! SYBR [®] Green reagents cannot be used for multiplex PCR.



1- and 2-Step RT-PCR

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

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

About the example experiment

To illustrate how to perform a Relative Standard Curve, this guide leads you through the process of designing and performing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software.

The objective of the Relative Standard Curve example experiment is to compare the expression of the FAS 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 kidney, liver, brain, and heart.
- The target is FAS.
- The endogenous control is HPRT.
- The reference sample is brain.
- One standard curve is set up for FAS. The standard used for the standard dilution series is a Human cDNA sample of known total concentration.
- One standard curve is set up for HPRT (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 (FAS), select assay ID Hs00907759_m1.
 - For the endogenous control assay (HPRT), select assay ID Hs99999999_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.	15
Define targets, samples, and biological replicates	16
Assign targets, samples, and biological groups	17
Set up the run method	19
Tips for designing your own experiment	20
For more information	21

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 2 in Booklet 1, *Getting Started with QuantStudio*TM 6 and 7 Flex Real-Time PCR System Software Experiments.

Define the experiment properties

2

Click **Experiment Setup** \blacktriangleright **Experiment Properties** to create a new experiment in the QuantStudioTM 6 and 7 Flex Software. Enter:

Field or Selection	Entry
Experiment Name	QS6_QuantStudio_384-Well_Relative_Standard_Curve_Example
Barcode	Leave field empty
User Name	Example User
Comments	Relative Standard Curve example
Instrument type	QuantStudio [™] 6 Flex System
Block	384-Well Block
Experiment Type	Relative Standard Curve
Reagents	TaqMan [®] Reagents
Ramp speed	Standard
Reagent information	NA

Save the experiment.

Your Experiment Properties screen should look like this:

How do you want to identify this expe	eriment?					
* Experiment Name: QS6_QuantStudio_384-Well_Relative_Standard_Curve_Example Comments: Relative Standard Curve Experiment Barcode: User Name: Example User						
* Which instrument type are you usin	g to run the experiment?					
✓ QuantStudio [™] 6 Flex System	QuantStudio™ 7 Flex System					
* Which block are you using to run the	e experiment?					
✓ 384-Well	96-Well (0.2mL)	Fast 96-Well (0.1mL)				
What type of experiment do you wa	nt to set up?					
Standard Curve	 Relative Standard Curve 	Comparative CT (ΔΔCT)	Melt Curve			
Genotyping	Presence/Absence					
* Which reagents do you want to use	to detect the target sequence?					
✓ TaqMan® Reagents	SYBR® Green Reagents	Other				
* What properties do you want for the	e instrument run?					
Standard	✓ Fast					
What is the reagent information?						
New Delete						
Type Name	Part Number	Lot Number	Expiration Date			

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	VIC	NFQ-MGB	

2. Samples

Sample name	Color
Brain	
Heart	
Kidney	
Liver	

- **3.** Dye to be used as a Passive Reference ROX
- 4. Custom Task Name Not applicable

5. Analysis Settings

Field	Select
Reference Sample	Brain
Endogenous Control	HPRT

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 🏹 Import from File
Target Name	Reporter	Quencher	Color	Sample Name Color
FAS	FAM 🗸	NFQ-MGB 🗸 🗸	- -	Brain
HPRT	VIC 🗸	NFQ-MGB 🗸 🗸		Heart
				Kidney
				liver
Pielogical Doplicato Cuoupo				Analysis Cattings
Biological Replicate Groups				Anaysis Settings
New Date				
Biological Group Name Color		Comments		
		Reference Sample: Brain		
			Endogenous Control: HPRT	
* Passive Reference				Custom Task Name
ROX		New Delete		
				Name Color Icon Char

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 **Coeffice and Set Up Standards** to open the Define and Set Up Standards dialog box.

To assign the targets and samples:

- **1.** Set up the standards.
- 2. For the first standard for the FAS target:
 - a. Click-drag to select wells A9-A16.
 - b. Check check box next to FAS in the Targets list.
 - c. Select S in the Task drop-down menu.
 - **d**. Enter 625 in the Quantity column.

- **e.** Repeat steps a through c for each of the standards for the FAS target, selecting the wells listed in the table below, and entering the corresponding quantity.
- **3.** Repeat step 2 for each standard for the HPRT target.

Target name	Well number	Task	Quantity	Sample name
FAS	A1 - A8	Negative	None	None
	E1 - E12	Unknown	Determined by run	Kidney
	E13 - E24	Unknown	Determined by run	Heart
	11 - 112	Unknown	Determined by run	Liver
	113 - 124	Unknown	Determined by run	Brain
	A9 - A16	Standard	625	None
	A17 - A24	Standard	2,500	None
	C1 - C8	Standard	312.50	None
	C9 - C16	Standard	1,250	None
	C17 - C24	Standard	5,000	None
HPRT	P1 - P8	Negative	None	None
	H1 - H12	Unknown	Determined by run	Kidney
	H13 - H 24	Unknown	Determined by run	Heart
	L1 - L12	Unknown	Determined by run	Liver
	L13 - L24	Unknown	Determined by run	Brain
	N1 - N8	Standard	165.25	None
	N9 - N16	Standard	625	None
	N17 - N24	Standard	2,500	None
	P9 - P16	Standard	312.50	None
	P17 - P24	Standard	1,250	None



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: 10 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				



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	Publication number
Consumables	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7 Flex</i> <i>Real-Time PCR System Software Experiments</i>	4489822
	Appendix A in Booklet 7, <i>QuantStudio[™] 6 and 7 Flex Real-Time PCR</i> System Software 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.	4489822
Amplification efficiency	Amplification Efficiency of TaqMan $^{\ensuremath{\mathbb S}}$ Gene Expression Assays Application Note	127AP05-03
Using Alternative Setup	Chapter 3 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7 Flex</i> <i>Real-Time PCR System Software Experiments</i>	4489822

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:

Assemble required materials	23
Prepare the template	23
Prepare the sample dilutions	24
Prepare the standard dilution series for FAS and HPRT assays	24
Prepare the reaction mix ("cocktail mix")	25
Prepare the reaction plate	26
Tips for preparing reactions for your own experiments	28
For more information	29

Assemble required materials

3

- Items listed in Booklet 1, *Getting Started with QuantStudio*[™] 6 and 7 Flex Real-Time PCR System Software 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×)

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

Prepare the template

	Prepare the template for the PCR reactions (both samples and standards) using one of the Invitrogen VILO kits, SuperScript [®] VILO [™] cDNA Synthesis Kit (Part no. 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> (Pub. no. 100002284) to:

- 1. Isolate total RNA from cells using an Ambion[®] sample preparation kit appropriate to the tissue or cell type.
- 2. Quantify and perform quality control on the RNA.
- 3. Convert the RNA to cDNA via reverse transcription.

Prepare the sample dilutions

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

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

Tube	Sample name	Diluent volume (µL)
1	Kidney	76
2	Heart	76
3	Liver	76
4	Brain	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
3	Liver	4
4	Brain	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
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:

Component	Volume (µL) for 1 reaction	Volume (µL) for 96 reactions (plus 10% excess)
TaqMan [®] Gene Expression Master Mix (2X)	5	530
FAS Assay Mix (20×)	0.5	53
Water	3.5	371
Total Reaction Mix Volume	9	954

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 96 reactions (plus 10% excess)
TaqMan [®] Gene Expression Master Mix (2X.)	5	530
HPRT Assay Mix (20×	0.5	53
Water	3.5	371
Total Reaction Mix Volume	9	954

- **4.** Mix the reaction in each tube by gently pipetting up and down, then cap each tube.
- 5. Centrifuge the tubes briefly to remove air bubbles.
- 6. Place the reaction mixes on ice until you prepare the reaction plate.Note: Do not add the sample or standard at this time.

Prepare the reaction plate

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

- A MicroAmp[®] Optical 384-Well Reaction Plate
- Reaction volume: 10 µL/well
- 96 Unknown wells U
- 80 Standard wells S
- 16 Negative control wells N
- 192 Empty wells

The following is an image of the plate layout for the example experiment:



QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments

To prepare the reaction plate components

- 1. For each target, prepare the negative control reactions:
 - **a.** To an appropriate volume 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	79.2	8.8
2	HPRT Reaction Mix	79.2	8.8

- **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)	Standard	Standard volume (µL)
1	FAS Std. 1	FAS Reaction Mix	79.2	FAS Std. 1	8.8
2	FAS Std. 2	FAS Reaction Mix	79.2	FAS Std. 2	8.8
3	FAS Std. 3	FAS Reaction Mix	79.2	FAS Std. 3	8.8
4	FAS Std. 4	FAS Reaction Mix	79.2	FAS Std. 4	8.8
5	FAS Std. 5	FAS Reaction Mix	79.2	FAS Std. 5	8.8
6	HPRT Std. 1	HPRT Reaction Mix	79.2	HPRT Std. 1	8.8
7	HPRT Std. 2	HPRT Reaction Mix	79.2	HPRT Std. 2	8.8
8	HPRT Std. 3	HPRT Reaction Mix	79.2	HPRT Std. 3	8.8
9	HPRT Std. 4	HPRT Reaction Mix	79.2	HPRT Std. 4	8.8
10	HPRT Std. 5	HPRT Reaction Mix	79.2	HPRT Std. 5	8.8

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)	Sample	Sample volume (µL)
1	FAS Kidney	FAS Reaction Mix	118.8	Kidney	13.2
2	FAS Heart	FAS Reaction Mix	118.8	Heart	13.2
3	FAS Liver	FAS Reaction Mix	118.8	Liver	13.2
4	FAS Brain	FAS Reaction Mix	118.8	Brain	13.2
5	HPRT Kidney	HPRT Reaction Mix	118.8	Kidney	13.2
6	HPRT Heart	HPRT Reaction Mix	118.8	Heart	13.2
7	HPRT Liver	HPRT Reaction Mix	118.8	Liver	13.2
8	HPRT Brain	HPRT Reaction Mix	118.8	Brain	13.2

- 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 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 TM 6 and 7 Flex Software.

For more information

For more information on	Refer to	Publication number
Assigning the reaction plate components	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and</i> 7 Flex Real-Time PCR System Software Experiments	4489822
Sealing the reaction plate	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and</i> 7 Flex Real-Time PCR System Software Experiments	4489822

Chapter 3 Prepare the Reactions *For more information*

3

This chapter explains how to run the example experiment on the QuantStudio ${}^{^{\rm TM}}$ 6 or 7 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 QuantStudioTM 6 or 7 Instrument is in operation.

Start the run

4

- 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[™] 6 and 7 Flex Real-Time PCR System Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software (to monitor an experiment started from another computer or from the QuantStudio[™] 6 or 7 Instrument touchscreen).
- From the QuantStudio[™] 6 or 7 Instrument touchscreen.

From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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[™] 6 and 7 Flex Real-Time PCR System 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 following is an image of 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 following is an image of the Temperature Plot screen as it appears during the example experiment.



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

View the Run Method

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

The following is an image of 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.

The following is an image of the View Run Data screen as it appears in the example experiment:

Run Data Report	
Experiment Name:	QS6_QuantStudio_384-Well_Relative_Standard_Curve_Example
Start Time:	12-10-2009 19:00:01 SGT
Stop Time:	12-10-2009 20:31:44 SGT
Run Duration:	91 minutes 42 seconds
User Name:	DEFAULT
Instrument Name:	
Firmware Version:	
Software Version:	N/A
Instrument Serial Number:	278880013
Sample Volume:	10.0
Cover Temperature:	105.0
Instrument Type:	
Block Type:	384-Well Block
Errors Encountered:	A 100 100 100 100 100 100 100 100 100 10

From the QuantStudio[™] 6 or 7 Instrument touchscreen

You can also view the progress of the run from the QuantStudio ${}^{\rm {\tiny TM}}$ 6 or 7 Instrument touchscreen.

The following are images of the Run Method screen on the QuantStudio TM 6 or 7 Instrument touchscreen:

Experiment view



Time view

S Time View Experiment View	
Run Started: July 06 2013 - 05:48PM Reaction Volume: 20 µL	Sample: 95.0 °C Heated Cover (Set Point): 105.0 °C (105.0 °C) Stage / Step / Cycle: 1 / 2 / 1
01.2	1.52
UIIJ	1:52
Remaining Tim	e 🔘 Elapsed Time
	July 06 2013 - 05:48PM Heated cover reached target temperature.



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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Assess amplification results using the Amplification Plot	42
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Adjust analysis settings	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 QuantStudioTM 6 and 7 Flex Real-Time PCR System Software calculates the quantity of an unknown target from the standard curve.

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

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

To view and assess the Standard Curve plot

Example

values

experiment standard curve

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

Note: If no data are displayed, click Analyze.

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

Menu	Selection
Target	All
Plot Color	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		

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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.

Ы	Plate La	ayout	Well Tab	ole													
	Show in T	ĩable ∀	Select Wel	ls ¥ Gi	roup By 🔻											🗄 Expand All	Collapse All
[#	Well	Omit	Flag	Samp	. Target Name	Task	Dyes A1		Ст		Ст Mean	CT SD	Quantity	Normalize	Normalize	Efficiency
	209 I	17			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.935			29.035	0.071	1,093.478		1.037	97.39 📥
	210 II	18			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.038			29.035	0.071	1,019.329		1.037	97.39
	211 II	19			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.009			29.035	0.071	1,039.701		1.037	97.39
	212 I	20			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.048			29.035	0.071	1,012.421		1.037	97.39
	213 I	21			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.073			29.035	0.071	995.587		1.037	97.39
	214 I	22			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.055			29.035	0.071	1,007.571		1.037	97.39
	215 I.	23			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.151			29.035	0.071	943.913		1.037	97.39
	216 I.	24			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.064			29.035	0.071	1,001.774		1.037	97.39
	229 J	13			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.973			29.035	0.071	1,065.29		1.037	97.39
	230 J	14			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.832			29.035	0.071	1,172.701		1.037	97.39
	231 J	15			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.955			29.035	0.071	1,078.69		1.037	97.39
	232 J	16			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	28.992			29.035	0.071	1,051.813		1.037	97.39
	233 J	17			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.029			29.035	0.071	1,025.679		1.037	97.39
	234 J	18			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.002			29.035	0.071	1,044.77		1.037	97.39
	235 J	19			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.044			29.035	0.071	1,015.042		1.037	97.39
	236 J.	20			1,000	RNase P	UNKNOWN	FAM-NFQ-MGB	29.04			29.035	0.071	1,018.366		1.037	97.39
	237 J.	21			1,000	RNase P	UNKNOWN	FAM-NEQ-MGB	29.009			29.035	0.071	1,039.876		1.037	97.39
	238 J	22			1,000	RNase P	UNKNOWN	FAM-NEQ-MGB	29.085			29.035	0.071	987.252		1.037	97.39
	239 J.	23			1,000	RNase P DNase D		FAM-NEQ-MGB	29.21			29.035	0.071	906.946		1.037	97.39
	240 J.	24			1,000	RNase P DNase D		FAMI-NEQ-MGB	29.071			29.035	0.071	990.01/		1.037	97.39
	203 K	.13			1,000	RNase P RNase D		FAMINEQ MGB	28,969			29,035	0.071	1,068.095		1.037	97.39
	204 K	15			1,000	RNase P RNase D		FAMINEQ-MGB	29.093			29,035	0.071	981.744		1.037	97.39
	200 K	15			1,000	RNase P RNase D		FAMINEQ-MGB	29.018			29,035	0.071	1,033.374		1.03/	97.39
	200 K	17			1,000	RNase P		EAM NEO MCB	20.904			29,035	0.071	1,037,409		1.03/	97.39
			•			Print and an and			- a riari		1	20 THE				1.1157	►
w	all Summ	aru	In I	Disto: 284		Set Liby 288	Analyzed: 29	9 Elagor	+ O	0m	itted by	Apabosis : O	Omitted M	anually: O	Samples I	ked: 4	Targets Lised: 2

9. Look at the values in the C_T column. In the example experiment, the C_T values fall within the expected range (>8 and <35).

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

- Slope/amplification efficiency values The amplification efficiency is calculated using the slope of the regression line in the standard curve. A slope close to –3.3 indicates optimal, 100% PCR amplification efficiency. Factors that affect amplification efficiency:
 - Range of standard quantities For more accurate and precise efficiency measurements, use a broad range of standard quantities, 5 to 6 logs (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 CT precision by omitting wells" on page 65). *Or*
- Rerun the experiment.

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. 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	 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 following is an image of the Plate Layout screen:

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	When you analyze your own Relative Standard Curve experiment, look for:
your own	• Outliers
experiments	• A typical amplification plot – The QuantStudio ^{TM} 6 and 7 Flex Real-Time PCI

- A typical amplification plot The QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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 following is an image of a typical amplification plot:



IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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.

Correct threshold values.

Threshold Set Correctly

1E01

The threshold is set in the exponential phase of the amplification curve.

Threshold settings above or below the optimum increase the standard deviation of the replicate groups.

Threshold Set Too Low

The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the exponential phase of the curve.

Threshold Set Too High

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





Correct baseline values

baseline.

Baseline Set Correctly 10 The amplification curve begins after the maximum 0.1 0.0 ΔRn 0.00 0.000 0.0000 0.00000 22 Cycle



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

- Omit wells (see "Improve CT precision by omitting wells" on page 65). *Or*
- Manually adjust the baseline and/or threshold (see "Adjust analysis settings" on page 61).

Assess the gene expression profile using the Gene Expression Plot

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

• **RQ vs Target** – Groups the relative quantification (RQ) values by target. Each sample is plotted for each target. You can view the plot as the linear, log10, Ln, and log2 graph types. The Gene Expression plot when viewed as a linear graph type looks like this:



RQ vs Sample - Groups the relative quantification (RQ) values by sample. Each target is plotted for each sample. You can view the plot as the linear, log10, Ln, and log2 graph types. The Gene Expression plot when viewed as a linear graph type looks like this:



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. experiment values

View the Gene Expression Plot

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 **E 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.

b
In the example experiment, the expression level of FAS in heart, kidney, and liver is displayed relative to its 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 in your own experiments When you analyze your own Relative Standard Curve experiment, look for differences in gene expression (as a fold change) relative to the reference sample.

Identify well problems using the Well Table

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

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

Example experiment values and flags	Review the Well Table to evaluate the $C_{\rm T}$ precision of the replicate groups.
View the well table	 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.

Z	Plate Layou	out	Well Ta	able										
	Show in Table	le 🔻	Select W	'ells 🔻	Group By 🔻								🗄 Expand All	🗏 Collapse All
1	# W	/ell	Omit	Flag	Sample Na	. Target Name	Task	Dyes	Ст	CT Mean	CT SD	Quantity Normal	liz Normaliz Ef	ficiency
			🗏 Brain -	- FAS - I	UNKNOWN									A
	205 I13				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.131	29.182	0.059	1,058.359	1.04	97.612
	206 I14				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.199	29.182	0.059	1,010.662	1.04	97.612 🚽
	207 I15				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.139	29.182	0.059	1,052.721	1.04	97.612
	208 I16				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.156	29.182	0.059	1,040.639	1.04	97.612
	209 I17				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.066	29.182	0.059	1,106.298	1.04	97.612
	210 I18				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.186	29.182	0.059	1,019.635	1.04	97.612
	211 I19				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.16	29.182	0.059	1,037.892	1.04	97.612
	212 I20				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.2	29.182	0.059	1,009.953	1.04	97.612
	213 I21				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.222	29.182	0.059	995.076	1.04	97.612
	214 I22				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.204	29.182	0.059	1,007.341	1.04	97.612
	215 I23				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.305	29.182	0.059	940.462	1.04	97.612
	216 I24				Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.217	29.182	0.059	998.492	1.04	97.612
			🗏 Brain -	- HPRT	- UNKNOWN									
	277 L13	}			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.252	30.438	0.205	475.135		87.858
	278 L14	ł			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.681	30.438	0.205	362.578		87.858
	279 L15	5			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.201	30.438	0.205	490.889		87.858
	280 L16)			Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.265	30.438	0.205	471.341		87.858
	281 L17				Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.352	30.438	0.205	446.114		87.858
	282 L18	3			Brain	HPRI	UNKNOWN	VIC-NEQ-MGB	30.232	30.438	0.205	481.19		87.858
	283 L19				Brain	HPRI	UNKNOWN	VIC-NEQ-MGB	30.746	30.438	0.205	348.027		87.858
	284 L2U	J			Brain	HPRI	UNKNOWN	VIC-NEQ-MGB	30.312	30.438	0.205	457.733		87.858
	285 L21				Brain	HPRI	UNKNOWN	VIC-NEQ-MGB	30.68	30.438	0.205	362.814		87.858
	286 L22	-			Brain	HPRI	UNKNOWN	VIC-NEQ-MGB	30.422	30.438	0.205	426,906		87.858
	287 T 28		4		Her Jir i	HURI	T INR INF HAIRI	on and blonge	an 200	211212	in ans			
We	ell Summary	y:	Ir	n Plate: 3	84 Set	Up: 192	Analyzed: 192	Flagged: ()		Omitted by Analysis: []	Omit	ted Manually: ()	Samples Used: 4	Targets 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 CT precision by omitting wells" on page 65).

Confirm accurate dye signal using the Multicomponent Plot

	The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.
Purpose	 In the Relative Standard Curve example experiment, you review the Multicomponent Plot screen for: ROX[™] dye (passive reference) FAM[™] dye (reporter for RNase P) VIC[®] dye (reporter for HPRT) 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. Display the unknown and standard wells one at a time 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 Show a legend for the plot (default). Note: This is a toggle button. When the legend is displayed, the button changes to With the plot default.
	ride die plot legend.

5. Check the FAM and VIC dye signals. In the example experiment, the FAM and VIC dyes signal increase throughout the PCR process, indicating normal amplification.



- **6.** Check the ROX dye signal. In the example experiment, the ROX dye signal remains constant throughout the PCR process indicating typical data.
- **7.** Select the negative control wells one at time and check for amplification. In the example experiment, there is no amplification in any of the negative control wells.



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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

below).

	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

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:

PCR F	ICR Filter — 🖉 🖉													
	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)							
-ilter	x1(470±15)	V												
	x2(520±10)		V											
ation	x3(550±11)			\checkmark										
Excit	x4(580±10)				\checkmark									
	x5(640±10)					\checkmark								
	x6(662±10)							=						
Melt I	Curve Filter ———													
				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)													
Filter	x2(520±10)													
ation	x3(550±11)													
Excita	x4(580±10)	x4(580±10)												
	x5(640±10)													
	x6(662±10)							~						

Tips for determining signal accuracy in your own experiment When you analyze your own Relative Standard Curve experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

View the endogenous control profile using the QC Plot

In the Relative Standard Curve experiment, the QC Plot screen displays the Endogenous Control Profile plot for all the targets in the experiment. The QC Plot serves as a tool to help users choose the best endogenous control for that experiment. The endogenous control profile plot is a visual display of the C_T 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 the 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.

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 HPRT.
 - 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 F 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. Note that the endogenous control, HPRT is expressed at the same level in all the four samples:

QC Plot							<	Target Table								
		æ				-	Я	Candidate Cor	ntrol	Tar	get		Color		Shape	
			<i>></i> 🖻		- "🛍		Π			FAS						
								V		HPRT				•	0	¥.
		Endogenous Control I	Profile													
30.0	•		•		•											
27.5					-											
25.0			_													
22.5																
20.0																
17.5							:									
년 15.0							:									
12.5																
10.0																
7.5																
5.0																
2.5																
0.0								View Replicate Re	sults Ta	ble View	Biological Gro	JDS				
	Brain	Heart	Kidney		Liver			Selected?		Comple	Tora	ot l	CT Meen		ACT Moon	
		Sample						Selectedr	Vidpor	o ample	EAC	et	CT Mean	017	12.00	
		- FAS - HPRT						V	Heart	y	FAS		20	525	12.38	6
									Liver		EAS		29	B.49	3.87	8
									Durin		540			100	0.07	<u>.</u>
Well Summ	arv: In Plate	: 384 Set Up: 192	An	abrzed: 192	F	lagged: (n -	Omitted by A	alvsis: I	n o	mitted Manualh	αO	Samples Use	d: 4	Targets U	sed: 2

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[™] 6 and 7 Flex Real-Time PCR System 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.

Summary				
lag Details				
Flag:	Description	Frequenc	cy Wells	5
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		
VOISE	Noise higher than others in plate	0		
6PIKE	Noise spikes	0		
VOSIGNAL	No signal in well	0		
DUTLIERRG	Outlier in replicate group	0		
XPFAIL	Exponential algorithm failed	0		
BI FATI	Baseline algorithm failed	0		-
'otal Wells: Vells Set Up:	384 Processed Wells: 192 Flagged Wells:	192 Manually Omitted Wells: 0 Analysis Omitted Wells:	0 Targets Used: 0 Samples Used:	2 4
Summary: In Plate:	384 Set Up: 192 Analyzed: 192	Flagged: 0 Omitted by Analysis: 0	Omitted Manually: [] Sample	s Used: 4 Targets

The following is an image of the QC Summary screen for the example experiment:

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

Flag	Description							
Pre-processing flag								
OFFSCALE	Fluorescence is offscale							
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							
Se	econdary analysis flags							
OUTLIERRG	Outlier in replicate group							
AMPNC	Amplification in negative control							
HIGHSD	High standard deviation in replicate group							



For more information

For more information on	Refer to	Publication number
Publishing data	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7</i> Flex Real-Time PCR System Software Experiments.	4489822

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[™] 6 and 7 Flex Real-Time PCR System 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 following is an image of the Analysis Settings dialog box for a Relative Standard Curve experiment:

Analysis Sett	ings for QS6_Q	JantStudio_384-W	/ell_Relative_	Standard_Curve_E	xample						
Ст Settings	Flag Settings	Relative Quantifica	tion Settings	Advanced Settings	Standard Curve Settings						
Review t Setting	he default settings f s, then change the	for analysis of targets in settings.	n this experiment.	To use different settin	gs for a target, select the targ	et from the table, deselect Use Default					
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. Baseline Threshold v Stage 2, Step 2 v Default CP Onthing and the setting and											
Default CT s	T Settings	calculate the CT for tar	aets without cust	om settings. To edit the	default settings, click Edit De	fault Settings					
Threshold	: AUTO Baseline	Start Cycle: AUTO	Baseline End C	vcle: AUTO Edit	Default Settings	CT Settings for FAS					
Target	Th	reshold	Baseline S	tart Base	ine End	r Settings to Use: 🗹 Default Settings					
FAS	AUT	го	AUTO	AUTO	<u>^</u>	Automatic Threshold					
HPRT	0.0	4	AUTO	AUTO		Threshold: 0.139964					
						✓ Automatic Baseline					
						Baseline Start Cycle: 3 🛊 End Cycle: 15 🛊					
auo to Libra	ru Lood fr	rom Libraru		Rouort	to Default Analycic Sett	inge Apply Applycic Sottinge Coper					
	Load II			<u>Revent</u>	to belaure Analysis Dett	Tappiy Analysis Sectings					

QuantStudio^M 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Relative Standard Curve and Comparative C_T 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*[™] 6 and 7 Flex Real-Time PCR System Software 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

Use the Baseline Threshold algorithm to determine the C_T values.

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

• 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[™] 6 and 7 Flex Real-Time PCR System Software.

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments

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 QS6_QuantStudio_384-Well_Relative_Standard_Curve_Example															
CT Settings Flag S	CT Settings Flag Settings Relative Quantification Settings Advanced Settings Standard Curve Settings														
Configure the flage	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	V	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	v	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														
Save to Library	Load from Library		<u>R</u> evert to Def	ault Analysis Settings	Apply Analysis	Settings Cancel									

The following is an image of the Flag Settings tab:

Relative Quantification Settings

Use the Relative Quantification Settings tab to:

- Change the type of analysis, singleplex or multiplex.
- Change the reference sample and/or endogenous control.

• Reject Outliers with ΔC_T values less than or equal to the entered value.

Note: The Outlier Rejection settings apply only to multiplex reactions.

- Select the algorithm to use to determine the relative quantification minimum and maximum values (error bars):
 - Confidence Level Select to calculate the RQ minimum and maximum values based on the selected confidence level. Select the confidence level to use.
 - Standard Deviations Select to calculate the RQ minimum and maximum values based on the selected number of standard deviations. Select the number of standard deviations to use.

Advanced Settings

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

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).

65

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

Σ	Plate L	ayout.	Well Ta	ble										
	Show in	Table 🔻	Select We	ells 🔻 Group By	•								🗄 Expand Al	I 🔲 Collapse All
1	#	Well	Omit	Flag Sample	Na Target Name	Task	Dyes	Ст	Ст Mean	CT SD	Quantity	Normaliz	Normaliz	Efficiency
ľ			🗉 Brain -	FAS - UNKNOW	V							,		A
	205 0	I13		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.131	29.182	0.059	1,058.359		1.04	97.612
	206 3	I14		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.199	29.182	0.059	1,010.662		1.04	97.612 🧮
	207 3	I15		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.139	29.182	0.059	1,052.721		1.04	97.612
	208	I16		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.156	29.182	0.059	1,040.639		1.04	97.612
	209 3	I17		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.066	29.182	0.059	1,106.298		1.04	97.612
	210	I18		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.186	29.182	0.059	1,019.635		1.04	97.612
- 1	211	I19		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.16	29.182	0.059	1,037.892		1.04	97.612
	212	I20		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.2	29.182	0.059	1,009.953		1.04	97.612
	213	I21		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.222	29.182	0.059	995.076		1.04	97.612
	214	I22		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.204	29.182	0.059	1,007.341		1.04	97.612
	215	I23		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.305	29.182	0.059	940.462		1.04	97.612
- 1	216	I24		Brain	FAS	UNKNOWN	FAM-NFQ-MGB	29.217	29.182	0.059	998.492		1.04	97.612
			🗏 Brain -	HPRT - UNKNOV	VN									
- 1	277	L13		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.252	30.438	0.205	475.135			87.858
- 1	278	L14		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.681	30.438	0.205	362.578			87.858
	279	L15		Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.201	30.438	0.205	490.889			87.858
	280 1	L16	닏	Brain	HPRT	UNKNOWN	VIC-NFQ-MGB	30.265	30.438	0.205	471.341			87.858
- 1	281	L1/		Brain	HPRI	UNKNOWN	VIC-NEQ-MGB	30,352	30,438	0.205	446.114			87.858
	282 1	L18		Brain	HPRI	UNKNOWN	VIC-NEQ-MGB	30,232	30,438	0.205	481.19			87.858
- 1	283	L19		Brain	HPKI		VIC-INEQ-MGB	30.746	30.438	0.205	348.027			87.858
	284 1	120		Brain	HPKI		VIC-NEQ-MGB	30.312	30,438	0.205	457.733			87.858
	285	122		Brain	HPKI		VIC-NEQ-MGB	30.68	30,438	0.205	302,814			87.858
	280	L22		Brain	HPKI		VIC-NEQ-MGB	30,422	30,438	0.205	420,900			87.858
	/8/1		1	er arri	HUNT	L INR DIE DAUM	OT INFLUMER	an 270	an 2139	0.205				
We	ell Sumn	nary:	In	Plate: 384	Set Up: 192	Analyzed: 192	Flagged: ()		Omitted by Analysis: ()	Omi	tted Manually: ()	Sam	ples Used: 4	Targets Used: 2

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	Publication number		
Amplification efficiency	Amplification Efficiency of TaqMan [®] Gene Expression Assays Application Note	127AP05-03		

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

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

3. Select **QuantStudio[™] 6 and 7** 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	QS6_QuantStudio_384- Well_Relative_Standard_Curve_Example_data
File Type	*.txt
Export File Location	C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\Export

Your Export screen should look like this:

Auto Export Fo	ormat : QuantStu	dio™ 6 and 7	✓ E	xport Data To: 💿 One File	🔿 Separate Files	🗹 Open f	file(s) when export is (complete
Export File Location: C:\Applie	ed Biosystems\Qi	uantStudio 6 and 7 Fle	x Softwar Browse	Export File Name: QS6_0	QuantStudio_384-\	Vell_Relative_Stand	da File Type: 📋 (*.t	txt) 🗸
Sample Setup	v Data 📝 Amp	lification 🗌 Multicomp	onent 🗌 Tech. Re	p. Results Bio. Rep.	. Results 🔽 Res	ults		
🖌 Skip Empty Wells 🛛 🖌 Skip	Omitted Wells							
Select Content		Wall	Il Position Som	ple Name Target Na	ma Task	Reporter	Quanchar	P
	~	vven vve	all FUSICIUM Sam	ple Name Target Na	ITTE TASK	Reporter	Quencher	
Airreids		1 A 1		FAS	NTC	FAM	NFQ-MGB	^
Vell		2 A2		FAS	NTC	FAM	NEQ-MGB	
		3 43		FAD	NTC	EAM	NEO MCR	
Well Position		4 A4		FAS	NTC	FAM	NEO MCB	
Consula Nama		5 A 5		FAD	NTC	EAM	NEO MCR	
Sample Name		7 47		FAS	NTC	FAM	NEO MCB	
Target Name		7 A7		FAD	NTC	EAM	NEO MCR	
in angee Name		8 A8		FAS	CTANDARD	FAM	NEQ-MGB	
 Task 		9 A9		FAD	STANDARD	EAM	NEO MCR	
		10 410		FAS	STANDARD	FAM	NEO MCB	
Reporter		12 412		FAD	STANDARD	EAM	NEO MCR	
El Ourante a		12 412		FAS	STANDARD	EAM	NEO MCB	
Quencher		13 A13		FAD	STANDARD	EAM	NEO MCR	
RO		19 419		FAS	STANDARD	EAM	NEO MCB	
		15 A15		FAD	STANDARD	EAM	NEO MCR	
RQ Min		17 417		FAS	STANDARD	EAM	NEO MCB	
		1/ A1/		FAD	STANDARD	EAM	NEO MCR	
		18 A18		FAS	STANDARD	FAP	INFQ-MGB	~
RQ Max	~	5 a 15						12410

QS6_QuantStudio_384-Well_Relative_Standard_Curve_Example_data.txt - Notepad	2
File Edit Format View Help	
<pre>* Block Type = 384-well Block Calibration Background is expired = Yes Calibration Background is expired = Yes Calibration Normalization FAM-ROX is expired = Yes Calibration Normalization FAM-ROX is expired = Yes Calibration Normalization YC-ROX performed on = 11-24-2009 Calibration Normalization YC-ROX performed on = 11-24-2009 Calibration Normalization YC-ROX performed on = 11-24-2009 Calibration Pure Dye FAM is expired = Yes Calibration Pure Dye MELTDOCTOR is expired = Yes Calibration Pure Dye MELTDOCTOR performed on = 12-07-2009 Calibration Pure Dye MELTDOCTOR performed on = 11-24-2009 Calibration Pure Dye ROX performed on = 11-24-2009 Calibration Pure Dye ROX performed on = 11-24-2009 Calibration Pure Dye ROX performed on = 11-24-2009 Calibration Pure Dye ROX performed on = 11-24-2009 Calibration Pure Dye ROX performed on = 11-24-2009 Calibration Pure Dye ROX performed on = 11-24-2009 Calibration Pure Dye ROX performed on = 11-24-2009 Calibration Pure Dye STR B Schred = Yes Calibration Pure Dye YCC sepformed on = 11-24-2009 Calibration Pure Dye YCC performed on = 11-24-2009 Calibration Pure Dye VCC sepformed on = 11-24-2009 Calibration ROI sepformed on = 11-24-2009 Calibration ROI performed on = 11-24-2009 Calibration Uniformity is expired = Yes Calibration Uniformity performed on = 11-24-2009 Calibration Uniformity is expired = Yes Calibration Uniformity is expired = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration Uniformity Sepfer = Yes Calibration UNIformity Sepfer = Yes Software YesFere = Yes Softwar</pre>	lex
Lampie Setup Sample Name Sample Color Biogroup Name Biogroup Reporter Reporter Quencher Quantity Comments RGB(255,0,0)" NTC F 1 A1 FAS RGB(255,0,0)" NTC F 2 A2 FAS RGB(255,0,0)" NTC F 3 A3 FAS RGB(255,0,0)" NTC F 4 A4 FAS RGB(255,0,0)" NTC F 5 A5 FAS RGB(255,0,0)" NTC F 6 A6 FAS RGB(255,0,0)" NTC F 6 A6 FAS RGB(255,0,0)" NTC F 7 A7 FAS RGB(255,0,0)" NTC F 6 A6 FAS RGB(255,0,0)" NTC F 7 A7 FAS RGB(255,0,0)" NTC F 7 A6 FAS RGB(255,0,0)" NTC F 8 <td>olor Target Name Target Color Task M NFQ-MGB M NFQ-MGB M NFQ-MGB M NFQ-MGB M NFQ-MGB M NFQ-MGB M NFQ-MGB M NFQ-MGB FAM NFQ-MGB</td>	olor Target Name Target Color Task M NFQ-MGB M NFQ-MGB M NFQ-MGB M NFQ-MGB M NFQ-MGB M NFQ-MGB M NFQ-MGB M NFQ-MGB FAM NFQ-MGB

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

PART 2 Running Comparative C_T Experiments

About Comparative C_T Experiments

This chapter covers:

IMPORTANT! First-time users of the QuantStudioTM 6 and 7 Flex Real-Time PCR System Software, please read Booklet 1, *Getting Started with QuantStudioTM 6 and 7 Flex Real-Time PCR System Software Experiments* and Booklet 7, *QuantStudioTM 6 and 7 Flex Real-Time PCR System Software 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 QuantStudioTM 6 and 7 Flex Software by pressing **F1**, clicking ? in the toolbar, or selecting **Help** \triangleright QuantStudioTM 6 and 7 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 6 and 7 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.

The Relative Standard Curve method determines the relative target quantity in samples. The QuanStudio[™] 6 and 7 Flex Software measures amplification of the target and of the endogenous control in samples, in a reference sample, and in a standard dilution series. For more information on Relative Standard Curve experiments, refer to Part I, Running Relative Standard Curve Experiments of this booklet.

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.

PCR options

- 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.

When performing real-time PCR, choose between:

- Singleplex and multiplex PCR (below) *and*
- 1-step and 2-step RT-PCR (page 72)

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.
- QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments

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 6 and 7 Flex Software.

The objective of the comparative C_T example experiment is to compare the expression of GH1, LPIN1, LIPC, GAPDH, and ACTB in liver, heart, brain, and lung tissues.

- The samples are liver, heart, lung, and brain tissues.
- The targets are GH1, LPIN1, LIPC, GAPDH, and ACTB.
- 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
 - GAPDH Assay Mix: Hs99999905_m1
 - ACTB Assay Mix: Hs99999903_m1



Chapter 7 About Comparative C_T Experiments *About the example experiment*

Design the Experiment

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

This chapter covers:

Define the experiment properties	75
Define targets, samples and biological replicates	76
Assign targets, samples and biological groups	78
Set up the run method	79
Tips for designing your own experiment	80
For more information	81

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 2 in Booklet 1, *Getting Started with QuantStudio*TM 6 and 7 Flex Real-Time PCR System Software Experiments.

Define the experiment properties

Click **Experiment Setup** \blacktriangleright **Experiment Properties** to create a new experiment in the QuantStudioTM 6 and 7 Flex Software. Enter:

Field or Selection	Entry
Experiment Name	QS6_QuantStudio_384-Well_Comparative_Ct_Example
Barcode	Leave field empty
User Name	Example User
Comments	Comparative $C_T (\Delta \Delta C_T)$ example
Instrument type	QuantStudio [™] 6 Flex System
Block	384-Well Block
Experiment Type	Comparative $C_T (\Delta \Delta C_T)$
Reagents	TaqMan [®] Reagents
Ramp speed	Fast
Reagent information	NA

Save the experiment.

Your Experiment Properties screen should look like this:



Define targets, samples and biological replicates

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

1. Targets

Target name	Reporter	Quencher	Color
ACTB	FAM	NFQ-MGB	
GAPDH	FAM	NFQ-MGB	
GH1	FAM	NFQ-MGB	
LPIN1	FAM	NFQ-MGB	
LIPC	FAM	NFQ-MGB	

2. Samples

Sample Name	Color
Brain	
Lung	
Liver	
Heart	

- **3.** Dye to be used as a Passive Reference ROX
- 4. Custom Task Name Not applicable
- 5. 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 De	lete		New Save to Library Import from Library Delete 🏹 Import from File			
Target Name	Reporter	Quencher	Color	Sample Name Color		
АСТВ	FAM 🗸	NFQ-MGB 🗸	· 📕 ·	Brain		
GAPDH	FAM 🗸	NFQ-MGB	· 🗾 🗸			
GH1	FAM 🗸	NFQ-MGB	· •			
LPIN1	FAM 🗸	NFQ-MGB	~	Heart		
LIPC	FAM 🗸	NFQ-MGB	· • •			
Biological Replicate Groups			Analysis Settings			
Biological Group Name Color		Comments				
			Reference Sample: Brain Endogenous Control: ACTB			
* Passive Reference				Custom Task Name		
ROX			New Delete			
			Name Color Icon Char			

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

Assign targets, samples and biological groups

Target name	Well number	Task	Sample name
ACTB	A1, A2, A3	Unknown	Brain
	C1, C2, C3	Unknown	Heart
	E1, E2, E3	Unknown	Lung
	G1, G2, G3	Unknown	Liver
	A24	Negative	None
GAPDH	A5, A6, A7	Unknown	Brain
	C5, C6, C7	Unknown	Heart
	E5, E6, E7	Unknown	Lung
	G5, G6, G7	Unknown	Liver
	C24	Negative	None
GH1	A9, A10, A11	Unknown	Brain
	C9, C10, C11	Unknown	Heart
	E9, E10, E11	Unknown	Lung
	G9, G10, G11	Unknown	Liver
	E24	Negative	None
LPIN1	A13, A14, A15	Unknown	Brain
	C13, C14, C15	Unknown	Heart
	E13, E14, E15	Unknown	Lung
	G13, G14, G15	Unknown	Liver
	G24	Negative	None
LIPC	A17, A18, A19	Unknown	Brain
	C17, C18, C19	Unknown	Heart
	E17, E18, E19	Unknown	Lung
	G17, G18, G19	Unknown	Liver
	I24 (Row I, Column 24)	Negative	None

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

Targets	₿ <		Plate La	ayout	W	'ell Tab	le																			
Name Task Custom Tasl	۹>																									
АСТВ Ц .	~	Ĭ	U) She	ow in W	(ells 🔻	Select	Wells	v 🖹	📰 View	/ Legen	d												1 .	W.		
GAPDH 🔽	~	Ē	1	2	3	4	5	6	7		0	10	11	12	13	14	15	16	17	19	10	20	21	22	23	24
GH1 🔽	~		- I	- C	Brain	T	нгаю	нгаю	на	0	2 HCDID	HERRIN	HIRD	12	1.5	HCBID	HERRIN	10	HCBID	HERRIN	HC2ID	20	21	22	2.5	24
LPIN1 V	~	A	•	-	-								-			and a	100		-	-	-					18
	~	в																								
		с							HADIT		Haarr	Haarr	Haarr		HADIT INN		Hearr Intel									N
Samples		D																								
Name		Е			en o		Har	Har	Har		Han	H	eer o						H	Han	H					N
✓ Brain		F																								
🗌 🔤 Lung		G			INA		Inter	INP	i war		i war	INA	inter		Liver	LINE"			- War		- war					13
Liver	-		-	-	-		-	-	-		-	-	-		-	-	-		-	-	-					
Heart		l"																								
		I																								N
		J																								
Piological Groupe		к																								
	Ň	L																								
		-																								
		P1																								
		Ν																								
		0																								
		Р																								
		w	/ells:	60	N 5																				31	9 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: 10 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	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:



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	Publication number
Consumables	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Experiments</i>	4489822
	Appendix A in Booklet 7, <i>QuantStudio™ 6 and 7 Flex Real-</i> <i>Time PCR System Software Experiments - Appendixes</i>	
Using the Standard Curve quantification methods	Booklet 2, Running Standard Curve Experiments.	4489822
Using the relative standard curve quantification method	Part 1 of this booklet	4489822
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 3 in Booklet 1, <i>Getting Started with QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Experiments</i>	4489822



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	83
Prepare the template	83
Prepare the sample dilutions	84
Prepare the reaction mix ("cocktail mix")	84
Prepare the reaction plate	85
Tips for preparing reactions for your own experiments	86
For more information	87

Assemble required materials

- Items listed in Booklet 1, *Getting Started with QuantStudio*[™] 6 and 7 Flex Real-Time PCR System Software 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)
 - GAPDH Assay Mix (20X)
 - GH1 Assay Mix (20X)
 - LIPN1 Assay Mix (20X)
 - LIPC Assay Mix (20X)

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

Prepare the template

	Prepare the template for the PCR reactions using the High-Capacity cDNA Reverse Transcription Kit or one of the Invitrogen VILO kits to carry out the reverse transcription.
Example experiment settings	For the Comparative C_T example experiment, the template for the PCR reactions is cDNA reverse-transcribed from total RNA samples using one of the Invitrogen VILO kits, SuperScript [®] VILO TM cDNA Synthesis Kit (Part no. 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* (Pub. no. 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
 - GAPDH Reaction Mix
 - GH1 Reaction Mix

- LPIN1 Reaction Mix
- LIPC 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 13 reactions (plus 10% excess)
TaqMan® Fast Universal PCR Master Mix (2X)	5.0	75.0
ACTB Assay Mix (20×)	0.5	7.5
Water	3.5	52.5
Total Reaction Mix Volume	9.0	135.0

- **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 GAPDH, GH1, LPIN1, and LIPC assays.Note: Do not add the sample at this time.

Prepare the reaction plate

Example experiment reaction plate components

- The reaction plate for the Comparative C_T example experiment contains:
 - A MicroAmp[®] Optical 384-Well Reaction Plate
 - Reaction volume: 10µL/well
 - The reaction plate contains:
 - 60 Unknown wells 🙂
 - 5 Negative control wells
 - 319 Empty wells

	Plate	Layout	vve	ell l'able																					
	🛄 sł	now in W	'ells 🔻	Select Wel	lls 🔻 🚦	📰 View Leg	jend																٠		X
	1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
4	U	AC	AC	U AC		U GA	U GA	U GA		U GH1	U GH1	U GH1		U LPI	U LPI	U LPI		U LIPC		U LIPC					N AC
В																									
	U	AC	AC	U AC		U GA	U GA	U GA		U GH1	U GH1	U GH1		U LPI	U LPI	U LPI									N GA
C																									
E	U	AC	AC	U AC		U GA	U GA	U GA		U GH1	U GH1	U GH1		U LPI	U LPI	U LPI		U LIPC		U LIPC					N GH1
F																									
:	U	AC	AC	U AC		U GA	U GA	U GA		U GH1	\rm GH1	U GH1		U LPI	U LPI	U LPI				U LIPC					N LPI
ŀ																									
I																									N LIPC
J																									
к																									
L																									
M																									
Ν																									
c																									
Р																									
,	Vells:	rels: 🗓 60 🖾 5 319 Empty																							

The following is an image of the plate layout for the example experiment:

To prepare the reaction plate components

- 1. Add 1 μ L of each cDNA to the appropriate wells.
- 2. Pipet 1 μ L of sterile water into the NTC wells.
- 3. Add 8 µL of the appropriate assay-specific cocktail to the wells.
- 4. Seal the reaction plate with optical adhesive film.
- 5. Centrifuge the reaction plate briefly to remove air bubbles.
- **6.** Confirm that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.
- 7. Until you are ready to perform the run, place the reaction plate at 4°C, in the dark.

Tips for preparing reactions for your own experiments

Tips for preparing templates	When you prepare your own Comparative C_T experiment, Life Technologies recommends the following templates:
	• Complementary DNA (cDNA) – cDNA reverse-transcribed from total RNA samples.
	 Genomic DNA (gDNA) – Purified gDNA already extracted from tissue or sample.
Tips for preparing the reaction mix	If your experiment includes more than one target assay, prepare the reaction mix for each target assay separately.
Tips for preparing the reaction plate

When you prepare your own Comparative C_T experiment, make sure the arrangement of the PCR reactions matches the plate layout displayed in the QuantStudioTM 6 and 7 Flex Real-Time PCR System Software.

For more information

For more information on	Refer to	Publication number
Assigning the reaction plate components	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7 Flex</i> Real-Time PCR System Software Experiments	4489822
Sealing the reaction plate	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio[™] 6 and 7 Flex</i> <i>Real-Time PCR System Software Experiments</i>	4489822



Chapter 9 Prepare the Reactions *For more information*

Run the Experiment

This chapter explains how to run the example experiment on the QuantStudio ${}^{\rm TM}$ 6 or 7 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 QuantStudioTM 6 or 7 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[™] 6 and 7 Flex Real-Time PCR System Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software (to monitor an experiment started from another computer or from the QuantStudio[™] 6 or 7 Instrument touchscreen).
- From the QuantStudio[™] 6 or 7 Instrument touchscreen.



From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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 6 and 7 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 following is an image of 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.

Current Temperatures Temperature Plot Cover Ð P h 8 i) ~ 🗹 📕 Sample **Temperature Plot** Block 110 100 90 80 70 Temperature 60 50 40 30 20 10 Temperature Plot 00:00:00 00:04:10 00:08:20 00:12:30 00:16:40 00:20:50 00:25:00 00:29:10 00:33:20 00:37:30 00:41:40 00:45:50 00:50:00 00:54:10 00:58:20 1 Hour View Ŧ Time Fixed View 🗖 1

The following is an image of 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 following is an image of the Run Method screen as it appears in the example experiment.

Run Method				Edit Run Method
Hold Stage	PCR S	tage		Adjust # of Cycles:
95.0 °C 00:20 1.9 °C/s	Number of Cycle Enable . Starting Cycle: 95.0 °C 1.9 °C/s 00:01	s: 40 ⇒ AutoDelta 1 ⇒ 1.6 °C/s 60.0 °C 00;20 €€€		Add Melt Curve Stage to End Add Holding Stage to End Add Infinite Hold to End <u>Send to Instrument</u>
Step1	Step1	Step2	_	
✓ Legend — Legend — Legend — Legend — Legend — Legend — Legend — Legend — Legend — Legend — Legend → Legend				
🔞 Data Collection On 📧 Data	a Collection Off 🔺 AutoDelta On	🔺 AutoDelta Off		

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments



View Run Data

Click View Run Data from the Run Experiment Menu.

The following is an image of the View Run Data screen as it appears in the example experiment:

Run Data Report	
Experiment Name:	QS6_QuantStudio_384-Well_Comparative_Ct_Example
Start Time:	10-02-2010 08:38:10 SGT
Stop Time:	10-02-2010 09:20:04 SGT
Run Duration:	41 minutes 53 seconds
User Name:	DEFAULT
Instrument Name:	Yankees_Rule
Firmware Version:	1.0.3
Software Version:	QuantStudio Software v1.0
Instrument Serial Number:	278880012
Sample Volume:	10.0
Cover Temperature:	105.0
Instrument Type:	
Block Type:	384-Well Block
Errors Encountered:	
	v

From the QuantStudio[™] 6 or 7 Instrument touchscreen You can also view the progress of the run from the QuantStudio ${}^{\rm TM}$ 6 or 7 Instrument touchscreen.

The following are images of the Run Method screen on the QuantStudioTM 6 or 7 Instrument touchscreen:

Experiment view



Time View

0	Time View	t#t	Experiment View							
F React	Run Started: July 0 ion Volume: 20 μL	6 2013	- 05:48PM	Sample: 95.0 °C Heated Cover (Set Point): 105.0 °C (105.0 °C) Stage / Step / Cycle: 1 / 2 / 1						
	Λ	1	.2	1.52						
	U	1	10	IJZ						
	Remaining Time Elapsed Time									
				July 06 2013 - 05:48PM						
				Heated cover reached target temperature.						



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:

Section 11.1 Review Results
Analyze the example experiment
Assess the gene expression profile using the Gene Expression Plot
Identify well problems using the Well Table
Assess amplification results using the Amplification Plot 101
Confirm accurate dye signal using the Multicomponent Plot 108
Determine signal accuracy using the Raw Data Plot 110
View the endogenous control profile using the QC Plot 112
Review the flags in the QC Summary 113
For more information 115
Section 11.2 Adjust parameters for re-analysis of your own experiments 117
Adjust analysis settings 117
Improve CT precision by omitting wells 120



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:

Gene Exp	ression															
Plot Set	tings															
Plot Type	RQ vs Target	Graph Type:	Linear 🔽 Orientat	<mark>ion:</mark> Vertical Ba	ars 💌											
Save o	current settings as	the default														
											Æ		A 🔖	2		
						R	Q vs Ta	arget			-	-	_			
650																
600																
550									53	0.871						
500																
450																
400																
C 350																1
02 300																
250																
200																
150																
100																
50																
0	1	2.232 1	.251 0.174	1	1.053	0.181	0.612	1	0.501	0.501	1	0.466	0.663	0.3		
		GAPON			0HN				°°	9°. 3 ^{%*}						
							Та	rget								
Legend																1
Brain	Heart Live	r Lung														
]
Well Summa	ary:	In Plate: 384	Set Up: 65	Analy	/zed: 65	F	lagged: 3	Omitte	ed by Analysis: ()	Omitted Manua	ally: O	Samp	les Used: 4		Targets Us	ed: 5



• **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 **E** Show a legend for the plot (default).

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

In the example experiment, as shown below, the expression level of each target gene in liver, heart, and lung is displayed relative to its respective expression level in the reference sample (brain). Since the reference sample is compared to itself, the relative expression level is 1. When the result is displayed in the Log10 graph type, the expression level of the reference sample appears as 0 in the graph (log10 of 1 = 0).



Assessing the gene expression plot your own experiments When you analyze your own Comparative C_T experiment, look for differences in gene expression (as a fold change) relative to the reference sample.

Identify well problems using the Well Table

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

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

Example experiment values and flags	Review the Well Table to evaluate the C_T precision of the replicate groups.
View the well table	 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 three outliers. You will omit these wells in the troubleshooting section ("Improve CT precision by omitting wells" on page 120).

 Plate Layout 	Well Table													
Show in Table 🔻	Select Wells 🔽	Group by 🔻											Expand 4	II 🗖 Colleone A
	Select web V	aroop by 🖡			_								C Caponari	
# Well	Omit	Flag Sa	mple Target	t Task	Dyes	Ст	Ст Mean	CT SD	ΔCT ΔCT Ι	Ие ∆Ст SE	ΔΔCτ	RQ	RQ	Min RQ M
153 69	Flagged We	ells A Live	r GH1	LINKNOW/N	EAM-NEO	36.234	35.576	0.642	1	6.385 0.	374 2	2.467	0.181	0.088
154 G10		Live	r GH1	UNKNOWN	FAM-NFQ	34.951	35.576	0.642	1	6.385 0.	374 2	2.467	0.181	0.088
155 G11		🔒 Live	r GH1	UNKNOWN	FAM-NFQ	35.543	35.576	0.642	1	6.385 0.	374 2	2.467	0.181	0.088
	🗏 Unflagged \	Wells												
1 A1		Brai	n ACTB	UNKNOWN	FAM-NFQ	17.339	17.281	0.050						
2 A2		Brai	n ACTB	UNKNOWN	FAM-NFQ	17.245	17.281	0.050						
3 A3		Brai	n ACIB	UNKNOWN	FAM-NEQ	17.260	17.281	0.050						
5 A5		Brai	n GAPDH	UNKNOWN	FAM-NEO	17.627	17.631	0.010		0.349 0.	030 (0.000	1.000	0.945
6 A6		Brai	n GAPDH	UNKNOWN	FAM-NFQ	17.622	17.631	0.010		0.349 0.	030 (0.000	1.000	0.945
7 A7		Brai	n GAPDH	UNKNOWN	FAM-NFQ	17.642	17.631	0.010		0.349 0.	030 (0.000	1.000	0.945
8 A8														
9 A9		Brai	n GH1	UNKNOWN	FAM-NFQ	31.215	31.200	0.211	1	3.919 0.	125 (0.000	1.000	0.786
10 A10		Brai	n GH1	UNKNOWN	FAM-NFQ	31.403	31.200	0.211	1	3.919 0.	125 (000.0	1.000	0.786
11 A11		Brai	n GH1	UNKNOWN	FAM-NFQ	30.982	31.200	0.211	1	3.919 0.	125 ().000	1.000	0.786
12 A12		Brai	n I DTN1	LINKNOW/N	EAM-NEO-	21 765	21.810	0.047		4 537 0	040 (000	1.000	0.926
13 A13	H	Brai	n IPIN1	UNKNOWN	FAM-NEO	21.839	21.819	0.047		4.537 0.	040 (0.000	1.000	0.926
15 A15		Brai	n LPIN1	UNKNOWN	FAM-NFQ	21.853	21.819	0.047		4.537 0.	040 (0.000	1.000	0.926
16 A16														
17 A17		Brai	n LIPC	UNKNOWN	FAM-NFQ	29.293	29.235	0.053	1	1.953 0.	042 (0.000	1.000	0.922
18 A18		Brai	n LIPC	UNKNOWN	FAM-NFQ	29.224	29.235	0.053	1	1.953 0.	042 (J.000	1.000	0.922
19 A19		Brai	n LIPC	UNKNOWN	FAM-NFQ	29.188	29.235	0.053	1	1.953 0.	042 ().000	1.000	0.922
20 A20														
22 A22														
23 A23														
24 A24			ACTB	NTC	FAM-NFQ	Undetermi								
25 B1														1
	<													>
Well Summary:	Ini	Plate: 384	Set Up: 65	Analyz	ed: 65	Flagged: 3		Omitted by Analy	ysis: () C	mitted Manually: ()		Samples Us	ed: 4	Targets 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 CT precision by omitting wells" on page 120).

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 .										



P	ate Lay	out	well Table																					
Ŭ	5 Show	in Wells 🏹	Select W	ells 🔻 🚦	🔡 View Le	gend																	10	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
Α	U AC.	. 🕕 АС	🚺 AC		U GA	. U GA	U GA		U GH1	U GH1	U GH1)		U LPI	U LPI	U LPI		U LIPC	U LIPC	U LIPC					N AC.
в																								
с	U AC.	. 🔲 АС	🔟 AC		U GA	. U GA	U GA		U GH1	U GH1	U GH1;		LPI	U LPI	U LPI									N GA.
D																								
Е	U AC.	U AC	🔟 AC		U GA	U GA	U GA		U GH1	U GH1	U GH1;		U LPI	U LPI	U LPI		U LIPC	U LIPC	U LIPC					N GH1
F																								
G	U AC.	U AC	U AC		U GA	. U GA	U GA		1	1	1		U LPI	U LPI	U LPI		U LIPC	U LIPC	U LIPC					N LPI.
н																								
Ι																								
J																								
к																								
L																								
м																								
N																								
0																								
Р																								
We	ells: 🚺	60 🖪 5	;																				3:	19 Empty
/ell S	umma	ry:	In	Plate: 384	ł	Set Up	: 65		Analyzed: 6	5	Flagg	ged: 3		Omitted by	Analysis: ()		Omitte	ed Manually	:0	Sa	mples Used	:4	Та	argets Used

The following is an image of the Plate Layout screen:

3. In the Amplification Plot screen, enter:

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

- **4.** View the baseline values:
 - a. From the Graph Type drop-down menu, select Linear.
 - **b.** Select the **Baseline** check box to show the start cycle and end cycle.

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



5. View the threshold values:

Menu	Select
Graph Type	Log
Target	LP1N1

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



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



- **6.** Locate any outliers:
 - **a**. From the Plot Type drop-down menu, select **C**_T **vs Well**.
 - **b.** Look for outliers from the amplification plot. In the example experiment, there are no outliers for LP1N1.



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- 11
- **7.** Repeat steps 2 through 6 for the GH1, TGFB1, LIPC, GAPDH, 18S, and ACTB wells. In the example experiment, there is one outlier for 18S. You will omit these wells in the troubleshooting section ("Improve CT precision by omitting wells" on page 120).

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

• Outliers

Tips for analyzing

your own

experiments

- A typical amplification plot The QuantStudio[™] 6 and 7 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[™] 6 and 7 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.

• Correct threshold values



• Correct baseline values



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

- Omit wells (see "Improve CT precision by omitting wells" on page 120). *Or*
- Manually adjust the baseline and/or threshold (see "Adjust analysis settings" on page 117).

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11



Confirm accurate dye signal using the Multicomponent Plot

	The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.
Purpose	 In the Comparative C_T example experiment, you review the Multicomponent Plot screen for: ROX[™] dye (passive reference) FAM[™] dye (reporter) Spikes, dips, and/or sudden changes
	Amplification in the negative control wells
View the Multicomponent Plot	 From the Experiment Menu pane, select Analysis ➤ Multicomponent Plot. Note: If no data are displayed, click Analyze. Display the unknown and standard wells one at a time 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.

4. Click **Figure 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 When you analyze your own Comparative C_T experiment, look for: dye accuracy in **Passive reference** – The passive reference dye fluorescence level should remain your own relatively constant throughout the PCR process. experiment **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. In the Comparative C_T example experiment, you review the Raw Data Plot screen for a About the example stable increase in signal (no abrupt changes or dips) from the appropriate filter. experiment View the Raw Data From the Experiment Menu pane, select Analysis > Raw Data Plot. 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 **Fraction 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

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

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



The filters used for the example experiment are:

PCR I	Filter —						(<u>a</u>					
	Load Save Revent 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)	\checkmark											
Filter	x2(520±10)		\checkmark										
ation	x3(550±11)			\checkmark									
Excita	x4(580±10)												
	x5(640±10)					\checkmark							
	x6(662±10)												
Melt	Curve Filter ———			Load Save Revertion	o Defaults			A					
				Emission Filter									
		m1(520±15)	m2(558±11)	m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)						
	x1(470±15)												
Filter	x2(520±10)												
ation	x3(550±11)												
Excita	x4(580±10)												
	x5(640±10)												
	x6(662±10)												

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

• Characteristic signal growth

accuracy in your own experiment

determining signal

Tips for

• 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 ACTB, GAPDH, GH1, and LPIN1 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.
	2. In the QC Plot screen, click Target Table .
	a. In the Candidate Control column, select the check box of the target of the endogenous control profile to plot. In the example experiment, the endogenous controls selected are ACTB, GAPDH, GH1, and LPIN1.
	b. Select a color for each target, from the Color drop-down menu.
	c. Select a shape for each target, from the Shape drop-down menu.
	3. Click the View Replicate Results Table.
	 Select the check box of the samples to plot. In the example experiment, all the four samples, Brain, Heart, Liver, and Lung are selected.
	5. Click 🧮 Show a legend for the plot (default).
	Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.



The following is an image of the QC Plot in the Comparative C_T example experiment:

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[™] 6 and 7 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 G9, G10, and G11 have data that triggered the HIGHSD 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 seven flagged wells.

3. In the Flag Details table, click each flag with a frequency >0 to display detailed information about the flag. In the example experiment. The HIGHSD flag appears three times, in the wells G9, G10, and G11, indicating high standard deviation in the replicate group.

Note: The HIGHSD flag appears because the C_T values exceed the expected range due to low expression of the GH1 gene in the Liver sample.

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

The following is an image of the QC Summary for the example experiment:

Summary						
ilag Details						
Flag:	Description		Frequency	Wells		
MPNC	Amplification in negative cont	trol	0			
ADROX	Bad passive reference signal		0			
FFSCALE	Fluorescence is offscale		0			
GHSD	High standard deviation in re	plicate group	3	G9, G10, G11		
AMP	No amplification		0			
ISE	Noise higher than others in p	late	0			
IKE	Noise spikes		0			
SIGNAL	No signal in well		0			
TLIERRG	Outlier in replicate group		0			
PFAIL	Exponential algorithm failed		0			
FAIL	Baseline algorithm failed		0			
OLDFAIL	Thresholding algorithm failed		0			
EATI	CT algorithm failed		0			
Flag Criteria: CT standard of Flagged Wells: G9 G10 G11	deviation > 0.5					
View HIGHSD	Troubleshooting Information					
ital Wells: ells Set Up:	384 Processed Wells: 65 Flagged Wells:	65 Mar 3 Ana	ually Omitted Wells: lysis Omitted Wells:	0 Targets U 0 Samples L	lsed: Jsed:	
1- Dista 294	5-11-55 A	where the first of the sector	Ourillard has Applying 0	Caribad Manasha 0	Secolar Useds 4	-

Possible flags

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

Flag	Description					
	Pre-processing flag					
OFFSCALE	Fluorescence is offscale					
Primary analysis flags						
BADROX	Bad passive reference signal					
NOAMP	No amplification					
NOISE	Noise higher than others in plate					
SPIKE	Noise spikes					
NOSIGNAL	No signal in well					
EXPFAIL	Exponential algorithm failed					
BLFAIL	Baseline algorithm failed					
THOLDFAIL	Thresholding algorithm failed					



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Flag	Description					
CTFAIL	C _T algorithm failed					
Secondary analysis flags						
OUTLIERRG	Outlier in replicate group					
AMPNC	Amplification in negative control					
HIGHSD	High standard deviation in replicate group					

For more information

For more information on	Refer to	Publication number
Publishing data	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and</i> 7 Flex Real-Time PCR System Software Experiment	4489822



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[™] 6 and 7 Flex Software are not suitable for your own experiment, you can change the settings in the Analysis Settings dialog box, then reanalyze your experiment.

View the analysis settings

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

The following is an image of the Analysis Settings dialog box for a Comparative C_T experiment:

	for QS6_QuantStudio_38	4-Well_Comparative_Ct_	Example	
CT Settings Fl	ag Settings Relative Quan	tification Settings Advance	d Settings	
Review the de Settings, the	fault settings for analysis of targ n change the settings.	ets in this experiment. To use di	ferent settings for a target,	select the target from the table, deselect Use Default
Data Step Sel Select the step ar analysis have bee Stage 2, Step	ection d stage to use for Cτ analysis. O n collected are displayed. 2 Υ	Only stage/step combinations for	which data suitable for CT	Algorithm Settings Baseline Threshold
Default CT Se	ttings			
Threshold: AU	TO Baseline Start Cycle: AU	TO Baseline End Cycle: AUT	0 Edit Default Sett	tings
Select a Targe	et			CT Settings for ACTB
Target	Thrashald	Deceline Start	Deceline Fed	
	Threshold	baseline Start	baseline Enu	CT Settings to Use: 🔽 Default Settings
АСТВ	AUTO	AUTO	AUTO	CT Settings to Use: ☑ Default Settings ☑ Automatic Threshold
ACTB GAPDH	AUTO	AUTO AUTO	AUTO AUTO	CT Settings to Use: Default Settings Automatic Threshold Threshold: 0.056851
ACTB GAPDH GH1	AUTO AUTO AUTO	AUTO AUTO AUTO	AUTO AUTO AUTO	Cr Settings to Use: Default Settings Automatic Threshold Threshold: 0.056851 Automatic Baseline
ACTB GAPDH GH1 LIPC	AUTO AUTO AUTO AUTO AUTO	AUTO AUTO AUTO AUTO AUTO	AUTO AUTO AUTO AUTO AUTO	Cr Settings to Use: Default Settings Automatic Threshold Threshold: 0.056851 Automatic Baseline Baseline Start Cycle: 30 End Cycle: 150
ACTB GAPDH GH1 LIPC LPIN1	AUTO AUTO AUTO AUTO AUTO AUTO AUTO AUTO	AUTO AUTO AUTO AUTO AUTO AUTO AUTO AUTO AUTO	AUTO AUTO AUTO AUTO AUTO AUTO	Cr Settings to Use: Default Settings Automatic Threshold Threshold: 0.056851 Automatic Baseline Baseline Start Cycle: 3 End Cycle: 15
ACTB GAPDH GH1 LIPC LPIN1	AUTO AUTO AUTO AUTO AUTO AUTO AUTO AUTO	AUTO AUTO AUTO AUTO AUTO AUTO AUTO AUTO AUTO	AUTO AUTO AUTO AUTO AUTO AUTO	Cr Settings to Use: Default Settings Automatic Threshold Threshold: 0.056851 Automatic Baseline Baseline Start Cycle: 3\$ End Cycle: 15\$
ACTB GAPDH GH1 LIPC LPIN1	AUTO AUTO AUTO AUTO AUTO AUTO	AUTO AUTO AUTO AUTO AUTO AUTO	AUTO AUTO AUTO AUTO AUTO AUTO	Cr Settings to Use: Default Settings Automatic Threshold Threshold: 0.056851 Automatic Baseline Baseline Start Cycle: 3 the End Cycle: 15 the



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*[™] 6 and 7 Flex Real-Time PCR System Software 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

Use the Baseline Threshold algorithm to determine the C_T values.

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

• 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[™] 6 and 7 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	Lico	Attributo	Condition	Value	Reject Well
MPNC	Amplification in negativ	✓	Ст	< *	35.000	
ADROX	Bad passive reference	v	Bad passive reference	> *	0.600	
FFSCALE	Fluorescence is offscale	V				
IGHSD	High standard deviatio	V	CT standard deviation	> *	0.500	
OAMP	No amplification	v	Amplification algorithm	< *	0.100	
OISE	Noise higher than oth	v	Relative noise	> *	4.000	
PIKE	Noise spikes	V	Spike algorithm result	>	1.000	
OSIGNAL	No signal in well	V				
UTLIERRG	Outlier in replicate group	V				
XPFAIL	Exponential algorithm f	V				
LFAIL	Baseline algorithm failed	~				
HOLDFAIL	Thresholding algorithm	v				
TFAIL	CT algorithm failed	v				

The following is an image of the Flag Settings tab:

Relative Quantification Settings

Use the Relative Quantification Settings tab to:

- Change the type of analysis, singleplex or multiplex.
- Change the reference sample and/or endogenous control.

• Reject Outliers with ΔC_T values less than or equal to the entered value.

Note: The Outlier Rejection settings apply only to multiplex reactions.

- Select the algorithm to use to determine the relative quantification minimum and maximum values (error bars):
 - Confidence Level Select to calculate the RQ minimum and maximum values based on the selected confidence level. Select the confidence level to use.
 - Standard Deviations Select to calculate the RQ minimum and maximum values based on the selected number of standard deviations. Select the number of standard deviations to use.

Advanced Settings

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

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

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

Improve C_T precision by omitting wells

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

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

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

Note: If no data are displayed, click Analyze.

- 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 G9, G10, and G11 have outliers.

4	Plate Layout	Well Table															_
	Show in Table	/ Select Wells V	Group by V	·											🗄 Exp	and All 📃 C	ollapse All
	# Well	Omit	Flag	Sample	Target	Task	Dyes	Ст	Ст Mean	CT SD	ΔCτ	∆Ст Ме	ΔCT SE	ΔΔCτ	RQ	RQ Min	RQ M
	149 G5			Liver	GAPDH	UNKNOWN	FAM-NFQ	19.113	19.217	0.092		0.026	0.071	-0.323	1.251	1.091	^
	150 G6			Liver	GAPDH	UNKNOWN	FAM-NFQ	19.250	19.217	0.092		0.026	0.071	-0.323	1.251	1.091	
	151 G7			Liver	GAPDH	UNKNOWN	FAM-NFQ	19.288	19.217	0.092		0.026	0.071	-0.323	1.251	1.091	
		🗏 Liver - GH1															
	153 G9		1	Liver	GH1	UNKNOWN	FAM-NFQ	36.234	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
	154 G10		1	Liver	GH1	UNKNOWN	FAM-NFQ	34.951	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
	155 G11		1	Liver	GH1	UNKNOWN	FAM-NFQ	35.543	35.576	0.642		16.385	0.374	2.467	0.181	0.088	
		🗏 Liver - LIPC															
	161 G17			Liver	LIPC	UNKNOWN	FAM-NFQ	21.973	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6
	162 G18			Liver	LIPC	UNKNOWN	FAM-NFQ	22.181	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6:
	163 G19			Liver	LIPC	UNKNOWN	FAM-NFQ	22.122	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6:
		🗏 Liver - LPIN	11														
	157 G13			Liver	LPIN1	UNKNOWN	FAM-NFQ	24.180	24.322	0.127		5.131	0.087	0.593	0.663	0.560	
	158 G14			Liver	LPIN1	UNKNOWN	FAM-NFQ	24.361	24.322	0.127		5.131	0.087	0.593	0.663	0.560	
	159 G15			Liver	LPIN1	UNKNOWN	FAM-NFQ	24.424	24.322	0.127		5.131	0.087	0.593	0.663	0.560	
		🗏 Lung - ACT	ГВ														
	97 E1			Lung	ACTB	UNKNOWN	FAM-NFQ	18.151	18.210	0.064							
	98 E2			Lung	ACTB	UNKNOWN	FAM-NFQ	18.202	18.210	0.064							
	99 E3			Lung	ACTB	UNKNOWN	FAM-NFQ	18.278	18.210	0.064							
		🗏 Lung - GAP	PDH														
	101 E5			Lung	GAPDH	UNKNOWN	FAM-NFQ	21.082	21.084	0.065		2.874	0.053	2.525	0.174	0.157	
	102 E6			Lung	GAPDH	UNKNOWN	FAM-NFQ	21.020	21.084	0.065		2.874	0.053	2.525	0.174	0.157	
	103 E7			Lung	GAPDH	UNKNOWN	FAM-NFQ	21.150	21.084	0.065		2.874	0.053	2.525	0.174	0.157	
		🗏 Lung - GH1	L														
	105 E9			Lung	GH1	UNKNOWN	FAM-NFQ	32.856	32.837	0.030		14.626	0.041	0.707	0.612	0.566	
	106 E10			Lung	GH1	UNKNOWN	FAM-NFQ	32.802	32.837	0.030		14.626	0.041	0.707	0.612	0.566	
	107 E11			Lung	GH1	UNKNOWN	FAM-NFQ	32.851	32.837	0.030		14.626	0.041	0.707	0.612	0.566	
		🗏 Lung - LIPO	0														
	113 E17			Lung	LIPC	UNKNOWN	FAM-NFQ	31.081	31.162	0.105		12.952	0.071	0.998	0.501	0.436	
	114 E18			Lung	LIPC	UNKNOWN	FAM-NFQ	31.281	31.162	0.105		12.952	0.071	0.998	0.501	0.436	~
		<															>
M	I Summany	In	Distor 294	C	Up; 65	Apple	di 65	Eboord: 7		Omitted by Ann	heries 0	Omitted	Manually: 0	Com	plac Usadu 4	Tan	oste Usodu 5

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

>	Plate Layout	Well Table															
	Show in Table 🔻	Select Wells 🔻	Group by 🔻											🗄 Exp	and All 🔳 🗆	ollapse All	
ľ	# Well	Omit	Flag Samp	ole Target	Task	Dyes	Ст	Ст Mean	CT SD	∆Ст	∆Ст Ме	ΔCT SE	ΔΔCτ	RQ	RQ Min	RQ M	
	149 65		Liver	GAPDH	UNKNOWN	FAM-NEO	19.113	19.217	0.092		0.026	0.071	-0.323	1.251	1.091	^	
	150 G6	H	Liver	GAPDH	UNKNOWN	FAM-NEO	19.250	19,217	0.092		0.026	0.071	-0.323	1.251	1.091		
	151 G7	i i	Liver	GAPDH	UNKNOWN	FAM-NFO	19.288	19.217	0.092		0.026	0.071	-0.323	1.251	1.091		
		Liver - GH1															
	153 G9		1 Liver	GH1	UNKNOWN	FAM-NFQ	36.234	35.576	0.642		16.385	0.374	2.467	0.181	0.088		
	154 G10		Liver	GH1	UNKNOWN	FAM-NFQ	34.951	35.576	0.642		16.385	0.374	2.467	0.181	0.088		
	155 G11		Liver	GH1	UNKNOWN	FAM-NFQ	35.543	35.576	0.642		16.385	0.374	2.467	0.181	0.088		
		🗏 Liver - LIPC															
	161 G17		Liver	LIPC	UNKNOWN	FAM-NFQ	21.973	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6	
	162 G18		Liver	LIPC	UNKNOWN	FAM-NFQ	22.181	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6	
	163 G19		Liver	LIPC	UNKNOWN	FAM-NFQ	22.122	22.092	0.107		2.901	0.078	-9.052	530.871	456.875	6	
		🗏 Liver - LPIN	1														
	157 G13		Liver	LPIN1	UNKNOWN	FAM-NFQ	24.180	24.322	0.127		5.131	0.087	0.593	0.663	0.560		
	158 G14		Liver	LPIN1	UNKNOWN	FAM-NFQ	24.361	24.322	0.127		5.131	0.087	0.593	0.663	0.560		
	159 G15		Liver	LPIN1	UNKNOWN	FAM-NFQ	24.424	24.322	0.127		5.131	0.087	0.593	0.663	0.560		
		🗏 Lung - ACT	B														
	97 E1		Lung	ACTB	UNKNOWN	FAM-NFQ	18.151	18.210	0.064								
	98 E2		Lung	ACTB	UNKNOWN	FAM-NFQ	18.202	18.210	0.064								
	99 E3		Lung	ACTB	UNKNOWN	FAM-NFQ	18.278	18.210	0.064								
		🗏 Lung - GAP	DH														
	101 E5		Lung	GAPDH	UNKNOWN	FAM-NFQ	21.082	21.084	0.065		2.874	0.053	2.525	0.174	0.157		
	102 E6		Lung	GAPDH	UNKNOWN	FAM-NFQ	21.020	21.084	0.065		2.874	0.053	2.525	0.174	0.157		
	103 E7		Lung	GAPDH	UNKNOWN	FAM-NFQ	21.150	21.084	0.065		2.874	0.053	2.525	0.174	0.157	_	
	405 50	Lung - GH1		0.14		5444.450	22.055	22.027	0.000			0.044	0.707	0.640	0.500		
	105 E9		Lung	GH1	UNKNOWN	FAM-NEQ	32.856	32.837	0.030		14.626	0.041	0.707	0.612	0.566		
	106 E10		Lung	GH1	UNKNOWN	FAM-NFQ	32.802	32.837	0.030		14.626	0.041	0.707	0.612	0.566		
	107 E11		Lung	GH1	UNKNOWN	FAM-NEQ	32.851	32.837	0.030	_	14.626	0.041	0./0/	0.612	0.566		
	110 517	= Lung - LIPC		1.10.0	LINIKALOMAN	EAM NEO	21.001	21.162	0.105		12.052	0.071	0.000	0.501	0.426		
	113 EI/		Lung	LIPC	UNKNOWN	FAMI-INFQ	31.001	31.102	0.105		12.952	0.071	0.998	0.501	0.430		
	114 E18		Lung	LIPC	UNKNOWN	FAM-NEQ	31.201	31.102	0.105		12.952	0.071	0.998	0.501	0.430	Ň	
L.		In	Plato: 294	Set Line 65	Applyced 65		Ebaardu 2	incerte 2 0)mitted by Analysis: 0		Omitted Manualhy 0		Sampler Uredu 4		Tarnets Urodi 5	

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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**[™] **6 and 7** 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	QS6_QuantStudio_384-Well_Comparative_Ct_Example_data
File Type	*.txt
Export File Location	C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\Export

Your Export screen should look like this:

Auto Export	Format : QuantStu	dio™ 6 and	7 💌	Export Data 1	o: 💿 One File 🔘	☑ Open file(s) when export is complete			
Export File Location: ms\Qua	antStudio 6 and 7	Flex Softwa	re\User Files\Export	Browse Export File	Name: jio_384-W	ell_Comparative_C	t_Example_data	a File Type: 🧊 (*.	.txt) 💙
Sample Setup	aw Data 🛛 🔽 Amp	olification	Multicomponent	Tech. Rep. Results	Bio. Rep. Resu	lts 🗸 Results			
🗹 Skip Empty Wells 🛛 🔽 Ski	o Omitted Wells								
Select Content			1	d	1	1		1	
		Well	Well Position	Sample Name	Delta Ct SD	Target Name	Task	Reporter	Q
✓ All Fields	<u></u>		1 A1	Brain		АСТВ		FAM	NF 🔨
Vel Well			2 A2	Brain				FAM	NF
			3 A3	Brain	0.05	ACTB	UNKNOWN	FAM	NF
Well Position			5 A5	Brain	0.051 GAPDH		UNKNOWN	FAM	NF =
			6 A6	Brain	0.05	1 GAPDH	UNKNOWN	FAM	NF
Sample Name			/ A/	Brain	0.05	I GAPDH	UNKNOWN	FAM	NF
Target Name			9 A9	Brain	0.21	/ GHI	UNKNOWN	FAM	NF
Target Name			10 A10	Brain	0.21	/ GHI	UNKNOWN	FAM	NF
✓ Task			11 A11	Brain	0.21	/ GHI	UNKNOWN	FAM	NF
			13 A13	Brain	0.06	9 LPIN1	UNKNOWN	FAM	NF
Reporter			14 A14	Brain	0.06	9 LPIN1	UNKNOWN	FAM	NF
			15 A15	Brain	0.06	9 LPIN1	UNKNOWN	FAM	NF
Quencher			1/ A1/	Brain	0.07	3 LIPC	UNKNOWN	FAM	NF
R RO			18 A18	Brain	0.07	3 LIPC	UNKNOWN	FAM	NF
V KQ			19 A19	Brain	0.07	3 LIPC	UNKNOWN	FAM	NF
RO Min			24 A24			ACTB	NIC	FAM	NF
			49 C1	Heart		ACTB	UNKNOWN	FAM	NF
RQ Max			50 C2	Heart		ACTB	UNKNOWN	FAM	NF 🗸
	~	<							>

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

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

QS6_QuantStudio_384-Well_Comparative_Ct_Example_data.txt - Notepad	
File Edit Format View Help	
* Block Type = 384-well Block	^
<pre>% calibration Background is expired = Yes % calibration Background performed one 00.24 2010</pre>	-
 Calibration Background performed on = 09-24-2010 Calibration PRM MELTOCTOP is expired - yes 	
* Calibration HRM MELTDOCTOR berformed on = 10-02-2010	
* Calibration Normalization FAM-ROX is expired = Yes	
* Calibration Normalization FAM-ROX performed on = 09-22-2010	
Calibration Normalization VIC-ROX is expired = Yes	
<pre>Calibration Normalization Vic-ROX performed on = 09-22-2010 Calibration Durp Dvg DVg ABV is expired = Vas</pre>	
* Calibration Pure Dve ABY performed on = 03-31-2010	
* Calibration Pure Dye ABYQ'is expired = Yes	
* Calibration Pure Dye ABYQ performed on = 03-27-2010	
* calibration Pure Dye Alio 700 hostored = Yes	
<pre>calibration Pure Dye DYE3 is expired = Yes</pre>	
* Calibration Pure Dye DyE3 performed on = 07-22-2010	
* Calibration Pure Dye DyE4 is expired = Yes	
* Calibration Pure Dye DYE4 performed on = 07-22-2010	
<pre>calibration Pure Dye FAM is expired = yes calibration Pure Dye FAM performed on = 00-22-2010</pre>	
<pre>calibration pure bye FAMO is expired = Yes</pre>	
* Calibration Pure Dye FAMQ performed on = 03-27-2010	
* Calibration Pure Dye JUN is expired = Yes	
* Calibration Pure Dye JUN performed on = 03-31-2010	
Calibration Pure Dye JUNO is expired = res	
* Calibration Pure Dye MELTDOCTOR is expired = Yes	
* Calibration Pure Dye MELTDOCTOR performed on = 10-02-2010	
* Calibration Pure Dye MP is expired = Yes	
Calibration Pure Dye MP performed on = 0/-22-2010 Calibration Pure Dye MUSTANCE is extended a very second secon	
<pre>calibration pure bye MUSTANGP performed on = 06-16-2010</pre>	
* Calibration Pure Dye NED is expired = Yes	
* Calibration Pure Dye NED performed on = 06-12-2010	
* Calibration Pure Dye NPR is expired = Yes	
<pre>* Calibration Pure Dye NPR performed on = 02-13-2010 * Calibration Pure Dye 0705 is expired = Ves</pre>	
\approx calibration pure by e 0705 performed on = 07-14-2010	
* Calibration Pure Dye QUASAR 705 is expired = Yes	
* Calibration Pure Dye QUASAR 705 performed on = 02-15-2010	
* Calibration Pure Dye QUASAR705 is expired = Yes	
<pre>* Calibration Pure Dye QUASAR/05 performed on = 04-00-2010 * Calibration Pure Dye POX is expired = Vas</pre>	
* calibration Pure Dyc ROX performed on = 09-22-2010	
* Calibration Pure Dye SYBR is expired = Yes	
* Calibration Pure Dye SYBR performed on = 07-22-2010	
* Calibration Pure Dye TAMRA is expired = Yes	
<pre>calibration Pure Dye tweetApperformed On = 02-13-2010 % Calibration Pure Dye VTC is expired = Ves</pre>	
* Calibration Pure Dye VIC performed on = 09-22-2010	
* Calibration Pure Dye VICQ is expired = Yes	
* Calibration Pure Dye VICQ performed on = 03-27-2010	
* Calibration ROI is expired = Yes	
Calibration Informity is expired = Ves	
	~

Design and Analyze a Gene Expression Study

The QuantStudioTM 6 and 7 Flex Real-Time PCR Development Software can combine the analysis of experiments that use the Relative Standard Curve or the Comparative $C_T (\Delta \Delta C_T)$ quantification methods into a Gene Expression study. A study provides a wider range for analyzing and comparing target behavior across multiple experiments.

Note: You can import different types of quantification experiments into a single Gene Expression study, but make sure the run method and experiment type are identical for all the experiments in that study. Also make sure that the experiments have been run on the same type of qPCR instrument.

Note: You can design an example study using the example experiments provided with the QuantStudioTM 6 and 7 Flex Real-Time PCR Development Software. This chapter explains how to design and analyze multiple Comparative C_T experiments as a study. When you design a Gene Expression study, make sure that each experiment in that Gene Expression study has a unique name. Absence of a unique name, leads to failure of the run.

This chapter covers:

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About the example study	126
Design a study	127
Analyze the example study	132
View the analysis settings	134
Assess amplification results using the Amplification Plot	138
Assess the gene expression profile using the Gene Expression Plot	140
Confirm accurate dye signal using the Multicomponent Plot	148
View the QC Plots	149
View the QC Summary	158
Compare analysis settings	160
Export the study	165
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About Gene Expression studies

In a Gene Expression study, you can	You cannot
 Specify the endogenous control(s) and reference sample for the study. Set individual efficiency values for each target. Select the control type when applicable. Set baseline and threshold values and confidence levels, or set the number of standard deviations for Comparative _{CT} Min./Max. Omit wells individually or together through their association with replicate 	 Create, add, or modify samples. Create, add, or modify targets. Change assay tasks. You can perform these operations in individual experiments.
groups (technical or biological).	

About the example study

In the Comparative C_T example study:

- Two reaction plates (experiments) are used.
- Experiments that you add to the study are two Comparative C_T experiments that have already been analyzed.
- The cDNA was prepared from total RNA that was isolated from the following 4 samples:
 - Heart
 - Liver
 - Brain
 - Lung
- Five targets (assays) are used:
 - LIPC: Hs00165106_m1
 - GAPDH: Hs99999905_m1
 - ACTB: Hs99999903_m1
 - LP1N1: Hs00299515_m1
 - GH1: Hs00236859_m1
- The reference sample is Brain.
- The endogenous control is ACTB.
- Each experiment in the study was designed for singleplex PCR, where the target and endogenous control assays are run in separate wells.
- For each experiment in the study, reactions were set up for 2-step RT-PCR:
 - The cDNA was reverse-transcribed from total RNA samples using Invitrogen VILO Kit.
 - The reactions were prepared using TaqMan[®] Fast Universal PCR Master Mix (2X).



Design a study

Create a new study	To create a new study in the QuantStudio ^{\mathbb{M}} 6 and 7 Flex Real-Time PCR Development Software, from the Home screen, click New Gene Expression Study .
Set up the study in the Study Proportion coroop	 In the Study Menu pane, select Setup > Study Properties In the Study Properties pane, click the Study Name field, then enter QS6_384-
Froperties screen	Well_Comparative_Ct_with_Bioreplicates_Study_Example.
	 Click the Comments field, then enter Example Comparative C_T Study for the Comparative Ct Experiments.
	4. In the Setup Experiments pane, click Add Experiment(s).
	 In the Open dialog box, browse to the QS6_384- Well_Comparative_Ct_Example_1.eds and QS6_384- Well_Comparative_Ct_Example_2.eds files at:
	C:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\QS6Flex
	The QuantStudio [™] 6 and 7 Flex Real-Time PCR Development Software displays the details of the experiment in the Properties pane.

Your Study Properties screen should look like this:

Study: QS6_384-Well_Comparative_Ct_v	ith_Bioreplicates_Study_Example	Type: RQ	Analyze Analysis Settings Export ?
Study Properties			
Study Name: QS6_384-Well_Comparative_ User Name (Optional): Example user Last Modified: Number of Experiments: 2	_with_Bioreplicates_Study_Example	Comments (Optional): Comparativ	re Ct Study Example
Set Up Experiments (2) Add Experiment(s) Remove Selected Experiment(Enter a filter query, then cick "Apply Filter." IF IF Experiment Name Image: Selected Experiment (Experiment Name Instrument Type IF Experiment Name Instrument Type IF Representer to the selected Experiment (Instrument Type	Edit Experiment(s) Hide Filter	Run Date	Apply Filter Remove Filter Properties: QS6_QuantStudio Comparative
QuantStudio Comparativ QuantStudio (TM) 5	4	10/05/201	Overview Samples Targets

Filter the experiments in the study

To narrow your search for an experiment, define and apply a filter:

- 1. In the left-most drop-down menu, select an experiment attribute to query.
- 2. In the center drop-down menu, select an operator for the query.
- 3. In the right-most field, enter the condition to look for, then click Apply Filter.



After you apply a filter, click Hide Filter/Show Filter to hide or show the filter tool, or click Remove Filter to remove the filter. **Define Replicates** In the Define Replicates screen, create biological replicate groups and use them to associate samples for the analysis. Biological replicates allow you to assess the representative nature of your results as they relate to the population being studied. Including biological replicates can give insight into any natural variation that is in the population. Example study The Gene Expression example study contains four biological replicate groups. These are lung, heart, brain, and liver. Each biological groups consists eight replicates. settings To define replicates 1. In the Study Menu pane, select **Setup** > **Define Replicates**. 2. Click Add Biological Group to open the Add Biological Replicate Group dialog box. **3.** Define the biological replicate group: a. Click the Biological Group Name field, then enter Heart. **b.** In the Color field, select the color. c. Click the Comments field, then enter Example Biological Replicate Group for the Example Comparative Ct Study. 4. Add technical replicates: a. From the Select Plate drop-down menu, select QuantStudio Comparative Ct Example 1.eds. b. In the plate layout, select wells A1, A3, A5, A7, A9, A11, A13, A15, A17, and A19, then click to add the technical replicate wells that are associated with the selected well to the biological group. c. Click OK. **d**. Perform steps a through c to add the remaining technical replicate wells associated with the selected well to the biological group.

5. On the Set Up Biological Replicates screen, select the Heart biological group that you just added to the study. The QuantStudio[™] 6 and 7 Flex Real-Time PCR Development Software displays the details of the biological group in the Properties pane.

Biological Re	licate Group															
ie biological ke	plicate droup						_									
ical Group Name:	Heart							51, 153	, 102	*						
ments (Optional):	Example Biolog	ical Replica	ate Group	for the	Examp	le Com	parativ	e Ct S	tudy							
technical replica	ites to the ne	w biolog	gical rep	icate g	jroup											
ach experiment in u select technical	the study, select replicates, the sol	the plate	, then sel	ect the	wells o	f the p	late th	at con	itain sa Is to th	mples	associ	iated w	vith th	e new biol	ical replicate group.	
	apheatesy and so	interest and	comacical		10 0000	clocod	rapilea				logical	replicat	to grou			
ect Plate: QS6_Qu	antStudio Com	parative	Ct Examp	ole 1 💊	•										Technical Replicate Groups Ac # of Replicates in Group: 5	Ided to Biological Replicate Group:
and and a second second	No.									NO	51 F		<u>`</u>		Sample	Target
Show in vireis V	View Legend							_		83	2		~		Brain	ACTB
1 2 3 4	5 6 7	8 9	10 11	12	13 1	4 15	16	17	18	19 2	0 21	22	23	24	Brain	LIPC
	10° 10° 10°	1	1 😁 🗃		1	an (***								12	Brain	GAPDH
															Brain	GH1
		Hear		<u>~</u> •				-	1827 1					12	Brain	LPIN1
er ver ver				ſ		1	1			57				12		
								Laver 1		100					>>	
		1	1 1		-			-								
			_						-						.<	
				+	_											
				+++	-											
				+++	-											
															Used on Plate(s):2	
					1										QS6_QuantStudio Compa QS6_QuantStudio Compa	rative Ct Example 1
															Goo_Quantistudio Compa	native cuexample 2
							1									
												2	10 Em	nntv		

Set Up Biological Repl	licate Gro	ups		
Add Biological Group	Edit Bio	logical Grou	p Delete Biolog	ical Group
Biological Group Name		Color	# of Replicates	Comments
Heart			5.000	
Liver			5.000	
Brain			5.000	
Lung			5.000	

- **6.** Click **Analyze**, then close the study:
 - a. Select File > Close.
 - **b.** At the prompt, click **Yes** to save the changes.

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c. In the Save Study as dialog box, click Save to accept the default file name and location. The example study is saved and closed, and you are returned to the Home screen. You can also save the study you create at C:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\QS6Flex.

Edit a Biological Replicate Group

- 1. Open the study of interest.
- 2. In the Study Menu pane, select **Setup > Define Replicates**.
- **3.** Click **Edit Biological Group** to open the Edit Biological Replicate Group dialog box.
- 4. Edit the group information:
 - a. From the Biological Group Name drop-down menu, select the group to edit.
 - b. To change the group name, click Edit, enter a new name, then click OK.
 - c. To change the color, select a color from the drop-down menu.
 - d. (Optional) Enter comments.
- 5. From the Select Plate drop-down menu, select the experiment of interest.
- 6. Add samples to the biological group:
 - **a.** In the Plate Layout, select the well(s) of the plate that contain samples to add to the biological group.
 - **b.** Click **b** to add the samples that are associated with the selected wells to the biological group.
- 7. Remove samples from the biological group:
 - **a.** In the Technical Replicate Groups Added to Biological Replicate Group pane, select a sample. You can select only one sample at a time.
 - **b.** Click **____** to remove the sample from the biological group.
- 8. Repeat steps 5 through 7 for the other experiments in the study.
- **9.** Click **OK** to save the changes and return to the study; click **Cancel** to exit the dialog box without saving the changes.

Remove Biological Replicate Groups

- 1. Open the study of interest.
- 2. In the Study Menu pane, select Setup > Define Replicates.
- **3.** In the Define Replicates screen, select the group to remove, then click **Delete Biological Group**.
- **4.** Click **Yes** to remove the group from the study; click **No** to keep the group in the study.

Tips for designing your own study

• Enter a study name that is descriptive and easy to remember. You can enter up to 100 characters in the Study Name field. The study name is used as the default file name.

You can only use the alpha-numeric, period (.), hyphen (-), underscore (_), and spaces () characters in the Experiment Name field.

- (*Optional*) Enter comments to describe the study. You can enter up to 1000 characters in the Comments field.
- Use the default user name, or enter a new user name, to identify the owner of the study. You can enter up to 100 characters in the User Name field.

Note: If security is enabled, the User Name field is automatically populated with the name you selected at log in.

- You can add an unlimited number of experiments (reaction plates) to the study. Click **Add Experiment (s)** or **Remove Selected Experiment(s)** to add or remove experiments to or from a study. Note the following:
 - Each experiment in the study must:
 - Have one or more common endogenous control(s). The endogenous control(s) must be present on each reaction plate within the study.

Note: The endogenous control gene for a given sample must be run on the same plate as the target gene for a given sample in order for a study to be created.

- Have identical thermal cycling parameters (the same number of steps and cycles). The 6 and 7 Flex Real-Time PCR Development Software cannot combine in the same study experiments that use Fast and Standard thermal cycling conditions.
- Have the same passive reference.
- Have the same experiment type.
- Have been run on the same instrument type.
- As the default, the QuantStudio[™] 6 and 7 Flex Real-Time PCR Development Software assigns the endogenous control and reference sample for a study based on the analysis settings of the first experiment that you added to the study.
- If experiments that contain biological replicate groups are added to a study, the QuantStudio[™] 6 and 7 Flex Real-Time PCR Development Software automatically merges the matching biological groups.
- The QuantStudio[™] 6 and 7 Flex Real-Time PCR Development Software automatically analyzes a study after you add more than one experiment to it. Consequently, to ensure that the software uses the correct settings, Life Technologies recommends that you review the analysis settings of your study after adding multiple experiments.
- When adding experiments to the study, **Ctrl-click** multiple experiments in the Open dialog box to add them to the study.
- Select an experiment that has been added to the study to view its properties in the Properties pane.
- Filter the experiments added to the study to simplify the list for easier review. See "Filter the experiments in the study" on page 127.

- Enter a biological replicate group name that is descriptive and easy to remember. You can enter up to 100 characters in the Biological Group Name field. You can only use the alpha-numeric, full-stop (.), hyphen (-), underscore (_), and space () characters in the Experiment Name field.
- (Optional) Enter comments to describe the biological replicate group. You can enter up to 1000 characters in the Comments field.
- You can add an unlimited number of technical replicates to a biological group.

IMPORTANT! A sample cannot belong to more than one biological group.

- Click-drag over the desired wells, or Ctrl-click or Shift-click in the plate layout to select multiple wells.
- Click the upper-left corner of the plate layout to select all wells.
- You can change the name of a biological replicate group, change its color identification and description, and add or remove technical replicates. See "Edit a Biological Replicate Group" on page 130.
- You can remove an existing biological replicate group. See "Remove Biological Replicate Groups" on page 130.

IMPORTANT! After you remove a biological replicate group from a study, you cannot restore it.

Analyze the example study

	This section explains how to use the QuantStudio [™] 6 and 7 Flex Real-Time PCR Development Software to analyze the Gene Expression example study. It also describes Life Technologies recommended best practices as you perform the analysis.							
	Note: The Comparative C_T Study Example.edm file illustrated in this chapter demonstrates the use of biological replicate groups. You can also create a study without the use of biological replicate groups.							
Access the example study	Access the example study that you created on your computer.							
	C:\Program Files\Applied Biosystems\QuantStudio 6 and 7 Flex Software\examples\QS6_384 Well Comparative Ct with Bioreplicates Study Example.edm							
View the experiment data	The Experiment Data and Well Results Data panes appear in the Amplification Plot, Multicomponent Plot, and Multiple Plots View screens.							
and well results	The Gene Expression screen displays the Replicate Results Data and the Well Results Data panes.							

Only the Well Results Data pane appears in the QC Plots View screen.

To display or hide columns in the Experiment Data pane– from the Show In Table drop-down menu, select or deselect one or more options: Experiments,
 # of Targets, # of Samples, Run Date.

• To display a subset of the study data in the plots– Select one or more rows in the Experiment Data pane or the Well Results Data pane, then select the **Hide unselected data from plot** check box appearing in the plot pane to display data only from the selected rows.

The Experiments Data pane lists each reaction plate (experiment) that is added to a study. The data that are displayed in the Well Results Data pane depend on which tab you select in the Experiment Data pane:

Tab	Description				Illustra	tion		
Well Table tab	When you select one or more experiments in the Experiment Data pane, the well table displays the wells that make up the selected experiment(s).	Experience Show	riment Data w In Table v Experiment S6_QuantStud S6_QuantStud S6_QuantStud Results Data Table Plat v In Table v	iio Comp iio Comp iio Comp iio Comp	# of Tar # 5 5 5	e of Sa 4	Run Date May 5, 2013 May 5, 2013	Collapse All
		#	Sample Brain Brain Brain Brain Brain Brain Brain	ACTB ACTB ACTB ACTB GAPDH GAPDH GAPDH GAPDH GAPDH	CS6 Quant QS6 Quant QS6 Quant QS6 Quant QS6 Quant QS6 Quant QS6 Quant QS6 Quant		Flag A	0.4552979: 0.4552979: 0.4552979: 0.993406. ♥





Tab	Description	Illustration					
Plate Layout tab	When you select one experiment in the Experiment Data pane, the plate layout displays the plate layout for the selected experiment. If you select more than one experiment in the Experiment Data pane, only the plate layout for the first experiment is displayed in the Plate Layout tab.	Experiment Data Show In Table T # Experiment # of Tar # of Sa Run Date 1 QS6_QuantStudio Comp 5 4 May 5, 2013 2 QS6_QuantStudio Comp 2 QS6_QuantStudio Comp 5 4 May 5, 2013					
		Well Results Data					
		Show in Wells Select Wells Image: Wew Legend Image: Wew 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 Image: Wey Legend Image: Wey Legend					

View the analysis settings

The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T) , flags, and relative quantification. If the default analysis settings in the QuantStudioTM 6 and 7 Flex Real-Time PCR Development Software are not suitable for your study, you can change the settings in the Analysis Settings dialog box, then reanalyze your study.

Note: In the Comparative C_T example study, the default analysis settings are used without changes.

To adjust the analysis settings

- 1. From the Study Menu pane, select Analysis.
- 2. Click Analysis Settings to open the Analysis Settings dialog box.
- **3.** Select the **Relative Quantification Settings** tab, then view the default reference sample and endogenous control. In the example study, the default reference sample is brain and the default endogenous control is 18S.
- **4.** Select the **C**_{**T**} **Settings** tab, then the **Flag Settings** tab. In the example study, the default analysis settings are used in each tab.



5. View and, if necessary, change the analysis settings. For more information on the changes to analysis settings, refer to "Tips for analyzing your own study" on page 135.

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*[™] 6 and 7 Flex Real-Time PCR System Software Experiments.

6. 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**.

7. Click Analyze.

The following is an image of the Analysis Settings dialog box for a Comparative C_T Study:

Analysis Settings	for QS6_384-Well_Compar	ative_Ct_with_Bioreplicat	es_Study_Example				
Ст Settings Fla	ag Settings Relative Quantifi	cation Settings					
Review the default settings for analysis of targets in this experiment. To use different settings for a target, select the target from the table, deselect Use Default Settings, then change the settings.							
Data Step Selection 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. Stage 2, Step 2							
Default CT Set Default CT setting	ttings s are used to calculate the CT for t	argets without custom settings. 1	o edit the default settings, d	ick Edit Default Settings.			
Threshold: AU	TO Baseline Start Cycle: AUTO	D Baseline End Cycle: AUTO	Edit Default Setting	35			
Select a Targe	t			CT Settings for ACTB			
Target	Threshold	Baseline Start	Baseline End	CT Settings to Use: 🗹 Default Settings			
ACTB	AUTO	AUTO	AUTO	Automatic Threshold			
GAPDH	AUTO	AUTO	AUTO	Threshold: 0.054707			
GH1	AUTO	AUTO	AUTO	✓ Automatic Baseline			
LIPC	AUTO	AUTO	AUTO	Baseline Start Cycle: 3 🗇 End Cycle: 15 💠			
LPIN1	AUTO	AUTO	AUTO				
	I						
Save to Library Load from Library Revert to Default Analysis Settings Analyze Cancel							

Tips for analyzing your own study

Unless you have already determined the optimal settings for your study, use the default analysis settings in the QuantStudioTM 6 and 7 Flex Real-Time PCR Development Software. If the default settings are not suitable for your study, change the settings as described below.

C_T Settings

• Data Step Selection

Use this feature to select multiple locations of analysis, in case several are chosen.

• Algorithm Settings

Use the Baseline Threshold algorithm to determine the C_T values.

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

• 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[™] 6 and 7 Flex Real-Time PCR Development 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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Analysis Settings for QS6_384-Well_Comparative_Ct_with_Bioreplicates_Study_Example										
CT Settings	CT Settings Flag Settings Relative Quantification Settings									
Configur	re the flags and filtering. In this panel yo	ou can enable, disable, ai	nd configure flags, and indicate	if a well is to be rejected w	hen a flag is raised.	2				
Flag	Description	Use	Attribute	Condition	Value	Reject Well				
SPIKE	Noise spikes	✓	Spike algorithm result	> 🗸	1.000					
BADROX	Bad passive reference		Bad passive reference	> 🗸	0.600					
NOAMP	No amplification	✓	Amplification algorith	< 🗸	0.100					
CTFAIL	Ct algorithm failed	~								
BLFAIL	Baseline algorithm failed	~								
EXPFAIL	Exponential algorithm	v								
THOLDFAIL	Thresholding algorith	V								
HIGHSD	High standard deviati	v	Ct standard deviation	> 🗸	0.500					
OUTLIERRG	Outlier in replicate gr	v								
NOSIGNAL	No signal in well	v								
NOISE	Noise higher than oth	✓	Relative noise	> •	4.000					
OFFSCALE	Fluorescence is offscale	✓								
AMPNC	Amplification in negati		Ст	< 🗸	35.000					
	Save to Library	Load from Librar	Revert to Default 4	Analysis Settings	nalyze Cancel					

The following is an image of the Flag Settings tab:

Relative Quantification Settings

Use the Relative Quantification Settings tab to:

- Change the reference sample and/or endogenous control. As the default, the QuantStudio[™] 6 and 7 Flex Real-Time PCR Development Software assigns the endogenous control and reference sample for a study based on the analysis settings of the first experiment added to it. You can add multiple endogenous controls to a study.
- Correct the amplification efficiency. You can enter a percentage value between 1% and 150% for each target. When you set an assay to have a value that differs from 100% efficiency, the software uses the relative standard curve algorithm.
- (For multiplex reactions) Specify the ΔC_T value at which to reject replicates (outlier rejection).
- 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.

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Assess amplification results using the Amplification Plot

In the comparative C_T example study, you review each target in the Amplification Plot screen for correct baseline and threshold values.

1. From the Study Menu pane, select Analysis > Amplification Plot.

Note: If no data are displayed, click Analyze.

- **2.** In the Experiment Data pane, select all of the experiments (click and drag to select all rows in the table).
- 3. In the Amplification Plot pane, set the parameters for the plot:
 - **a.** From the Plot Type drop-down menu, select Δ **Rn vs Cycle**.
 - b. From the Plot Color drop-down menu, select Well.
 - c. Click E Show a legend for the plot.

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

- **d.** From the Target drop-down menu, select **ACTB** to highlight all ACTB wells in the study.
- 4. View the baseline values:
 - a. From the Graph Type drop-down menu, select Linear.
 - b. Select the Show 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 fluorescence is detected. In the example study, the baseline is set correctly.

Note: The data shown in the screenshot below is of the example experiment. Results vary depending on the experiment setup.



5. View the threshold values:

- a. From the Graph Type drop-down menu, select Log.
- **b.** Deselect the **Show Baseline** check box, then select the **Show Threshold** check box to show the threshold.
- **c.** Verify that the threshold is set correctly. In the example study, the threshold is in the exponential phase.

Amplification						
Plot Settings						
Plot Type: 🛆 Rn vs Cycle 💌 Graph Type: Log 💌 Plot Color: Well 💌						
Save current settings as the default						
	Đ	Ð	A	ih 📈		=
	·	·	_		. 🛄	
Amplification						
1601						
1600		10000000	estate a			
0.1 1.056851	inum	1				
						1
0.0001						
0.00001						
0.000001						
0.0000001						
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 Cvcle	34		36	38	40	42
L rates						
						^
						~
Target: ACTB Threshold: V Auto 056851 V Auto Baseline						
Show: 🗹 Threshold — 🔲 Baseline Start: Well 🔳 Target 🔺 Baseline End: Well 🔳 Target 🔺						
65 Wells Selected		 Image: A set of the	Hide u	nselected	data from	n plot

6. Repeat steps 3 through 5 for the remaining targets.

Tips for assessing amplification in your own study

- Ensure that your study meets the following requirements:
 - A typical amplification plot See the amplification plot examples in the Chapter 5 and Chapter 11.
 - **Correct baseline and threshold values** See the threshold examples and the baseline examples in Chapter 5 and Chapter 11.

If your study does not meet these requirements, you can:

- Manually adjust the baseline and/or threshold. See "View the analysis settings" on page 134.
- Omit individual wells from the analysis. See "Omit wells from the analysis" on page 164.



Assess the gene expression profile using the Gene Expression Plot

The Gene Expression Plot screen displays the results of the relative quantification calculations in the gene expression profile. Three plots are 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 following graph types: linear, log10, Ln, log2.
• RQ vs Sample – (displayed only when the Technical Replicates tab is selected in the Replicate Results Data pane) – 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.
• RQ vs BioGroup – (displayed only when the Biological Replicates tab is selected in the Replicate Results Data pane) – Groups the relative quantification (RQ) values by biological replicate group. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.
In the Comparative C_T example study, you review each target in the Gene Expression Plot screen for the expression level (or fold change) of the target sample relative to the reference biological replicate group.
 From the Study Menu pane, select Analysis > Gene Expression. In the Gene Expression Plot pane, set the parameters for the plot: a. In the Plot Type drop-down menu, select RQ vs BioGroup. b. In the Graph Type drop-down menu, select Log10. c. In the Orientation drop-down menu, select Vertical Bars. d. Click In Show a legend for the plot

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

In the example study, the expression levels of multiple targets in the biological replicate group are displayed relative to the expression levels of the same targets in the reference biological replicate group (universal). Because 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).



- 3. Select multiple endogenous controls:
 - a. In the Replicate Results Data pane, select the Technical Replicates tab.

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b. From the Endo Controls drop-down menu, select **GAPDH**, **GH1**, and **LIPC**, then deselect **ACTB**.

Note: You can also select the endogenous controls in the Analysis Settings dialog box. See "View the analysis settings" on page 134.

Replicate Results Data												
Teo	chnical Replicat	es Biolog	ical Replicat	es								
Sho	ow In Table	Add Bi	oGroup	En	ndo Controls 🗙 Ref Sample 🔻							
#	Omit	Sample	Target		ACTB		Ст Ме	ΔCτ SE	Δ/			
1		Brain	ACTB	~	GAPDH					^		
2		Brain	ACTB	\checkmark	GH1							
3		Brain	GAPDH	H	LIDC							
4		Brain	GAPDH	<u> </u>	LIPC	0	.3144583	0.03370482				
5		Brain	GH1		LPIN1							
6		Brain	GH1	_	31.09304	4 1	3.828029	0.1315035	0.0			
7		Brain	LIPC		28.92929	8 1	1.664282	0.0410995	-1			
8		Brain	LIPC									
9		Brain	LPIN1		21.7320	3	4.467014	0.0398248	-1			
10		Brain	LPIN1									
11		Heart	ACTB		18.13637	7						
12		Heart	ACTB									
13		Heart	GAPDH							_		
14		Heart	GAPDH		17.31571	8 -0	.8206588	0.0384727		Y		
	<								>			

	Replica	ite Result	a Data															
2	Techni	cal Replicates	Biologia	cal Replicates														
í	Show	In Table 🔻	Add Bio	Group Endo	Controls	Ref Sar	onle 🔻											h
	onow	ATT TODIC Y			controls	Y Ker da	npic y											
	# O	nit S	ample	Target CT	Mean	∆Ст Ме	ΔCT SE	ΔΔCT	RQ	RQ Min	RQ Max						•	-
	1	Br	ain /	ACTB 1	7.265015													^
	2	📃 Br	ain (GAPDH 1	7.579475	0.31446025	0.0337048	1.1351172	0.45529792	0.43082792	0.48115778							
	3	E Br	ain (GH1 3:	1.093044	13.828031	0.1315035 0	.0095444	0.9934061	0.80079186	1.2323498							
	4	📃 Br	ain l	LIPC 2	8.929298	11.664284	0.0410995	-1.1238338	2.179253	2.0372863	2.3311126							
	5	Br Br	ain l	LPIN1 2	1.732025	4.4670115	0.03982485	-1.1272669	2.1844451	2.0464113	2.3317897							
	6	He He	art /	ACTB 1	8.136375													2
	7	He	art (GAPDH 1	7.315718 -	-0.82065696	0.0384727	0.0	1.0	0.93888897	1.0650887							
	8	E He	art (GH1	31.95486	13.818485	0.0868147	0.0	1.0	0.86736786	1.1529133							
	9	He	art I	LIPC 3	0.924492	12.788117	0.05548723	0.0	1.0	0.9130676	1.0952091							
	10	E He	art I	LPIN1 23	3.730654	5.5942783	0.0346004	0.0	1.0	0.9448669	1.0583501							~
	11	Lì	er /	ACTB 19	9.201887													
1	12	Liv Liv	er (GAPDH 19	9.181273 -	-0.02061506	0.05293959	0.8000419	0.5743325	0.5265987	0.62639314							
1	13	Lh Lh	er (GH1 3	5.452633	16.254082	0.23876446	2.435596	0.18484707	0.12329348	0.27713096							
	14	Liv Liv	er l	LIPC 2	1.993778	2.7918909	0.0500186	-9.996226	1021.32495	940.93475	1108.5834							~
-	LT																	
	Well R	esults Dat	a –															
ſ	Show	In Table 🔻	Group R	esults By 🔻											ſ	Expand All	Collapse Al	
U													4					_
	#	Sample	Target	Experim	Omit	Flag	RQ	RQ Min	RQ Max	Flags	Ст	ΔCT	∆Ст Ме	∆C⊤ SE	ΔΔCT			
		Brain	ACTB	QS6 QuantS							17.2657	97			NaN			^
		Brain	ACTB	QS6 QuantS							17.170	82			NaN			
		Brain	ACTB	QS6 QuantS							17.1861	25			NaN			-
		Brain	ACTB	QS6 QuantS							17.4040	72			NaN			-
		Brain	ACTB	OS6 OuantS							17.2161	24			NaN			
		Brain	АСТВ	OS6_QuantS							17.3471	49			NaN			
		Brain	GAPDH	OS6_QuantS			0.455297	92 0.430827	92 0.48115	778	17,5089	68	0.31446025	5 0.0337048.	1.1351172			
		Brain	GAPDH	OS6_QuantS	Ē		0.455297	2 0 430827	92 0 48115	778	17.504	08	0.31446025	5.0.0337048	1 1351172			
		Brain	GAPDH	OS6_QuantS	П		0.455297	92 0.430827	92 0.48115	778	17.5238	08	0.31446025	5 0.0337048.	1.1351172			

4. Click **Analyze**. In the example study shown below, all samples for the endogenous controls **GAPDH**, **ACTB**, and **LIPC** have no values (with the exception of the C_T Mean value), and the RQ values for the remaining samples change. For example, in the example study, the RQ value for the brain sample changes from 1.0 (with 18S as the endogenous control) to none with the new endogenous controls.

>[Repli	cate Resi	ilts Data														
1	Tech	inical Replical	es Biol	ogical Replicates													
r	Chos	v In Tabla	Add B	DioGroup	ndo Control	Dof Co	mple T										
L	anov		V Add B			Ker Sa											
	# (Omit	Sample	Target	Ст Mean	∆Ст Ме	ΔCT SE	ΔΔCT	RQ	RQ Min	RQ Max						
	1		Brain	ACTB	17.265017	-8.602256	0.08189089	-0.006942	1.0048238	0.8922643	1.1315829	Ð					^
	2		Brain	GAPDH	17.579475	5											
	3		Brain	GH1	31.093044	ł											
	4		Brain	LIPC	28.929298												
	5		Brain	LPIN1	21.73203	-4.1352425	0.0/94393/	-1.1342099	2.1949832	1.9560475	2.4631054	4					
	6		Heart	ACTB	18.1363/	-8.595313	0.06349747	0.0	1.0	0.9119953	1.0964969	9					
			Heart	GAPDH	21.05496												
	0		Heart	LIDC	30 02440												
	10		Heart	LIPC	23 730658		0.06456639	0.0	1.0	0 91058207	1.0981987	7					
	11		Liver	ACTB	19,201889	-5.7585125	0.13895898	2,8368008	0.13997093	0.11432851	0.1713646	5					
	12		Liver	GAPDH	19.181273												
	13		Liver	GH1	35.452633	1											
	14		Liver	LIPC	21.993778	8											
	15		Liver	LPIN1	24.238232	-0.7191273	0.14233042	2.2819052	0.20562604	0.16713315	0.25298434	4					
1	16		Lung	ACTB	18.253523	-9.9706745	0.03890908	-1.375361	2.5943282	2.4519393	2.7449856	5					
	17		Lung	GAPDH	21.026237	'											
	18		Lung	GH1	32.66009)											<u> </u>
		aculta Dati															
ř	veirk	esuits Data															
E	Show	In Table 🔻	Group Res	ults By 🔻											Expand All	Collapse A	
4	#	Sample	Target	Experim	Omit Fla	g RQ	RQ Min	RQ Max	Flags	Ст	ΔСт Д	∆Ст Ме	∆C⊤ SE	ΔΔCT			
		Brain	ACTB	OS6 QuantS						17.265797				NaN			^
		Brain	ACTB	OS6 QuantS						17.17082				NaN			
		Brain	ACTB	OS6 QuantS						17.186125				NaN			
		Brain	ACTB	OS6 QuantS						17.404072				NaN			
		Brain	ACTR	OS6 Quants						17.210124				NaN			
		Brain	GAPDH	OS6 Quants		0.455	29792 0.43082	792 0.4811577	18	17.508968	0	.31446025	0.0337048	1.1351172			
		Brain	GAPDH	OS6 QuantS	n	0.455	29792 0.43082	792 0.4811577	78	17,50408	0	.31446025	0.0337048.	1.1351172			
		Brain	GAPDH	OS6 QuantS		0.455	29792 0.43082	2792 0.4811577	78	17.523808	0	.31446025	0.0337048.	1.1351172			~
Т					-												7

Tips for assessing the gene profile in your own study

Look for:

- Differences in gene expression (as a fold change) relative to the reference sample.
- Standard deviation in the replicate groups (C_T SD values).

If needed, you can omit outliers. See "Omit replicates from the analysis" on page 147.

Note: To display a subset of the study data in the Gene Expression Plot pane, select one or more rows in the Technical Replicates tab or the Biological Replicates tab, then select **Hide unselected data from plot** to display data only from the selected rows.

QuantStudio^m 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments



View the replicate results data and the well results data

The Replicate Results Data pane lists each reaction plate (experiment) that is added to a study. The results of the study are arranged by technical or biological replicate association.

The data that are displayed in the Well Results Data pane depend on which tab you select in the Replicate Results Data pane:

Technical Replicates Tab	Biological Replicates Tab					
This tab arranges the results of the relative quantification analysis by technical replicate group. The ViiA [™] 7 Software displays the results for each sample/target combination as a row in the table. You can view the members of a technical replicate group by	This tab arranges the results of the relative quantification analysis by biological replicate group. The QuantStudio ^{TM} 6 and 7 Flex Software displays the results for each biological group as a row in the table (each row displays a biological sample with its target).					
selecting the appropriate row in the table. When a row is selected, the Well Results Data pane displays the wells that make up the selected technical replicate group.	selecting the appropriate row from the table. When a row is selected:					
Replicate Results Data Technical Replicates Bloogical Replicates Brain ACTB 1 Brain ACTB 17.265017 2 Brain Brain ACTB 1 Brain ACTB 17.265017 2 Brain Brain ACTB 1 Brain ACTB 17.265017 3 Brain Brain ACTB 1 Brain ABrain Life/2820.04109551 6 Heatt 7 Heatt 6 Heatt 9 Heatt 10 Life/2820.04109551 6 Heatt 9 Heatt 11 Life/280.0400051 6 Heatt 9 Heatt 10 Heatt 11 Life/280.040007005293955 12 Life/20H1 13 Life/2740.0400705293955 13 Life/260H1 <tr< td=""><td> The Biological Replicate Details table displays the technical replicate groups that make up the selected biological replicate group. The Well Results Data pane displays the individual members of the technical replicate groups that make up the selected biological replicate groups that make up the selected biological replicate group. Replicate Results Data replicate group. </td></tr<>	 The Biological Replicate Details table displays the technical replicate groups that make up the selected biological replicate group. The Well Results Data pane displays the individual members of the technical replicate groups that make up the selected biological replicate groups that make up the selected biological replicate group. Replicate Results Data replicate group. 					
# Sample Target Experim Omit Flag RQ Brain ACTB OS6 QuantS Brain GAPDH OS6 QuantS O.45529792 Brain GAPDH OS6 QuantS U	Biological Replicate Details Edit BioGroup Delete BioGroup # Omit Sample Target Cr Mean ACT Ve ACT SE 1 Brain ACTB 17.265017 -8.602256 0.0818901 2 Brain ACTB 17.579475 > Vell Results Data Person > > Vell Results Data Experim Omit Flag RQ # Brain ACTB 056.0uantS Person Person Person # Show In Table Group Results By Experim Omit Flag RQ # Brain ACTB 056.0uantS Person Person					

Column descriptions

The table below provides definitions for the column headings that appear in the tables in the Technical Replicates and Biological Replicates tabs.

Note: To show or hide columns in a table, select or deselect the column name from the Show In Table drop-down menu.

Column	Description							
ΔC_T	The calculated ΔC_{T} value for the replicate group associated with the test sample.							
	Note: The ΔC_T value is calculated only for multiplex experiments and is calculated at the well level (that is, the individual technical replicate level) by subtracting the target C_T value from the endogenous control $_{CT}$ value.							
ΔC_T Mean	The arithmetic average of the technical replicate C_T values for the sample replicate group.							
_	Note: The ΔC_T Mean value is calculated at the reaction plate level and represents the mean difference between the target $_{CT}$ values and the endogenous control C_T values for all the technical replicates for that sample that are present on the plate.							
ΔC_T SE	The Standard Error of the mean associated with the reported Mean ΔC_{T} value.							
	Note: The ΔC_T SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level _{CT} value variation between the target and the endogenous control.							
	Note: If you select the Standard Deviation option in the RQ Min/Max calculations on the Relative Quantification Settings in the Analysis Settings dialog box, ΔC_T SD, that is the Standard Deviation values are calculated by the QuantStudio TM 6 and 7 Flex Software.							
$\Delta\Delta C_{T}$	The calculated $\Delta\!\Delta_{CT}$ value for the replicate group associated with the reference sample.							
# Replicates	The number of biological replicate groups in the study.							
Biological Group	The name of the biological replicate group.							
C _T	Threshold cycle; the PCR cycle number at which the fluorescence meets the threshold in the amplification plot.							
C_{T} Mean	The arithmetic average of the technical replicate _{CT} values.							
Experiment	The name of the experiment file (for example, heart.eds).							
Flag	The number of QC flags that the well generated as listed in the $ ightarrow$ symbol.							
Omit (Replicate	Indicates the omission status of the members of the associated technical or biological replicate group(s):							
Results Data pane)	• A check mark (✓) indicates that all replicates have been removed from the analysis.							
	• A hyphen (-) indicates that one or more replicates have been removed from the analysis.							
Omit (Well Results Data pane)	Indicates the omission status of the well. A check mark (\checkmark) indicates that the well has been removed from the analysis.							
RQ	The calculated relative level of gene expression for the replicate group that is associated with the test sample.							

Column	Description
RQ Max	The maximum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box.
	Note: The maximum includes the variability associated with the endogenous control and targets in only the test samples.
RQ Min	The minimum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box.
	Note: The minimum includes the variability associated with the endogenous control and targets in only the test samples.
Sample	The sample associated with the data displayed in the row.
Target	The target assay associated with the data displayed in the row.
Well	The location of the well in the reaction plate.

About the example study, you review the Replicate Results Data pane and the Well Results Data pane to evaluate the C_T precision of the replicate groups and view related RQ information.

View the results data

- 1. From the Study Menu pane, select **Analysis** Gene Expression.
- **2.** Click \leq at the top left of the Replicate Results Data pane.
- 3. View the technical replicates:
 - **a.** Click the **Technical Replicates** tab. The table displays the results by technical replicate group.
 - **b.** In the Technical Replicates table, select the following groups:
 - Brain/GAPDH (row2)
 - Brain/LIPC (row 4)
 - Brain/LPIN1 (row 5)

The Well Results Data pane displays all wells that make up the selected groups.

- 4. View the values in the Well Results Data pane:
 - a. From the Group Results By drop-down menu, select Target.

b. View the C_T , ΔC_T Mean, and ΔC_T SE values to evaluate the C_T precision of the replicate groups. In the example study, the low ΔC_T SE values indicate these replicates have good C_T precision.

>	Replic	ate Resul	ts Data															
	Techn	ical Replicate	s Biolog	ical Replicates														
I	Show	In Table	Add Bi	oGroup End	o Controls	▼ Ref Sar	nple 🔻											
	# O	mit	Sample	Target (Ст Mean	∆Ст Ме	ΔCT SE	ΔΔCτ	RQ	RQ Min	RQ Max							
	1	E	Brain	ACTB	17.265017													^
	2	E E	Brain	GAPDH	17.579475	0.3144583	0.03370482	1.135117	0.45529795	0.43082792	0.48115784							
	3	E 6	Brain	GH1	31.093044	13.828029	0.13150350	.0095443	0.9934062	0.8007919	1.2323499							
	4	E E	Brain	LIPC	28.929298	11.664282	0.0410995	-1.1238337	2.1792529	2.0372863	2.3311124							
	5	E 6	Brain	LPIN1	21.73203	4.467014	0.0398248	-1.1272674	2.1844459	2.0464118	2.3317904							
	6	- H	leart	ACTB	18.136377													
	7		leart	GAPDH	17.315718	-0.8206588	0.0384727	0.0	1.0	0.9388889	1.0650887							
	8		leart	GH1	31.95486	13.818484	0.08681472	0.0	1.0	0.8673678	1.1529135							
	9	H	leart	LIPC	30.924492	12.7881155	0.0554872	0.0	1.0	0.9130675	1.0952092							
	10		leart	LPIN1	23.730658	5.594281	0.0346003	0.0	1.0	0.94486696	1.0583501							
	11		.iver	ACTB	19.201889													
	12		.iver	GAPDH	19.181273	-0.020617	0.05293955	0.8000416	0.5743326	0.5265989	0.62639326							
	13		.iver	GH1	35.452633	16.25408	0.238/6446	2.4355955	0.18484/12	0.12329351	0.2//131							
. L	14		Iver	LIPC	21.993778	2.791889	0.0500186	-9.996226	1021.32495	940.9349	1108.5833							~
ł																		
	Well R	esults Da	ta															
	Show	In Table 😗	Group F	Results By 🔻												Expand All	Collapse	All
	#	Sample	Target	Experim	. Omit	Flag	RQ	RQ Min	RQ Max	Flags	Ст	∆Ст	∆Ст Ме	ΔCT SE	ΔΔCT			
		Brain	ACTB	OS6 Quant	s 🗆						17,2657	97			NaN			~
1		Brain	ACTB	OS6 Quant	s 🗆						17.170	82			NaN			
1		Brain	ACTB	OS6 Quant	s 🗌						17.1861	25			NaN			
1		Brain	ACTB	OS6 Ouant	s 🗆						17.4040	72			NaN			_
1		Brain	ACTB	OS6 Quant	s 🗌						17.2161	24			NaN			
1		Brain	ACTB	OS6 Ouant	s 🗆						17.3471	49			NaN			
1		Brain	GAPDH	OS6 Quant	s 🗌		0.4552979	92 0.430827	92 0.481157	78	17.5089	68	0.31446025	0.0337048	1.1351172			
1		Brain	GAPDH	OS6 Quant	s 🗆		0.4552979	92 0.430827	92 0.481157	78	17.504	08	0.31446025	0.0337048.	1.1351172			
		Brain	GAPDH	QS6 Quant	<u>s</u>		0.4552979	92 0.430827	92 0.481157	78	17.5238	08	0.31446025	0.0337048.	1.1351172			~

Tips for viewing replicate results in your own study

- Select the **Technical Replicates** tab or the **Biological Replicates** tab to organize and view the sample data according to the associated technical replicate group or biological replicate group.
- View all wells for a technical or biological replicate group by selecting the appropriate row in the table. When a row is selected, the Well Results Data pane displays the wells that make up the group. **Ctrl-click** to select multiple rows.
- Change the endogenous control by clicking **Endo Control**, then selecting a new target.
- Change the reference sample by clicking **Ref Sample**, then selecting a new sample.
- Add biological replicate groups by clicking Add BioGroup.
- Omit biological or technical replicates from the analysis. See "Omit replicates from the analysis" on page 147.

Note: The QuantStudio 384 Well Comparative Ct with Bioreplicates Study Example.edm file demonstrates the use of biological replicate groups. You can also create example study that does not use biological replicate groups.

Omit replicates from the analysis

To omit a technical or biological replicate from the analysis:

- 1. From the Study Menu pane, select **Analysis** Gene Expression.
- **2.** Select the **Technical Replicates** or **Biological Replicates** tab according to the type of replicate that you want to omit.
- **3.** In the replicate table, scroll to the biological or technical replicate of interest, then select the corresponding check box in the Omit column.



4. Click **Analyze** when you finish omitting wells.

IMPORTANT! You cannot omit *all* technical replicates that belong to a reference sample or a reference biological group, or that serve as the endogenous control for a study.

Note: You can also omit the biological replicates in the Biological Replicate Details table at the bottom of the Biological Replicates tab.

Confirm accurate dye signal using the Multicomponent Plot

	The Multicomponent Plot screen displays the complete spectral contribution of each dye over the duration of the PCR run in a selected well of any experiment that is added to the study.
About the example study	 In the comparative C_T example study, you review the Multicomponent Plot screen for: ROX[™] dye (passive reference) FAM[™] dye (reporter) Spikes, dips, and/or sudden changes Amplification in the negative control wells
View the	1. From the Study Menu pane, select Analysis > Multicomponent Plot .
Multicomponent Plot	 In the Experiment Data pane, select the QuantStudio Comparative Ct Example 1.eds experiment.
	3. Display the unknown wells one at a time in the Multicomponent Plot pane:a. Click the Plate Layout tab.
	b. Select one well in the plate layout; the well is shown in the Multicomponent Plot pane.
	Note: If you select multiple wells, the Multicomponent Plot pane displays the data for all selected wells simultaneously.
	4. In the Multicomponent Plot pane, set the parameters for the plot:
	a. From the Plot Color drop-down menu, select Dye.
	b. Click Show a legend for the plot .
	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 study, the FAM dye signal increases throughout the PCR process, which indicates normal amplification.
	6. Check the ROX dye signal. In the example study, the ROX dye signal remains constant throughout the PCR process, which indicates typical data.



7. Repeat steps 2 through 6 for the remaining experiments in the study.

Tips for confirming dye-signal accuracy in your own studies

- **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 fluorescence** There should not be any spikes, dips, and/or sudden changes in the fluorescence.
- **Negative control wells** There should not be any amplification in the negative control wells.

If your study does not meet the guidelines above, you can omit individual wells from the analysis. See "Omit wells from the analysis" on page 164.

View the QC Plots

The QC Plots screen displays the endogenous control and replicate analysis results for each reaction plate (experiment) added to the study. The following plots are derived from the experiments added to a study:

- Endogenous Control Profile
- Box Plot
- Technical Replicates Correlation
- Biological Replicates Correlation



View the Endogenous Control Profile plot

The QC Plots screen displays the Endogenous Control Profile plot for the endogenous controls used in the experiments added to a gene expression study. The endogenous control profile plot displays how much of the endogenous control is expressed in a sample. The sample is plotted on the X-axis, and the C_T is plotted on the Y-axis. The expression is viewed as a color and shape combination in the plot.

In the example study, you view four potential endogenous controls expressed in four samples. The potential endogenous controls are:

- ACTB
- GH1
- LIPC
- LPIN1

The samples are brain, heart, lung, and liver.

- 1. From the Study Menu pane, select **Analysis** > **QC Plots**.
- 2. In the QC Plots pane, click Endogenous Control Profile.
- **3.** In the **Candidate Endogenous Controls** pane, select the check boxes of those Targets whose profile you want to view in the plot pane. In the example study, the **Target** ACTB is chosen to be the endogenous control because it is expressed at similar levels in three out of four of the given samples.
- 4. In the Replicate Results Data pane, view results by replicate group.
 - **a**. Click the **Technical Replicates** tab. The table displays the results by technical replicate group.
 - **b.** Click the **Biological Replicates** tab. The table displays the results by biological replicate group.



View the box plotsThe Box Plot displays the C_T distribution of a particular Target in samples. You can see
the individual C_T values/ raw data with this plot.

In the example study, you view the box plots of five targets in four different samples:

- LPIN1
- GAPDH
- LIPC
- GH1
- ACTB

The samples are Heart, Liver, Lung, and Brain.

- 1. In the QC Plots pane, click **Box Plots** to access the Replicate Results Data pane.
- **2.** In the Replicate Results Data pane, click the **Technical Replicates** tab. The table displays the results by technical replicate group.
- **3.** Click the **Biological Replicates** tab. The table displays the results by biological replicate group.
- 4. Click the Experiments tab to select the experiment whose Box plot to view.
- **5.** View the values in the Well Results Data pane. View the C_T , ΔC_T Mean, and ΔC_T SE values to evaluate the C_T precision of the replicate groups. In the example study, the low ΔC_T SE values indicate these replicates have good C_T precision.



View the Technical Replicates Correlation plot The Technical Replicates Correlation plot displays the correlation between the target genes in one or more samples.

The Technical Correlation Group plot is made of two components, the scatter plot and the heat map.

Scatter Plot

The scatter plot shows the distribution of ΔC_T of targets for different samples.

If a correlation (represented by R² or the coefficient of determination) exists among the targets in the samples, the targets appear on or along the line of reference. If the correlation is weak or not present, the targets appear scattered in the plot, away from the line of reference.

The line of reference is fixed in the QuantStudio[™] 6 and 7 Flex Real-Time PCR Development Software.



Scatter Plot

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If:

- R² =/≈ 1, then the correlation is strong
- R² < 1, then the correlation is weak
- R² = 0, then there is no correlation

Heat maps

The heat map shows the variation of coefficient determination for different scatter plots. Each cell of the heat map represents a different scatter plot, and therefore a different



value for R². The cells inclined to red represent a lower R² value; the cells inclined to green represent a higher R² value.

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In the example study, you view the scatter plots and heat maps of five targets in four different samples:

- LPIN1
- GAPDH
- LIPC
- GH1
- ACTB

The samples are Brain, Heart, Kidney and Lung.

1. In the QC Plots pane, click **Technical Replicates Correlation** to access the Heat Map.



In the Heat Map, click the cell with a correlation value, R² =/≈ 1. The corresponding scatter plot pane displays the scatter plot of the targets in that technical replicate group along the line of reference.



3. Click the cell with a correlation value, $R^2 = /\approx 0$. The corresponding scatter plot pane displays the scatter plot of the targets in that technical replicate group away from the line of reference.



View the Biological Group Correlation plot

3

The Biological Group Correlation plot displays the correlation between the target genes in one or more biological group samples. Biological groups provide a broader set of samples, with the same targets.

Note: If the experiments in your study do not use biological replicate groups, see "Define Replicates" on page 128 to create a new biological group.

The Biological Group Correlation plot is also made of two components, the scatter plot and the heat map.

Scatter Plot

The scatter plot shows the distribution of ΔC_T of targets for different biological groups. If a correlation (represented by R² or the coefficient of determination) exists among the targets in the biological groups, the targets appear on or along the line of reference. A weak correlation or no correlation is represented by the targets being scattered in the plot and away from the line of reference.



Note: The line of reference is fixed in the

QuantStudio[™] 6 and 7 Flex Real-Time PCR Development Software.

- If:
 - R² =/≈ 1, then the correlation is strong
 - R² < 1, then the correlation is weak
 - R² = 0, then there is no correlation





Heat maps

The heat map shows the variation of coefficient determination for different scatter plots. Each cell of the heat map represents a different scatter plot, and therefore a different value for R². The dull green cells represent a lower R² value; the bright green cells represent a higher R² value.

In the example study, you view the scatter plots and heat maps of eight targets across four biological groups:

- LPIN1
- GAPDH
- LIPC
- GH1
- ACTB

Technical replicate samples of Brain cDNA belong to the biological group Brain, those of Lung cDNA belong to biological group Lung, technical replicate samples of Liver cDNA belong to the biological group Liver, and those of Heart cDNA belong to the biological group Heart.

To view the Biological Group Correlation plot:

1. In the QC Plots pane, click **Biological Group Correlation** to access the heat map.

	🛄 Show i	n Wells 🔻 📲 View Leg	gend		•	
		Heart	Lung	Brain	Liver	1.00
	Heart	1.0	0.957	0.985	0.5494	0.9015 0.803
	Lung	0.957	1.0	0.9779	0.5737	0.7045 0.6061 0.5076
	Brain	0.985	0.9779	1.0	0.6041	0.4091 0.3106
	Liver	0.5494	0.5737	0.6041	1.0	0.2121 0.1136 0.0152

In the Heat Map, click the cell with a correlation value, R² =/≈ 1. The corresponding scatter plot pane displays the scatter plot of the targets in that biological replicate group along the line of reference.



3. Click the cell with a correlation value, $R^2 = /\approx 0$. The corresponding scatter plot pane displays the scatter plot of the targets in that biological replicate group away from the line of reference.



Tips for viewing your own scatter plots and heat maps

When you analyze your study, look for scatter plots and heat maps that display a correlation value that is $=/\approx 1$ for samples that come from the same source or tissue (technical or biological replicates). If the replicates do not correlate well, that could be a sign that there is a problem with a sample.

If your study does not meet the guidelines above, you can omit individual wells from the analysis. See "Omit wells from the analysis" on page 164.

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View the QC Summary

The QC Summary screen displays a list of the QuantStudio[™] 6 and 7 Flex Real-Time PCR Development Software flags, and it includes the flag frequency and location for any experiment that is added to a study.

About the example study and the Comparative C_T example study, you review the QC Summary screen for any flags generated by the study data. In the example study, several wells produced data that generated flags.

View the QC Summary 1. From the Study Menu pane, select **Analysis** • **QC Summary**.

Note: If no data are displayed, click Analyze.

2. In the Flags Summary table, look in the Frequency column to determine which flags appear in the study. In the example study. The EXPFAIL flag appears 10 times and the NOAMP flag appears once.

Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the study.

- **3.** For each flag that appears in the study, click the flag row to display details about the flag in the Flag Details table. In the example study, the NOAMP flag indicates no amplification and the EXPFAIL flag indicates that the exponential algorithm failed.
- **4.** Consider removing the NOAMP well from the analysis. See "Omit wells from the analysis" on page 164.

Flag Summary															
Flag					Name					Free	quency				
Experiment Flag	s - 2 of 11 Foun	d													
SPIKE				N	loise spikes					0					
BADROX				ad passive ref	ference signa				0	0					
NOAMP				lo amplification 1											
CTFAIL				T algorithm fa	iled				0						
BLFAIL				E	Baseline algorithm failed 0										
EXPFAIL				E	xponential alg	orithm failed				10	10				
HIGHSD				H	ligh standard	deviation in r	eplicate group)		0					
NOSIGNAL				N	lo signal in we	ell				0					
NOISE				N	loise higher th	nan others in	plate			0					
OFFSCALE				F	luorescence is	s offscale				0					
AMPNC				A	mplification in	negative con	ntrol			0					
Replicate Flags -	0 of 2 Found														
THOLDFAIL				1	Thresholding a	lgorithm faile	d			0					
OUTLIERRG				C	Outlier in replic	ate group				0					
A T															
Flag Details															
F lag:	NOAMP-No a	mplification													
Flag Detail:	The sample d	id not amplify	1												
Flag Criteria:	Amplification	algorithm res	ult < 0.1												
-	View NOAMP 1	Froubleshooti	na												
	Information	ino dible shie o di	iiig.												
Show In Table	Group Res	ults By 🔻												Expand All	Collapse All
# Sample	Target	Experim	Flag	RQ	RQ Min	RQ Max	Flags	Ст	∆Ст	∆Ст Ме	ACT SE	ΔΔCT	Well	Omit	
Liver	GH1	OS6 QuantS		0.18484712	0.12329351	0.277131	NOAMP	Undetermi		16.25408	0.23876446	2.4355956	G11		

Possible flags

For Comparative C_T studies, the flags listed below may be generated by the study data.
If a flag does not appear in the study, its frequency is 0. If the frequency is >0, the flag appears somewhere in the study, and the associated well position is listed in the Wells column.

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
Se	econdary analysis flags
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group

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Tips for using flags to evaluate your study
 In the Flag Summary table, click each flag that has a frequency >0 to display details about the flag in the Flag Details table. If needed, click the troubleshooting link in the Flag Details table to view information on correcting the flag.
 Note: In the Flag Details table, the numbers on each flag symbol indicate the number of flags generated for that well. For example, ▲ indicates that two flags

have been generated for that well.You can change the flag settings. For more information, see "Flag Settings" on page 136:

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags that are applied by the QuantStudio[™] 6 and 7 Flex Real-Time PCR Development Software.
- You can omit individual wells from the analysis. See "Omit wells from the analysis" on page 164.

Compare analysis settings

Use the Compare Settings screen to perform a side-by-side comparison of analysis settings for a comparative _{CT} study. You can change one or more of the analysis settings, then compare the new results with the previous results. For example, you can compare the effects of:

- Using multiple endogenous controls versus a single endogenous control.
- Changing the amplification efficiency of a specific target versus keeping it at 100%.

About the example
studyIn the comparative CT example study, you change the endogenous control to LPIN1,
then compare results.

Modify comparison
criteria1. From the Study Menu pane, select Analysis > Compare Settings. When the
Compare Settings screen is initially displayed (before you make any changes):

- In the Settings Comparison pane, the green column is titled "Current Analysis Settings," the white column is titled "Comparison Analysis Settings," and the Use Comparison Analysis Settings button is under the green column.
- In the Results Comparison pane, values in the white columns and values in the green columns are the same.
- The Gene Expression Comparison plot is the same plot that is displayed in the Gene Expression screen (Analysis > Gene Expression).

Gene Ex	pression			< Settings	Compariso	n								
	Gene Expres	sion Compa	rison	Settings		Current	: Analysis Settin	gs Edit	Compari	son Analysis Se	ettings Edi	t		
1,200				Target S	ettings (Thre	shold, Baseline	Start, Baseline	End)				_		^
1 150				GH1		AUTO,	AUTO, AUTO		AUTO, J	AUTO, AUTO				
1,100				GAPD	н	AUTO,	AUTO, AUTO		AUTO,	AUTO, AUTO				
1,100 ·				LPIN1		AUTO,	AUTO, AUTO		AUTO, A	AUTO, AUTO				
1,050				ACTB		AUTO,	AUTO, AUTO		AUTO, A	Αυτο, Αυτο				
1.000				LIPC		AUTO,	AUTO, AUTO		AUTO, A	Αυτο, Αυτο				
				Flag Sett	ings									_
950 -				SPIKE		USED S	pike algorithm	result>1.0	USED SI	oke algorithm	result>1.0			
900	•			BADR	JX ID	USED B	ad passive refe	rence algorith	IM USED B	od passive refe	rence algoritr	im		
850				NUAM	P	USED A	Amplification alg	orithm result<	USED A	mplification alg	orithm result.	<0		
000				DIEAT	L	USED			USED					
800				EYDEA		USED			USED					
750				EXF17		0520			0320					×
700									Use	Comparison .	Analysis Set	tinas		
650				.										
ø														
D2 600				Results C	omparison	1								1
550 ·				Entern filte	, august the set	eliele "Apple El	tor "							
500				Enter a filte	r query, then	г сіск Арріу Ні	uer.							
450				IF Sample	~ =		*					Apply F	iter Rem	ove Filter
												-		
400				Show In	Table 🔻	Hide Compari	son Filter							
350				(
300				#	Sample	Target	Ст Меа	Ст Меа	∆Ст Ме	∆Ст Ме	ΔCT SE 1	∆C⊤ SE 2	ΔΔC⊤ 1	ΔΔСт 2
250				1	Brain	ACTB	17.265	17.265						^
				2	Brain	GAPDH	17.579	17.579	0.314	0.314	0.034	0.034	1.135	1.
200				3	Brain	GH1	31.093	31.093	13.828	13.828	0.132	0.132	0.01	(
150 -				4	Brain	LIPC	28.929	28.929	11.664	11.664	0.041	0.041	-1.124	-1.
100				5	Brain	LPIN1	21.732	21.732	4.467	4.467	0.04	0.04	-1.127	-1.
50				6	Heart	ACTB	18.136	18.136						
50				/	Heart	GAPDH	17.316	17.316	-0.821	-0.821	0.038	0.038	0	
0		<u>ه</u> ر		8	Heart	UDC	31.955	31.955	13.818	13.818	0.08/	0.08/	0	
	Bialt	's. Me.	Luns	10	Heart	LIPC LDIN1	30.924	30.924	12.788	5 504	0.055	0.055	0	
		Sample			inearc	LFINI	23.731	23./31	3.394	5.594	0.055	0.055	0	~
		Gampie					<		Ш					>

2. In the Settings Comparison pane, click **Edit** in the green column to open the Comparison Analysis Settings dialog box.

- **3**. In the Comparison Analysis Settings dialog box, change the endogenous control:
 - a. Select the **Relative Quantification Settings** tab.
 - **b.** In the Endogenous Control(s) pane, select **LIPN1** from the Endogenous Control drop-down menu.



c. Click **Analyze** to analyze the data and close the dialog box.

ettings Flag Settings Relative Quantifical	tion Settings					
Comparative CT Analysis Settings						2
Select the type of applying to perform						
Multiplex Singlepley						
Paterance Cample(c)						
Colorisation of the second sec			and a state			
Biological Replicate Group Reference Sample:		groups or this st	uay.		Technical Replicate Group Reference Sample:	Heart
						loure
Called the terret to use of the and a sector to the						
Select the target to use as the endogenous control to	r this experime	nt.				
Endogenous Control: ACTB						
Efficiency						
	Target	Efficien	Efficien	Override	Std/Pcc	
	ACTR	100.0	0.0	Override	Sturist	
Enter percentage values between 1 and 150%	GAPDH	100.0	0.0	V		
	GH1	100.0	0.0	Image: A start of the start		
	LIPC	100.0	0.0	1		
	L DTN11	100.0	0.0			
Outlier Rejection						
Select to reject replicates with $\Delta C\tau$ values less than o	r equal to the v	value entered be	low. These anal	ysis settings ap	oply only to multiplex reactions.	
Reject Replicates with specified ΔCT						
107 < 1.0						
201 2 1.0						
RQ Min/Max Calculations						
Select an algorithm to determine RQ Min and Max valu	les (error bars)					
Onfidence Level: 95.0 ♥ %						
O Standard Deviations: 1 V						

- **4.** In the Settings Comparison pane, click **Use Comparison Analysis Settings** under the green column, then compare the results:
 - In the Settings Comparison pane, the white column is titled "Comparison Analysis Settings," the green column is titled "Current Analysis Settings," and the Use Comparison Analysis Settings button is under the white column.
 - In the Results Comparison pane, values in the white columns are based on the default analysis settings, and values in the green columns are based on the modified analysis settings. In the example experiment, changing the endogenous control to 18S affects the RQ values. To view the RQ values, scroll to the left. If desired, you can click and drag the RQ column headings so that they appear first in the table.
 - The Gene Expression Comparison plot displays the default analysis settings (the values from the white columns).

Note: The Gene Expression Comparison plot has limited functions. For example, you cannot change to log scale and you cannot view by target.

Note: The default analysis settings are the settings automatically made by the software when the study is initially analyzed.

Gene Expression	< Settings	Compariso	n								
Gene Expression Comparison	> Settings		Current	Analysis Settin	gs Edit	Compari	son Analysis Se	ettings Edi	t		
825 800 775 750 725	GH1 GAPI LPIN ACTI	Settings (Thre DH 1 3	shold, Baseline (AUTO, / AUTO, / AUTO, / AUTO, / AUTO, /	Start, Baseline AUTO, AUTO AUTO, AUTO AUTO, AUTO AUTO, AUTO	End)	AUTO, AUTO,	AUTO, AUTO AUTO, AUTO AUTO, AUTO AUTO, AUTO				
700	E Eba Set	tings	AUTO, A	AUTO, AUTO		AUTO, I	AUTO, AUTO				
650 625 600 675 550	SPIK BADI NOA CTF/ BLFA	E ROX MP IL IL	USED Sp USED Ba USED Ar USED USED USED	pike algorithm ad passive refe mplification alg	result>1.0 rence algorith orithm result<	USED S Im USED B <0 USED A USED USED USED	oike algorithm ad passive refe mplification alg	result>1.0 rence algoriti orithm result	ייי יישר כוויי		
525 500 475 470		Use Comparison Analysis Setting									¥
G 425 400	Results	Comparison									
375	Enter a filt	er query, then	click "Apply Filt	er."					Apply Fi	Iter	ovo Filtor
300 275 260	Show In	Table 🔻 🚺	Hide Comparis	on Filter							
225	#	Sample	Target	Ст Меа	Ст Меа	∆Ст Ме	∆Ст Ме	ΔCT SE 1	ΔCT SE 2	ΔΔCτ 1	∆∆Ст 2
175	1 2 3	Brain Brain Brain	ACTB GAPDH GH1	17.265 17.579 31.093	17.265 17.579 31.093	-4.467 -4.153 9.361	0.314	0.04	0.034	1.127 2.262 1.137	1.
120	4 5	Brain Brain	LIPC LPIN1	28.929 21.732	28.929 21.732	7.197	11.664 4.467	0.036	0.041	0.003	-1. -1.
50 25	. 6 . 7 . 8	Heart Heart Heart	ACTB GAPDH GH1	18.136 17.316 31.955	18.136 17.316 31.955	-5.594 -6.415 8.224	-0.821 13.818	0.035 0.04 0.088	0.038	0	
Bally Hest June -	9 10	Heart Heart	LIPC LPIN1	30.924 23.731	30.924 23.731	7.194	12.788 5.594	0.057	0.055	0	~
Sample				<							>

5. From the Study Menu pane, select **Analysis** > **Gene Expression** to view the gene expression plot using the modified analysis settings.

Note: In the Gene Expression screen you can view the modified data in log scale, by target, and so on. See "Assess the gene expression profile using the Gene Expression Plot" on page 140.

- **6.** (*Optional*) View the data in the other analysis screens. All other analysis screens for the study display the data using the modified analysis settings.
- 7. Close the study.
 - Save your changes before closing the study. Or
 - Close the study without saving your changes. If you do not save your changes, the software reverts to the default analysis settings the next time you open the study.



Tips for managing vour own	• Edit the comparison analysis settings as desired. For information on editing the settings, see "View the analysis settings" on page 134.
comparison	 After making your first round of changes to the analysis settings, you can continue making changes using one of the following methods:
	 (Recommended) Revert to the saved analysis settings, then make new changes. To do this: In the Settings Comparison pane, click Use Comparative Analysis Settings (now under the white column) to revert to the saved analysis settings, then repeat steps 2 through 6 above. This method ensures that you do not lose the saved analysis settings.
	Note: If you have made changes, but have not saved them, the software reverts to the default analysis settings when you click Use Comparative Analysis Settings . The default analysis settings are the settings automatically made by the software when the study is initially analyzed.
	 Continually compare new settings with previous settings. To do this: In the Settings Comparison pane, alternate clicking Edit in the white and green columns, then repeat steps 3 through 6 above. This method does not allow you to return to your saved settings; subsequent comparisons are made with the previous analysis settings, building upon any changes that you have already made.
Revert to the default analysis settings	IMPORTANT! The default analysis settings are defined by the software. If you make changes to the analysis settings and save the study, the saved changes are lost when you revert to the default analysis settings.
5	1. In the Settings Comparison pane, click Edit next to the settings you want to revert to the default: <i>Current Analysis Settings</i> or <i>Comparison Analysis Settings</i> .
	2. In the Analysis Settings dialog box, revert to defaults and reanalyze the data:
	a. Click Revert to Default Analysis Settings.
	b. At the prompt, click Yes .
	Warning You have selected to revert to Default Analysis Settings. Are you sure you want to continue?
	c. Click Analyze to analyze the data and close the dialog box.
	3. In the Settings Comparison pane, click Use Comparison Analysis Settings . In the Results Comparison pane, values for the settings you selected to edit in step 1 ("Current Analysis Settings" or "Comparison Analysis Settings") are generated according to the default analysis settings.
Omit wells from	You can use the Well Table to omit individual wells from the analysis. To omit a well:
the analysis	1. From the Study Menu pane, select one of the following analysis screens:
	Analysis > Amplification Plot
	 Analysis > Multicomponent Plot
	 Analysis > Multiple Plots View
	2. In the Experiment Data pane, select the experiment that contains the well to omit.

3. In the Well Results Data pane, click the **Well Table** tab, then select the check box in the Omit column for the well to omit.

Note: You cannot omit all technical replicates that belong to a reference sample or a reference biological group, or that serve as the endogenous control.

Export the study

Note: If you are using RealTime StatMiner[®] Software to analyze the exported data, make sure you have assigned a sample to all the wells containing data in the individual experiments. If a sample is not assigned, the RealTime StatMiner[®] Software reports an error during import.

You can export the data within a study from the Analysis screen. To export a study:

- 1. On the Analysis screen, click Export to access the Export Properties tab.
- 2. Define export properties.
 - a. Select the data to export. Options are:
 - Amplification Data
 - Results
 - Technical Analysis Result
 - BioGroup Analysis Result
 - QC Summary
 - **b.** Select **One File** or **Separate Files** from the drop-down menu to export all data to one file or in separate files for each data type respectively.
 - c. Enter the export file properties and file name.
 - **d.** Select the file type from the **File Type** drop-down menu. You can choose from ***.txt**, ***.xls**, and ***.xlsx**.
 - e. Enter the Export File Location. The default location is C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\Export.
 - f. Select the Open file(s) when export is complete check box to automatically open the file when export is complete.

For the example study, enter:

Field or Selection	Entry
Select Data to export	Amplification Data
Select one file or separate files	One File
Export File Name	QS6_384- Well_Comparative_Ct_with_Bioreplicates_Study_Example_data
File Type	*.txt
Export File Location	C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\Export



Field or Selection	Entry
Open file(s) when export is complete	Unchecked
Save current settings as the default	Unchecked
7900 Format	Unchecked

The following is an image of the Export Data screen:

Export Data							
Select the type of export. Click "Sta	data to export, select whether to export one file or separate files, then enter export file properties. (Optional) Click "Customize Export" to change the export Export" to export your data.	format and to select fields to					
Export Propertie	Customize Export						
1. Select data	export: Amplification Data Results Technical Analysis Result BioGroup Analysis Result QC Summary						
 Select one f Enter export 	or separate files: One File Select to export all data to one file or in separate files for each data type. ile properties:						
Export File Name: QS6_384-Well_Comparative_Ct_with_Bioreplicates_Study_Example_data File Type: 👔 (
Export File Loc	ion: C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\Export	Browse					
Dpen file(s)	hen export is complete						
Save current set	ngs as the default 🔲 7900 Format	Start Export Cancel					

3. To change the export format, complete the tasks on the Customize Export tab.

Note: To complete the tasks on the Customize Export tab you must have at least one type of data to export.

- **a.** Select the data from the Export Properties tab. The type of data that you selected in the Export Properties tab appears in the Customize field in the Customize Export tab.
- **b.** Select the data type content.
- **c.** Select the Tabs or Commas radio button to select the Field Separator (Delimiter).
- **d.** Select the Open file(s) when export is complete check box to automatically open the file when export is complete.

For the example study, enter:

Field or Selection	Entry
Customize	Amplification Data
Field Separator (Delimited)	Commas
Open file(s) when export is complete	Unchecked
Save current settings as the default	Unchecked
7900 Format	Unchecked

The following is an image of the Export Data screen for customized export of Amplification Data:

export your data.								
omize Export								
)ata 🗙			File Name: Q	S6 384-We	ell Comparative Ct	with Biore	plicates Study Exam	nple data 🛛 File Type: 📳
				_			_ /_	
t Amplification Data	a Export							
Experim Well	Cvcle	Sample	Target	Rn	ΔRn	Omitted	Sample	
OS6 Quan A1		1 Brain	ACTB		0.002		10	A
OS6 Ouan A1		2 Brain	ACTB		0.001	H	10	
OS6 Ouan A1		3 Brain	ACTB		0.001	Ē	10	
OS6 Ouan A1		4 Brain			0.002	П	10	
OS6 Ouan A1		5 Brain			9.776026E-4	Ē	10	
QS6 Quan A1		6 Brain	ACTB		-6.325745		10	
QS6 Quan A1		7 Brain	ACTB		-0.002	Π	10	
QS6_Quan A1		8 Brain	ACTB		-0.001		10	
QS6_Quan A1		9 Brain	ACTB		-0.001		10	
0.05 0 14		10 Brain	ACTB		-0.001		10	
QS6_Quan A1	OS6 Quan A1		ACTB		-0.001		10	
QS6_Quan A1 QS6_Quan A1			ACTR		4 064515		10	
QS6_Quan A1 QS6_Quan A1 QS6_Quan A1		12 Brain	ACTB		-1.501313			
QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1		12 Brain 13 Brain	ACTB		7.3627447		10	
QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1		12 Brain 13 Brain 14 Brain	ACTB ACTB		7.3627447		10 10	
QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1		12 Brain 13 Brain 14 Brain 15 Brain	ACTB ACTB ACTB ACTB		7.3627447 0.003 0.010		10 10 10	
QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1		12 Brain 13 Brain 14 Brain 15 Brain 16 Brain	ACTB ACTB ACTB ACTB ACTB		7.3627447 0.003 0.010 0.023		10 10 10 10	
	Data ♥ t Amplification Data Experim Well QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1 QS6_Quan A1	Data ♥ Data ♥ t Amplification Data Export Experim Well Cycle QS6_Quan A1 QS6_Quan A1	Data ▼ Table Cycle Sample Cycle	Data ▼ Fie Name: Q Data ▼ Fie Name: Q t Amplification Data Export Experim Well Cycle Sample Target QS6_Quan A1 2 Brain ACTB QS6_Quan A1 3 Brain ACTB QS6_Quan A1 5 Brain ACTB QS6_Quan A1 6 Brain ACTB QS6_Quan A1 7 Brain ACTB QS6_Quan A1 7 Brain ACTB QS6_Quan A1 9 Brain ACTB QS6_Quan A1 9 Brain ACTB QS6_Quan A1 9 Brain ACTB	Data ▼ File Name: QS6_384-Wi The Name: QS6_384-Wi Amplification Data Export Experim Well Cycle Sample Target Rn QS6_Quan A1 2 Brain ACTB QS6_Quan A1 2 Brain ACTB QS6_Quan A1 4 Brain ACTB QS6_Quan A1 5 Brain ACTB QS6_Quan A1 6 Brain ACTB QS6_Quan A1 7 Brain ACTB QS6_Quan A1 9 Brain ACTB QS6_Quan A1 9 Brain ACTB QS6_Quan A1 9 Brain ACTB QS6_Quan A1 9 Brain ACTB QS6_Quan A1 9 Brain ACTB	Capate Yold oble. Imize Export Data ▼ File Name: Q56_384-Well_Comparative_Ct Amplification Data Export Experim Well Cycle Sample Target Rn ARn Q56_Quan A1 1 2 Sequan A1 2 Brain ACTB 0.001 Q56_Quan A1 2 Brain Q56_Quan A1 5 Brain Q56_Quan A1 5 Brain Q56_Quan A1 6 Brain Q56_Quan A1 7 Brain Q56_Quan A1 9 Brain Q56_Quan A1 9 Brain Q56_Quan A1 9 Brain Q56_Quan A1 9 Brain	Data ✓ File Name: QS6_384-Well_Comparative_Ct_with_Blore Data ✓ File Name: QS6_384-Well_Comparative_Ct_with_Blore t Amplification Data Export Experim Well Cycle Sample Target Rn Omitted QS6_Quan A1 2 Brain ACTB 0.001	Data ✓ File Name: Q56_384-Well_Comparative_Ct_with_Biorepicates_Study_Example Data ✓ File Name: Q56_384-Well_Comparative_Ct_with_Biorepicates_Study_Example t Amplification Data Export: Experim Well Cycle Sample Target Rn ARn Omitted Sample Q56_Quan A1 1 Brain ACTB 0.001 10 10 Q56_Quan A1 2 Brain ACTB 0.001 10 10 Q56_Quan A1 5 Brain ACTB 0.002 10 10 Q56_Quan A1 5 Brain ACTB 9.776026E4 10 10 Q56_Quan A1 6 Brain ACTB -6.325745 10 10 Q56_Quan A1 8 Brain ACTB -0.001 10 10 Q56_Quan A1 9 Brain ACTB -0.001 10 10 Q56_Quan A1 9 Brain ACTB -0.001 10 10 Q56_Quan A1 9 Brain ACTB -0.001 <td< td=""></td<>

- 4. Select the **Save current settings as the default** check box to save the export settings that you have modified. Alternatively, select the **7900 Format** check box to save the export settings in the 7900 format.
- 5. Click Start Export.

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For more information

For more information on	Refer to	Publication number
Calculating Relative Quantification Values	User Bulletin #2: Relative Quantitation of Gene Expression.	4303859
Consumables	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7 Flex</i> <i>Real-Time PCR System Software Experiments</i>	4489822
	Appendix A in Booklet 7, <i>QuantStudio[™] 6 and 7 Flex Real-Time PCR</i> System Software Experiments - Appendixes	
Publishing data	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7 Flex</i> Real-Time PCR System Software Experiments.	4489822

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USER GUIDE



QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Genotyping Experiments

Booklet 4

Publication Number 4489822 **Revision** A



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About Genotyping Experiments

This chapter covers:

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5' nuclease assay	6
Minimizing non-specific fluorescence	7
Reading and analyzing the plates	7
About the example experiment	8

IMPORTANT! First-time users of the QuantStudioTM 6 and 7 Flex Real-Time PCR System Software please read Booklet 1, *Getting Started with QuantStudio*TM 6 and 7 Flex Real-Time PCR System Software Experiments and Booklet 7, *QuantStudio*TM 6 and 7 Flex Real-Time PCR System Software 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[™] 6 and 7 Flex Real-Time PCR System Software by pressing F1, clicking ? in the toolbar, or selecting **Help > QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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 software calculates the delta Rn (Δ Rn) value per the following formula:

 $\Delta Rn = Rn (post-PCR read) - Rn (pre-PCR read), where Rn = normalized readings.$

About TaqMan [®] SNP Genotyping assays	A Genotyping assay detects variants of a single nucleic acid sequence, without quantifying the target. The presence of two probes in each reaction allows Genotyping of the two possible variants at the single nucleotide polymorphism (SNP) site in a target sequence.
	Each TaqMan [®] SNP Genotyping Assay consists of a single, ready-to-use tube containing:
	Two sequence-specific primers for amplifying the polymorphism of interest
	 Two allele-specific TaqMan[®] MGB probes for detecting the alleles for the specific polymorphism of interest
About TaqMan [®]	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 [™] dye-labeled probe corresponds to the second nucleotide inside the square brackets of the context sequence in the AIF. For the context sequence ATCGATT[G/T]ATCC, the VIC [®] dye-labeled probe binds to the G allele, and the FAM [™] dye-labeled probe to the T allele.
	• A minor groove binder (MGB), which increases the melting temperature (T _m) for a given probe length and allows the design of shorter probes. The use of shorter probes results in greater differences in T _m values between matched and mismatched probes, and more robust genotyping.
	• A non-fluorescent quencher (NFQ) at its 3' end, which allows for detection of the reporter dye fluorescence with greater sensitivity than with a fluorescent quencher.

5' nuclease assay

The figure below is a schematic depiction of the 5' nuclease assay. During PCR:

- Each TaqMan[®] MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites.
- When the oligonucleotide probe is intact, the proximity of the quencher dye to the reporter dye quenches the reporter signal.
- AmpliTaq Gold[®] DNA polymerase extends the primers bound to the genomic DNA template.

- AmpliTaq Gold[®] DNA polymerase (with its 5' nuclease activity) cleaves probes that are hybridized to the target sequence.
- Cleavage of the probes hybridized to the target sequence separates the quencher dye from the reporter dye, resulting in increased fluorescence by the reporter. The fluorescence signal generated by PCR amplification indicates which alleles are present in the sample.



Minimizing non-specific fluorescence

In TaqMan[®] assays, fluorescence from nonspecifically bound probes is reduced, because nucleotide mismatches between a probe and a sequence reduce the chances that the probe will be cleaved. The probe's short length means that a one-base-pair mismatch will a have a larger negative effect on the binding. The mismatched probe will not bind tightly to the allele; the AmpliTaq Gold[®] DNA polymerase will likely displace the probe without cleaving the dye.

Reading and analyzing the plates

The QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Genotyping Experiments

in each sample well on an Allelic Discrimination Plot, which contrasts the reporter dye intensities of the allele-specific probes. Finally, the QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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 QuantStudioTM 6 and 7 Flex Real-Time PCR System 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
$VIC^{\textcircled{R}}$ dye-labeled probe fluorescence only	Homozygosity for Allele 1
$FAM^{\scriptscriptstyleTM}$ 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 QuantStudioTM 6 and 7 Flex Real-Time PCR System Software.

The objective of the example Genotyping experiment is to investigate SNP rs8039, where possible genotypes are AA, AC, and CC. In the example, two unknown genomic DNA (gDNA) samples were genotyped using TaqMan[®] Drug Metabolism Genotyping Assay ID C_11711420_30. The reactions were set up so that the PCR

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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*.

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Design the Experiment

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

This chapter covers:

Define the experiment properties.	11
Define SNPs and samples	12
Assign markers, samples, and controls	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 2 in Booklet 1, *Getting Started with QuantStudio*TM 6 and 7 Flex Real-Time PCR System Software Experiments.

Define the experiment properties

2

Click **Experiment Setup** → **Experiment Properties** to create a new experiment in the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software. Enter:

Field	Entry
Experiment Name	QuantStudio_384-Well_SNP_Genotyping_Example
Barcode	Leave field empty
User Name	Example User
Comments	Genotyping example
Instrument type	QuantStudio [™] 6 Flex System
Block	384-Well Block
Experiment Type	Genotyping
Reagents	TaqMan [®] Reagents
Ramp speed	Standard
Reagent information	NA

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:

Experiment: QuantStudio_384-W	ell_SN Type: Genotyping	Reagents: Ta	qMan® Reagents 🛛 😪
* Experiment Name: QuantStudio_384-Well_S Barcode: User Name: Example User	:NP_Genotyping_Example	Comments: Genotyping Example	~
Which instrument type are you usin	g to run the experiment?		
✓ QuantStudio™ 6 Flex System	QuantStudio™ 7 Flex System		
* Which block are you using to run the	e experiment?		
✓ 384-Well	96-Well (0.2mL)	Fast 96-Well (0.1mL)	
What type of experiment do you wa	nt to set up?		
Standard Curve	Relative Standard Curve	Comparative Ct (ΔΔCt)	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	e instrument run?		
✓ Standard	Fast		
Include: VPre-PCR Read Amplification	Post-PCR Read		
What is the reagent information?			
New Delete			
Type Name	Part Number	Lot Number	Expiration Date

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	Sample name Color		Color
Sample 1		Sample 11		Sample 21	

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Genotyping Experiments

Sample name	Color	Sample name	Color	Sample name	Color
Sample 2		Sample 12		Sample 22	
Sample 3		Sample 13		Sample 23	
Sample 4		Sample 14		Sample 24	
Sample 5		Sample 15		Sample 25	
Sample 6		Sample 16		Sample 26	
Sample 7		Sample 17		Sample 27	
Sample 8		Sample 18		Sample 28	
Sample 9		Sample 19		Sample 29	
Sample 10		Sample 20		Sample 30	

- **3.** Dye to be used as a Passive Reference ROX
- 4. Custom Task Name Not applicable

Your Define screen should look like this:

SNPs									
New Edit Save to Library Import from Library Delete									
CND Assau Name	NCDI CND Deference	Contast Coquence	Allala 1	Reporter	Quanchar	Allala 2	Deportor	Quanchar	Color
	NCBI SINF Reference	context Sequence		vic	NEO-MGR		FAM		COIOT
SIVE ASSAY 1			Allele 1	110					
SNP Assay 2			Allele 1	VIC	NFQ-MGB	Allele 2	FAM	NFQ-MGB	
Samples									
oumpies	_								
New Save to Library Import from I	.ibrary Delete <table-cell> I</table-cell>	mport from File							
Sample Name 0	Color								
Sample 1	*								~
Sample 2	Sample 2								
Sample 3	Samole 3								
Sample 4									
sampie 5 Notes and the second se									
Passive Reference Custom Task Name									
ROX V Dekte									
				Name		Color	lc	on Char	

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

Assign markers, samples, and controls

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

• SNP Assay 1

Target name	Well number	Task	Sample
SNP	A3, A7, A11	Unknown	Sample 3
Assay 1	C1, C5, C9	Unknown	Sample 4
	C3, C7, C11	Unknown	Sample 5
	E1, E5, E9	Unknown	Sample 6
	E3, E7, E11	Unknown	Sample 7
	G1, G5, G9	Unknown	Sample 8
	G3, G7, G11	Unknown	Sample 9
	11, 15, 19	Unknown	Sample 10
	13, 17, 111	Unknown	Sample 11
	К1, К5, К9	Unknown	Sample 12
	K3, K7, K11	Unknown	Sample 13
	M1, M5, M9	1/1	Sample 14
	M3, M7, M11	Unknown	Sample 15
	01, 05, 09	1/2	Sample 16
	03, 07, 011	2/2	Sample 17
SNP Assay 1	А1, А5, А9	Negative	NTC

• SNP Assay 2

Target name	Well number	Task	Sample
SNP	A15, A19, A23	Unknown	Sample 1
Assay 2	C13, C17, C21	Unknown	Sample 2
	C15, C19, C23	Unknown	Sample 18
	E13, E17, E21	Unknown	Sample 19
	E15, E19, E23	Unknown	Sample 20
	G13, G17, G21	Unknown	Sample 21
	G15, G19, G23	Unknown	Sample 22
	113, 117, 121	Unknown	Sample 23
	115, 119, 123	Unknown	Sample 24
	K13, K17, K21 K15, K19, K23		Sample 25
			Sample 26
	M13, M17, M21	1/2	Sample 27
	M15, M19, M23	Unknown	Sample 28
	013, 017, 021	1/1	Sample 29
	015, 019, 023	Unknown	Sample 30
SNP Assay 2	A13, A17, A21	Negative	NTC



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: 10 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



Your Run Method screen should look like this:

For more information

For more information on	Refer to	Publication number
Consumables	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7 Flex</i> <i>Real-Time PCR System Software Experiments</i>	4489822
	Appendix A in Booklet 7, <i>QuantStudio™</i> 6 and 7 Flex Real-Time PCR System Software Experiments - Appendixes	
Data collection	Chapter 2 in Booklet 1, Getting Started with QuantStudio ^{m} 6 and 7 Flex Real-Time PCR System Software Experiments	4489822
Amplification efficiency	Amplification Efficiency of TaqMan $^{\circledast}$ Gene Expression Assays Application Note	127AP05-03
Using Alternative Setup	Chapter 3 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7 Flex</i> <i>Real-Time PCR System Software Experiments</i>	4489822

Chapter 2 Design the Experiment *For more information*

2

Prepare the Reactions

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

This chapter covers:

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Prepare the sample dilutions	19
Prepare the reaction mix ("cocktail mix")	20
Prepare the reaction plate	20
Example experiment reaction plate components	20
To prepare the reaction plate: dried gDNA	21
To prepare the reaction plate: wet gDNA	22
Tips for preparing reactions for your own experiments	22
Tips for preparing samples	22
Tips for preparing the reaction mix	22
Tips for preparing the reaction plate	22
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Assemble required materials

3

- Items listed in Booklet 1, *Getting Started with QuantStudio*[™] 6 and 7 Flex Real-Time PCR System Software Experiments
- Samples Sample 1 Sample 30
- Example experiment reaction mix components:
 - TaqMan® Genotyping Master Mix (2X)
 - SNP 1 Assay Mix (20×)
 - SNP 2 Assay Mix (20×)

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

Prepare the sample dilutions

For the example experiment, two targets are assigned to 36 wells each. Each well contains 20 ng of Coriell DNA diluted from $100 \text{ ng/}\mu\text{L}$ of stock.

To prepare the sample dilutions:

1. Label a separate microcentrifuge tube for each sample to be diluted.

- **2.** Add 5µL of sample stock to each empty tube.
- 3. Add 45μ 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)	36 Reactions + 10% excess (µL)			
	Dry	Wet	Dry	Wet		
TaqMan $^{ extsf{B}}$ Genotyping Master Mix (2X)	10.0	10.0	400.0	400.0		
SNP Assay Mix (20×)	1.0	1.0	40.0	40.0		
H ₂ 0, DNase-free	9.0	7.0	360.0	280.0		
Total Reaction Mix Volume	20.00	18	800.0	720.0		

- **3**. Gently pipet 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 The r experiment • 2 reaction plate

- The reaction plate for the Genotyping example experiment contains:
 - A MicroAmp[®] Optical 384-Well Reaction Plate
 - Reaction volume: 10 µL/well
 - 72 Unknown wells 🛄

components
2 IP P	3 Sample 3 Sample 5 U SNP	4 5 Sample 4 Sample 6	6 7 Sample 3 Sample 5 Sample 5 Sample 7	8 9	10 11 Sample Sample	12 13	14 15 Sample 1 10 2 Sample 1 1 10	16	1 Sam
Р.,, 44. Р.,, 19.,,	Sample 3	Sample 4	Sample 3	Sangle	Sample 1	S Sample	2. Sample 1 2. Sample 1 3. Sample 1 3. Sample 1 3. Sample 1 3. Sample 1 3. Sam		Sam,
■ 4 IP	Sample 5	Sangle 4	Sample 5	Sample	4 Sample 1 Tan I Skip	Sample	2 Sample 1 2 1 SNP		Samı U
e 4 e 6 lP	Sample 5	Sample 4	Sample 5	Sample	4 Sample 5	5 Sample	2 Sample 1/		Sam
e 6 IP	Sample 7	Sample 6	Sample 7						
e 6 IP	Sample 7	Sample 6	Sample 7						
		U SNP	U SNP	Sample	6 Sample 7	7 Sample	19 Sample 21		Samp U
e 8 4P	Sample 9	Sample 8	Sample 9	Sample	8 Sample 9	Sample	21 Sample 2:	2	Samp
10 19	Sample 11	Sample 10	Sample 11	Sample	10 Sample 1	1 Sample	23 Sample 24		Samp C
	Sample 13	Sample 12	Sample 13	Sample	12 Sample 1	3 Sample	25 Sample 26		Samp 🗸
	e 10 NP	e 10 e 12 Sample 13	e 10 Sample 11 Sample 10 Sample 10 Sample 12 Sample 13 Sample 12	Sample 1 Sample 2 Sample 2 Sample 2 # 10 SAP I SAP I SAP # 10 Sample 11 Sample 10 SAP # 10 SAP I SAP I SAP # 10 SAP I SAP I SAP # 10 SAP I SAP I SAP # 12 Sample 13 Sample 12 Sample 13	No.u Sample 11 Sample 10 Sample 10 e 10 Sample 11 Sample 10 Sample 11 NBm Sample 11 Sample 10 Sample 11 1 Sample 10 Sample 10 1 Sample 10 Sample 10	No. Sample 11 Sample 11 Sample 11 Sample 10 Sample 11 Sample 10 Sample 10 Sample 11 Sample 10 Sample 10 </td <td>NP III SNP e 10 Sample 11 Sample 10 III SNP III SNP III SNP III SNP e 10 Sample 11 III SNP III SNP III SNP III SNP e 10 Sample 11 III SNP III SNP III SNP e 10 Sample 10 III SNP III SNP e 11 Sample 13 Sample 12 Sample 13</td> <td>No. No. N</td> <td>NP. III SND II</td>	NP III SNP e 10 Sample 11 Sample 10 III SNP III SNP III SNP III SNP e 10 Sample 11 III SNP III SNP III SNP III SNP e 10 Sample 11 III SNP III SNP III SNP e 10 Sample 10 III SNP III SNP e 11 Sample 13 Sample 12 Sample 13	No. N	NP. III SND II

The following is an image of the plate layout:

To prepare the reaction plate: dried gDNA

1. Pipet 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 20 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.				
YDINA	3. Transfer 18 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 prepar	ing 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[™] 6 and 7 Flex Real-Time PCR System 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	Publication number
Assigning the reaction plate components	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and</i> 7 Flex Real-Time PCR System Software Experiments	4489822
Sealing the reaction plate	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and</i> 7 <i>Flex Real-Time PCR System Software Experiments</i>	4489822

This chapter explains how to run the example experiment on the QuantStudio[™] 6 or 7 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 6 or 7 Instrument is in operation.

Start the run

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

Note: To collect real-time data during a run, click the ¹/₂₀ button on the Run Method screen in the Experiment Setup menu.

Monitor the run

Monitor the example experiment run:

- From the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software (to monitor an experiment started from another computer or from the QuantStudio[™] 6 or 7 Instrument touchscreen).
- From the QuantStudio[™] 6 or 7 Instrument touchscreen.

From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software

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

View the Amplification Plot

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software for potential problems.

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

The following is an image of 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 following is an image of 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 following is an image of the Run Method screen as it appears in the example experiment.



View run data

Click View Run Data from the Run Experiment Menu.

The following is an image of the View Run Data screen as it appears in the example experiment.

Run Data Report	
Experiment Name:	QuantStudio_384-Well_SNP_Genotyping_Example
Start Time:	09-17-2010 06:57:35 SGT
Stop Time:	09-17-2010 08:30:35 SGT
Run Duration:	93 minutes 0 seconds
User Name:	DEFAULT
Instrument Name:	Yankees_Rule
Firmware Version:	1.0.3
Software Version:	QuantStudio Software v1.0
Instrument Serial Number:	278880012
Sample Volume:	10.0
Cover Temperature:	105.0
Block Type:	384-Well Block
Errors Encountered:	× ×

From the QuantStudio[™] 6 or 7 Instrument touchscreen You can also view the progress of the run from the QuantStudio ${}^{\rm TM}$ 6 or 7 Instrument touchscreen.

The following are images of the Run Method screen on the QuantStudio $^{\text{TM}}$ 6 or 7 Instrument touchscreen:

Experiment View



Time View

C Time View III Experiment Vie	w
Run Started: July 06 2013 - 05:48PM Reaction Volume: 20 µL	Sample: 95.0 °C Heated Cover (Set Point): 105.0 °C (105.0 °C) Stage / Step / Cycle: 1 / 2 / 1
01.2	1.52
UIIJ	JIJZ
Remaining T	ime 🔵 Elapsed Time
	July 06 2013 - 05:48PM Heated cover reached target temperature.



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	ion 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	36
	Identify well problems using the Well Table	39
	Confirm accurate dye signal using the Multicomponent Plot	42
	Determine signal accuracy using the Raw Data Plot	44
	Review the flags in the QC Summary	46
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Sect	ion 5.2 Adjust parameters for re-analysis of your own experiments	49
	Adjust analysis settings	49
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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.
- 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 negative controls.

Note: Including controls helps to improve the clustering algorithm, particularly in situations where a limited number of samples are run.

To view and assess the allelic discrimination plot

- 1. From the Experiment menu pane, select Analysis > Allelic Discrimination Plot.
- 2. Click the Plate Layout tab, then click any empty well to select it.

Note: In the Allelic Discrimination Plot, the software highlights all wells that are selected in the Plate Layout tab. If the plot displays a single color for all wells, then all wells in the plate layout are selected.

3. In the allelic discrimination plot, select **SNP Assay 1** from the SNP Assay menu, then enable Autocaller.

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

Genotype	Symbol	Location
Homozygous for Allele 1 of the selected SNP assay.	● (red)	X-axis of the plot
Homozygous for Allele 2 of the selected SNP assay.	😑 (blue)	Y-axis of the plot
Heterozygous for both alleles of the selected SNP assay (Allele 1 and Allele 2).	• (green)	Midway between the homozygote clusters
A negative control.	■ (black)	Bottom-left corner of the plot
Undetermined.	★ (black)	Anywhere on plot

b

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 negative controls should be selected. The presence of an unknown among the negative 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 [™] 6 and 7 Flex Software displays in the plot.	
Plot Type drop- down menu	Determines the type of plot (Cartesian or Polar) that the QuantStudio [™] 6 and 7 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. A cooms in. Sooms 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 50.	



The following is an image of the Allelic Discrimination plot for the example experiment:

Troubleshoot clustering on the Allelic Discrimination Plot

Do all controls have the correct genotype?

In the example experiment and in your own experiments, confirm that data points cluster as expected.

Clustering in positive controls

- 1. From the well table, select the wells containing a positive control to highlight the corresponding data points (symbols) in the Allelic Discrimination Plot.
- **2.** Check that the data points for the positive controls cluster along the expected axis of the plot. For example, if you select the Positive Control Allele 1/Allele 1, then the controls should cluster along the X-axis.
- **3.** Repeat steps 1 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 negative controls, and not unknown samples.

Samples clustered with negative control

ხ

Samples that clustered with the negative 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	For the example experi called:	ment, confirm that the QuantStudio [™] 6 and 7 Flex Software
layout values	• 54 samples as Alle	le 1 homozygous (鱼)
	• 12 samples as Alle	le 2 homozygous (🔍)
	• 24 samples as hete	rozygous (🔍)
	• 0 samples as unde	termined (X)
	Confirm that no wells o experiment does not di	of the reaction plate triggered QC flags (\blacktriangle). The example splay any flags.
View the layout	 Click the icon layout. 	beside the Allelic Discrimination Plot to maximize the plate
	2. Click 👩 Show in wells to display. Reparameters.	Wells, then select or deselect a parameter that you want the epeat this step until the plate layout contains all of the desired
	Parameter	Description

The name of the sample applied to the well.

Parameter	Description
Task	The task assigned to the well:
	🔟 – Unknown
	📉 – Negative Control
	🔄 – Positive Control - 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
	 ■ Negative Control
	X Undetermined
Flag	The number of QC flags the well triggered as listed in the \blacktriangle symbol.



The following is an image of the plate layout of the example Genotyping experiment.

Tips for troubleshooting plate setup in your own experiment

5

You can adjust your view of the plate layout:

- Note the location of any samples that trigger QC flags (▲). Understanding the position of errors can aid in diagnosing any failures that may occur.
- You can select the entire reaction plate, areas of the reaction plate, or specific wells:
 - Click the upper left corner of the reaction plate to select all 384 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 i (Zoom In), i (Zoom Out), and i (Fit Plate) buttons to magnify or compress the view of the wells shown.
- Use the \trianglelefteq 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 The Amplification Plot screen displays amplification of all samples in the selected wells. Use the amplification plots to confirm the results of the experiment: plots • 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_{Rn} 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 $\Delta Rn vs$. 1. From the Experiment Menu pane, select Analysis > Amplification Plot. Cycle plot **Note:** If no data are displayed, click **Analyze**.

2. Select the plot type and format:

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



QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Genotyping Experiments

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





- **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.

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.

ExampleFor the example experiment, look for wells that triggered QCexperiment valuesexperiment has no flags.and flagsFor the example experiment has no flags.	\mathcal{L} flags ($\underline{\land}$). The example
View the well table 1. Select the Well Table tab.	
2. Click the Flag column header to sort the data so that the appear at the top of the table.	wells that triggered flags
3. Confirm the integrity of the controls:	
 a. From the Group By menu, select Task to organize t function on the reaction plate. 	he table rows by their
b. Confirm that each of the controls do not display fla	ngs (<mark>1</mark>).
c . Click the l icon to collapse the negative and positi	ive controls.



The following is an image of 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 $_{\Lambda}$ 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, Negative 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 _{Rn}	Normalized signal (R_n) of the reporter dye of the associated allele for the SNP evaluated by the well.
Pass Ref	The signal of the passive reference dye for the well.

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Column	Description
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
	 ■ Negative 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 [™] 6 and 7 Flex Real-Time PCR System 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 QuantStudioTM 6 and 7 Flex Real-Time PCR System Software does not display a corresponding column for the flag.

A ($\underline{\land}$) 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 [™] 6 or 7 Instrument 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 _{Rn} 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 a _{Rn} greater than the limit defined in the analysis settings indicating possible amplification.
NOAMP	The well did not produce an $_{Rn}$ 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.

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Genotyping Experiments

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.

Tips for analyzing your own experiments

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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 (\blacktriangle) 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 (<u>)</u> in the Flag column, note the data and the flag(s) triggered by the associated well.
- Select areas of the table or wells of a specified type by:
 - Left-clicking the mouse and dragging across the area you want to select an area of the table.
 - Selecting Sample, SNP Assay, or Task from the Select Wells menu in the Well Table tab to select wells of a specific type using the well-selection tool.
- Group the rows of the plate layout by selecting an option from the Group By menu. You can then collapse or expand the lists either by clicking the +/- icon next to individual lists, or by clicking Collapse All or Expand All.
- Omit a well from the analysis by selecting the **Omit** check box for that well. To include the well in the analysis, deselect the **Omit** check box.

Note: You must reanalyze the experiment each time you omit or include a well.

Confirm accurate dye signal using the Multicomponent Plot

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

Purpose	 In the example experiment, you review the Multicomponent Plot screen for: ROX[™] dye (passive reference) FAM[™] dye (reporter) VIC[®] dye (reporter) Spikes, dips, and/or sudden changes Amplification in the negative control wells
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 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
	 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. Wells with the negative control should not show amplification. The example experiment does not have negative controls.

Tips for confirming dye accuracy in your own experiment

5

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.
- **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 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 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 42. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM[™] dye filter.



			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)						
×3(550±11)						
x4(580±10)						
x5(640±10)						
x6(662±10)						
lt Curve Filter ———			Load Save Revert &	o Defaults		
lt Curve Filter ———			Load Save Revert & Emission Filter	9 Defaults		
It Curve Filter ———	m1(520±15)	m2(558±11)	Load Save Revert & Emission Filter m3(586±10)	Defaults m4(623±14)	m5(682±14)	m6(711±12)
It Curve Filter x1(470±15)	m1(520±15)	m2(558±11)	Load Save Revert & Emission Filter m3(586±10)	Defadir m4(623±14)	m5(682±14)	m6(711±12)
t Curve Filter	m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
x1(470±15) x2(520±10) x3(550±11)	m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
x1(470±15) x2(520±10) x3(550±11) x4(580±10)	m1(520±15)	m2(558±11)	Load Save Revert Emission Filter m3(586±10)	Defaults m4(623±14) 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	m5(682±14)	m6(711±12)
tt Curve Filter	m1(520±15)	m2(558±11)	Load Save Revert Emission Filter m3(586±10)	Defaults m4(623±14)	m5(682±14)	m6(711±12)
tt Curve Fifter x1(470±15) x2(520±10) x3(550±11) x4(580±10) x5(640±10) x6(662±10)	m1(520±15)	m2(558±11)	Load Save Revert h Emission Filter m3(586 ± 10)	Mathematical States Sta	m5(682±14)	m6(711±12)

The filters are:



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

- Characteristic signal growth
- No abrupt changes or dips

Review the flags in the QC Summary

The QC Summary screen displays a list of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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 \triangle icon. The flag details appear in the QC Summary.

In the example experiment, there are no flags.

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.

- **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.

201	Description	Frequency	Wolle	
ay.	Bad partice reference signal	lo	Wells	
	Bad passive reference signal	0		
FSCALE	Fluorescence is orrscale	0		
SIGNAL	Number of dusters outside expected range	0		
JOTER#	Desitive central failed	0		
	Construction rates and character	0		
DNC	Amplification in pagative control	0		
PINC	Amplification in negative control	0		
AMP	No amplification	0		
196	Noise nigher than others in plate	0		
NE	Noise spikes	U		
FAIL	Exponential algorithm failed	0		
AIL	Baseline algorithm failed	0		
OLDFAIL	Thresholding algorithm failed	0		
AIL	Lor algorithm raied	<u>۲</u>		
AIL	Let algorithm taled			

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

Flag	Description
	Pre-processing flag
OFFSCALE	Fluorescence is offscale
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
Se	econdary analysis flags
AMPNC	Amplification in negative control
CLUSTER#	Number of clusters outside expected range
PCFAIL	Positive Control failed
SMCLUSTER#	Small number of samples in clusters

For more information

For more information on	Refer to	Publication number
Publishing data	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7</i> Flex Real-Time PCR System Software Experiments	4489822



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 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:

- Call Settings
- C_T Settings
- Flag Settings
- Advanced Settings

The following is an image of the Analysis Settings dialog box for a Genotyping experiment:

		Well_SNP_Genotypir	ng_Example		
Call Settings C	T Settings Flag Settin	gs Advanced Setting	js		
Review the de Default Sett	fault settings for analysis of ings , then change the settir	the SNP assays in this exp Igs.	eriment. To use different	settings for a SNP assay	y, select the SNP assay in the table, deselect Use
🦟 Data Analysis	Settings				
🔘 Analyze Data	from Post-PCR Read Only		Analyze I	Data from Pre-PCR Re	ad and Post-PCR Read
🔘 Analyze Real-	Time Rn Data		🔘 Analyze I	Real-Time Rn - Median	n(Rna to Rnb)
🔿 Analyze Real-	Time dRn Data				
SNP Assay		Autocaller	Keen Manual C	Quality Value	Call Settings for SNP Assay 1
Select a SNP Assay	Assay Analysis Type	Autocaller	Keep Manual C	Quality Value	Call Settings for SNP Assay 1 Apply Call Settings: ✓
SNP Assay	Assay Analysis Type Default	Autocaller Yes	Keep Manual C No	Quality Value	Call Settings for SNP Assay 1 Apply Call Settings: Call Settings Apply Call Settings Autocaller Enabled
Select a SNP / SNP Assay SNP Assay 1 SNP Assay 2	Assay Analysis Type Default Default	Autocaller Yes Yes	Keep Manual C No No	Quality Value 95 95	Call Settings for SNP Assay 1 Apply Call Settings: Autocaller Enabled Keep Manual Calls from Previous Analysis
Select a SNP / SNP Assay SNP Assay 1 SNP Assay 2	Assay Analysis Type Default Default	Autocaller Yes Yes	Keep Manual C No No	Quality Value 95 95	Call Settings for SNP Assay 1 Apply Call Settings: Default Settings Autocaller Enabled Keep Manual Calls from Previous Analysis Quality Value: 95.0
Select a SNP / SNP Assay SNP Assay 1 SNP Assay 2	Assay Analysis Type Default Default	Autocaller Yes Yes	Keep Manual C No No	Quality Value 95 95	Call Settings for SNP Assay 1 Apply Call Settings: Default Settings Autocaller Enabled Keep Manual Calls from Previous Analysis Quality Value: 95.0
Select a SNP / SNP Assay SNP Assay 1 SNP Assay 2	Assay Analysis Type Default Default	Autocaller Yes Yes	Keep Manual C No No	Quality Value 95 95	Call Settings for SNP Assay 1 Apply Call Settings: Autocaller Enabled Keep Manual Calls from Previous Analysis Quality Value: 95.0

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

Note: You can save the changes to the analysis settings to the Analysis Settings Library for later use. For more information, see About the Analysis Settings Library in Booklet 1, *Getting Started with QuantStudio*[™] 6 and 7 Flex Real-Time PCR System Software 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 check box.
 - 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

Use the Baseline Threshold algorithm to determine the C_T values.

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

• 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[™] 6 and 7 Flex Real-Time PCR System 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.

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Genotyping Experiments

4. Click **Apply Analysis Settings** in the Analysis Settings dialog box. If the run status is complete, the data are reanalyzed.

Analysis Settin	gs for QuantStudio_384-	Well_SNP_Genotyping	_Example					
Call Settings	CT Settings Flag Setting	s Advanced Settings						
Configure t	he flags and filtering. In this par	el you can enable, disable, a	and configure flags, and indi	icate if a well is to be re	jecte	ed when a flag is raised.	(2
Flag	Description	Use	Attribute	Condition		Value	Reject Well	
BADROX	Bad passive referenc.	. 💌	Fluorescence	<	~	500.000		^
OFFSCALE	Fluorescence is offsca	le 🔽						
NOSIGNAL	No signal in well							
CLUSTER#	Number of clusters o.	🗹						
PCFAIL	Positive control failed	V						
SMCLUSTER	Small number of sam.	🗹	Number of data poin	≤	~	2.000		
AMPNC	Amplification in nega.	. 🗹	Ст	<	~	35.000		Ξ
NOAMP	No amplification		Amplification algorith	<	~	0.100		
NOISE	Noise higher than ot.	. 🗹	Relative noise	>	~	4.000		
SPIKE	Noise spikes		Spike algorithm result	>	~	1.000		
EXPFAIL	Exponential algorith	V						
BLFAIL	Baseline algorithm fail.	🗹						
THOLDFAIL	Thresholding algorith.	🗹						
	1		I	i			I	
Save to Library	Load from Library		Revert to De	efault Analysis Seti	ting	s Apply Analys	sis Settings	ancel

The following is an image of the Flag Settings tab:

Advanced Settings

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

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	Publication 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[™] 6 and 7** 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	QuantStudio_384-Well_SNP_Genotyping_Example_data
File Type	*.txt
Export File Location	C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\Export

Your Export screen should look like this:

	Quanton			Export Data			e open ne(o)		-pre
ort File Location: ms\Qua	antStudio 6 and 1	7 Flex Soft	ware\User Files\Export	Browse Export File	Name: QuantStud	io_384-Well_SN	P_Genotyping_Exa File	e Type: 📋 (*.txt)	
Sample Setup	aw Data 🛛 📝 Am	plification	Multicomponent	Results					
Skip Empty Wells 🛛 🗹 Skij	o Omitted Wells								
elect Content		Well	Well Position	Sample Name		Task	Allele1 Delta	Allele2 Delta	
All Fields	^	wen	1 A1	Sample Name	CND Assault	NTC	Allelei Delta	Allelez Delta	4
Airfields			I AI	Camala 2	SNP Assay 1	NTC	0.034	0.062	ł
Well			3 A3	Sample 3	SNP Assay 1	UNKNOWN	2.309	0.392	
			5 A5	Comple D	SNP Assay 1	NTC	0.035	0.058	1
Well Position			7 A7	Sample 3	SNP Assay 1	UNKNOWN	2.2/3	0.286	1
			9 A9	Comple D	SNP Assay 1	NTC	0.040	0.058	ł
Sample Name			11 ATT	Sample 3	SNP Assay 1	UNKNOWN	1.818	0.141	ł
SND Accov Namo			13 A13		SNP Assay 2	NIC	0.044	0.042	1
Sive Assay Ivallie			15 A15	Sample 1	SNP Assay 2	UNKNOWN	1.311	0.241	ł
Task	_		1/ A1/		SNP Assay 2	NIC	0.046	0.050	Į.
			19 A19	Sample 1	SNP Assay 2	UNKNOWN	1.081	0.136	2
Allele1 Delta Rn			21 A21		SNP Assay 2	NIC	0.044	0.039	Į.
			23 A23	Sample 1	SNP Assay 2	UNKNOWN	1.000	0.170	Į.
Allele2 Delta Rn			49 C1	Sample 4	SNP Assay 1	UNKNOWN	0.153	2.520	Į.
Dass Rof			51 C3	Sample 5	SNP Assay 1	UNKNOWN	2.463	0.343	1
r dabarter			53 (5	Sample 4	SNP Assay 1	UNKNOWN	0.121	2.265	Į.
Ouality(%)			55 C/	Sample 5	SNP Assay 1	UNKNOWN	2.211	0.233	ĺ.
·····			57 (9	Sample 4	SNP Assay 1	UNKNOWN	0.117	2.285	f
Call			59 C11	Sample 5	SNP Assay 1	UNKNOWN	2.203	0.214	1
	×	<							Ľ

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

📮 QuantStudio_384-Well_SNP_Genotyping_Example_data.txt - Notepad	
File Edit Format View Help	
<pre>slock type = 384-well Block calibration Background performed on = 08-25-2010 calibration HRM MELTOCTOR is expired = Yes calibration HRM MELTOCTOR is expired = Yes calibration Normalization FAM-ROX performed on = 03-22-2010 calibration Normalization FAM-ROX performed on = 07-22-2010 calibration Pure Dye ABY performed on = 03-31-2010 calibration Pure Dye ABY performed on = 03-31-2010 calibration Pure Dye ABY performed on = 03-31-2010 calibration Pure Dye ABY performed on = 03-27-2010 calibration Pure Dye ABY performed on = 04-06-2010 calibration Pure Dye ATTO 700 performed on = 04-22-2010 calibration Pure Dye ATTO 700 performed on = 07-22-2010 calibration Pure Dye ATTO 700 performed on = 07-22-2010 calibration Pure Dye ATTO 700 performed on = 07-22-2010 calibration Pure Dye PVE1 is expired = Yes calibration Pure Dye PVE1 is expired = Yes calibration Pure Dye PVE1 is expired = Yes calibration Pure Dye FAM performed on = 07-22-2010 calibration Pure Dye FAM performed on = 03-27-2010 calibration Pure Dye BUN performed on = 03-27-2010 calibration Pure Dye BUN performed on = 03-27-2010 calibration Pure Dye MELTDOCTOR performed on = 03-12-2010 calibration Pure Dye MELTDOCTOR performed on = 03-21-2010 calibration Pure Dye MELTDOCTOR performed on = 06-12-2010 calibration Pure Dye MELTDOCTOR performed on = 06-12-2010 calibration Pure Dye MED performed on = 06-12-2010 calibration Pure Dye MED performed on = 06-12-2010 calibration Pure Dye MED performed on = 07-22-2010 calibration Pure Dye MED</pre>	
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carror action on thormaticy is expired = tes	× .

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



QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Presence/ Absence Experiments

Booklet 5

Publication Number 4489822 **Revision** A



For Research Use Only. Not for use in diagnostic procedures.

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About Presence/ Absence Experiments

This chapter covers:

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About the example experiment	7

IMPORTANT! First-time users of the QuantStudioTM 6 and 7 Flex Real-Time PCR System Software please read Booklet 1, *Getting Started with QuantStudioTM 6 and 7 Flex Real-Time PCR System Software Experiments* and Booklet 7, *QuantStudioTM 6 and 7 Flex Real-Time PCR System Software 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[™] 6 and 7 Flex Real-Time PCR System Software by pressing F1, clicking ? in the toolbar, or selecting Help > QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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 TM 6 and 7 Instruments collect 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 software 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 [™] 6 and 7 Instruments provide 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 <i>no amplification control</i> (<i>NAC</i>).
	If the run method includes amplification, real-time data are plotted in an amplification plot.
No IPC, singleplex setup	Omit the IPC from your Presence/Absence experiment. PCR reactions contain one primer/probe set. PCR reactions do not contain the IPC. With this setup, there are two well types:
	• Unknown wells – Wells contain sample template; the presence of the target is not known.

• **Negative controls** – Wells contain water or buffer instead of sample template.

About the instrument run

With Presence/Absence experiments, the instrument runs can include:

- **Pre-PCR read** Perform the pre-PCR read on the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software before PCR amplification to collect baseline fluorescence data.
- Amplification Perform amplification on the QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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 6 and 7 Flex Real-Time PCR System Software.

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 (Part no. 4318930). The DNA is extracted from each of the four samples of ground beef or from the bacteria found in the ground beef.
- The target is a pathogen.
- The experiment is designed for duplex PCR, where each reaction contains two primer/probe sets. One set detects the pathogen sequence, TGFB (primer set and FAM[™] dye-labeled probe to detect the TGFB sequence). The other primer/probe set detects the IPC primer set and VIC[®] dye-labeled TaqMan[®] probe detects the IPC template.

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Presence/Absence Experiments

Design the Experiment

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

This chapter covers:

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

Note: To automatically export the analyzed data to a specified location, select the Auto Export task from the Export screen, before running the experiment. For more information on Auto Export, refer to Chapter 2 in Booklet 1, *Getting Started with QuantStudio*TM 6 and 7 Flex Real-Time PCR System Software Experiments.

Define the experiment properties

2

Click **Experiment Setup** ▶ **Experiment Properties** to create a new experiment in the QuantStudio[™] 6 and 7 Flex Software. Enter:

Field or Selection	Entry
Experiment Name	QuantStudio_384-Well_Presence- Absence_Example
Barcode	Leave field empty
User Name	Example User
Comments	Presence/Absence example
Instrument type	QuantStudio [™] 6 Flex System
Block	384-Well Block
Experiment Type	Presence/ Absence
Reagents	TaqMan [®] Reagents
Ramp speed	Standard
Reagent information	NA

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 e	xperiment?		-
Experiment Name: QuantStudio_384-We Barcode: User Name: Example User	II_Presence-Absence_Example	Comments: Presence/ Absence Experiment	~
Which instrument type are you u United instrument type are you u United instruction United instruction United instruction Instruction United instructinstructing	sing to run the experiment? QuantStudio™ 7 Flex System		
Which block are you using to run	the experiment?		
✓ 384-Well	96-Well (0.2mL)	Fast 96-Well (0.1mL)	
* What type of experiment do you	want to set up?		
Standard Curve	Relative Standard Curve	Comparative Cτ (ΔΔCτ)	Melt Curve
Genotyping	Presence/Absence		
* Which reagents do you want to u	se to detect the target sequence?		
✓ TaqMan® Reagents	Other		
• What properties do you want for	the instrument run?		
✓ Standard	Fast		
Include: 🗹 Pre-PCR Read 🔽 Amplification	Post-PCR Read		
What is the reagent information?			
New Delete	Dest Number	Lot Number	Everytice Date
Type Nam	- Fart Number	Loc Nomber	Expiration Date

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	NFQ-MGB	

2. Samples

Sample Name	Color
NTC	
NAC	
Sample (+)	
Sample (-)	

- **3.** Dye to be used as a Passive Reference ROX
- 4. Custom Task Name

Not applicable

Your Define screen should look like this:

Targets			Samples						
New Save to Library Import from Library De	lete		New Save to Library Import fr	om Library Delete	Import from File				
Target Name	Reporter	Quencher	Color	Sample Name	Color				
TGFB	FAM 🗸	NFQ-MGB 🗸 🗸	~	NTC	-				
IPC	VIC 🗸	NFQ-MGB 🗸 🗸		NAC	v				
				Sample (+)					
				Sample (-)	~				
* Passive Reference				Custom Task Name					
ROX				New Delete					
				Name	Color	lcon Char			

Assign targets and samples

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

Target Name	Well Number	Task	Sample
TGFB	A1 - P3 (Columns 1 - 3)	Negative	NTC
IPC		IPC	
TGFB	A4 - P12 (Columns 4 - 12)	Unknown	Sample
IPC		IPC	(-)
TGFB	A13 - P15 (Columns 13 - 15)	NTC	NAC
IPC		Blocked IPC	
TGFB	A16 - P24 (Columns 16 - 24)	Unknown	Sample
IPC		IPC	[+]

Your Assign screen should look like this:

Targets 🔼	<	Plate Layout	Well Table										
Name Task Custom Task Image: TGFB Image: Task Image: Task Image: Task Image: Task Image: Task		Show in We	ills 🔻 Select W	ells 🔻 🔛 📰	View Legend						•		
		1	2	3	4	5	6	7	8	9	10	11	
	A	NTC I IPC N TGFB	NTC I IPC N TGFB	NTC	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	^
	в	NTC I IPC N TGFB	NTC	NTC I IPC N TGFB	Sample (-)	Sample (-) IPC IGF8	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	=
Samples 🔺	c	NTC I IPC N TGFB	NTC	NTC I IPC N TGFB	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	
Name NAC NTC Sample (+)	D	NTC I IPC N TGFB	NTC I IPC N TGFB	NTC	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	
Sample (-)	E	NTC I IPC N TGFB	NTC I IPC N TGFB	NTC	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	
	F	NTC			Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	Sample (-)	~
	v	Vells: 🚺 0 📧	0 👖 288	N 48 📈 48	3							0 Emp	ty

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
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) 	Step2	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



Your Run Method screen should look like this:

For more information

For more information on	Refer to	Publication number
Consumables	Chapter 3 in Booklet 1, <i>Getting Started with QuantStudio™</i> 6 and 7 Flex Real-Time PCR System Software Experiments	4489822
	Appendix A in Booklet 7, <i>QuantStudio[™] 6 and 7 Flex Real-Time PCR</i> System Software Experiments - Appendixes	
Data collection	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio</i> [™] 6 and 7 Flex Real-Time PCR System Software Experiments	4489822
Amplification efficiency	Amplification Efficiency of TaqMan [®] Gene Expression Assays Application Note	127AP05-03
Using Alternative Setup	Chapter 3 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7</i> Flex Real-Time PCR System Software Experiments	4489822



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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Prepare the reaction mix ("cocktail mix")	15
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	Assemble required materials Prepare the reaction mix ("cocktail mix") Prepare the reaction plate For more information

Assemble required materials

3

- Items listed in Booklet 1, *Getting Started with QuantStudio*[™] 6 and 7 Flex Real-Time PCR System Software Experiments
- Samples DNA extracted from ground beef (100 ng/µL)
- Example experiment reaction mix components:
 - TaqMan[®] Universal PCR Master Mix
 - 10X IPC Mix
 - 50X IPC DNA
 - 20X Primer/ Probe Mix

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

Prepare the reaction mix ("cocktail mix")

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

- Sample (+)
- Sample (-)
- 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 160 reactions (144 wells + 10% excess) (µL)
Cocktail Mix 1 for Sample (+)	TaqMan [®] Universal PCR Master Mix (2.0×)	12.50	2000.0
	10× IPC Mix	2.50	400.0
	50X IPC DNA	0.50	80.0
	20X Primer/ Probe Mix	1.25	200.0
	Water/ Buffer	5.75	920.0
	Diluted unknown 1	2.5	400
	Total reaction mix volume	25.0	4000.0
		_	
Cocktail Mix 2 for Sample (-)	TaqMan $^{\otimes}$ Universal PCR Master Mix (2.0×)	12.50	2000.0
	10× IPC Mix	2.50	400.0
	50× IPC DNA	0.50	80.0
	20X Primer/ Probe Mix	1.25	200.0
	Water/ Buffer	5.75	920.0
	Diluted unknown 2	2.5	400
Cocktail Mix 3 for NTC/ IPC+	TaqMan [®] Universal PCR Master Mix (2.0×)	12.50	662.5
	10× IPC Mix	2.50	132.5
	50× IPC DNA	0.50	26.5
	20X Primer/ Probe Mix	1.25	66.25
	Water/ Buffer	8.25	304.75
	Total reaction mix volume	25.0	1325.0
	TaqMan [®] Universal PCR Master Mix (2.0X)	12.50	662.5
Cocktail Mix 4 for NAC/ IPC-	TaqMan [®] Universal PCR Master Mix (2.0×)	12.50	662.5
	10× IPC Mix	2.50	132.5
	50× IPC DNA	0.50	26.5
	20X Primer/ Probe Mix	1.25	66.25
	IPC Block	2.5	132.5
	Water/ Buffer	5.75	304.75
	Total reaction mix volume	25.0	1325.0

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 384-Well Reaction Plate
- Reaction volume of 20 µL/well
- 144 Sample (+) wells 🛄 🔟
- 144 Sample (-) wells 🛄 🗹
- 48 NTC/ IPC+ 🔟 💋
- 48 NAC/IPC- 🔟 🔳

The following is an image of the plate layout:

Σ	P	late Layo	put	Well Tab	ble																				
	Č	🧾 Show	in Wells	V Sele	ct Wells 🖣	/ 💼	View Lege	end															•	8)	-
		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																								
	в	T pr		T ne																		ill me			
	c	T me	T me																						
	D	T me	T mr																						
	Е	T me	T me	T m/																					
	F	T me	T mr																						
	G	T. me																							
1	н	T me		T n/																					
	Ι		T m/																						
	J	T me	T m/																						
	к	T me	T mr	T m/																					
	L	T me	T mr																						
	м	T me	T mr																						
	N	T me																							
	0	T. me																							
	Р	T ne	T m/																						
	We	ells: 🕕	0 🖪 0	1 28	88 📘 4	8 🚬 48	3																	I) Empty

To prepare the reaction plate:

- 1. Add 25 µL of Cocktail mix 1 to wells A16–P24.
- 2. Add 25 µL of Cocktail mix 2 to wells A4–P12.
- **3.** Add 25 µL of Cocktail mix 3 to wells A13–P15.
- 4. Add 25 μL of Cocktail mix 4 to wells A1–P3.
- 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	Publication number
Assigning the reaction plate components	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and</i> 7 Flex Real-Time PCR System Software Experiments	4489822
Sealing the reaction plate	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and</i> 7 Flex Real-Time PCR System Software Experiments	4489822

This chapter explains how to run the example experiment on the QuantStudio ${}^{\rm TM}$ 6 or 7 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 QuantStudioTM 6 or 7 Instrument is in operation.

Start the run

4

- 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[™] 6 and 7 Flex Real-Time PCR System Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software (to monitor an experiment started from another computer or from the QuantStudio[™] 6 or 7 Instrument touchscreen).
- From the QuantStudio[™] 6 or 7 Instrument touchscreen.

From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software

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

View the Amplification Plot

You can view the progress of the run in real time. During the run, periodically view all the three plots available from the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software for potential problems.

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

The following is an image of 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.

Temperature Plot Current Temperatures Cover Ð P i) • 4 ~ 🗹 📕 Sample **Temperature Plot** Block 110 100 90 80 70 Temperature 60 50 40 30 20 10 Temperature Plot 0 00:58:20 01:02:30 00:33:20 00:37:30 00:41:40 00:45:50 00:50:00 00:54:10 01:06:40 01:10:50 01:15:00 01:19:10 01:23:20 01:27:30 1 Hour View -Time Fixed View 🗖

The following is an image of 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 following is an image of the Run Method screen as it appears in the example experiment.



View run data

Click View Run Data from the Run Experiment Menu.

The following is an image of the View Run Data screen as it appears in the example experiment.

Run Data Report	
Experiment Name:	QuantStudio_384-Well_Presence-Absence_Example
Start Time:	01-20-2010 18:35:18 SGT
Stop Time:	01-20-2010 20:06:20 SGT
Run Duration:	91 minutes 1 seconds
User Name:	DEFAULT
Instrument Name:	
Firmware Version:	
Software Version:	N/A
Instrument Serial Number:	278880001
Sample Volume:	20.0
Cover Temperature:	105.0
Instrument Type:	
Block Type:	384-Well Block
Errors Encountered:	× ×

From the QuantStudio[™] 6 or 7 Instrument touchscreen You can also view the progress of the run from the QuantStudio ${}^{{}^{\rm TM}}6$ or 7 Instrument touchscreen.

The following are images of the Run Method screen on the QuantStudio $^{\text{TM}}$ 6 or 7 Instrument touchscreen:

Experiment View



Time View

0	Time View	t#t	Experiment View	
F React	Run Started: July 0 ion Volume: 20 μL	6 2013	- 05:48PM	Sample: 95.0 °C Heated Cover (Set Point): 105.0 °C (105.0 °C) Stage / Step / Cycle: 1 / 2 / 1
	Λ	1	.?	1.52
	U			1.52
		(Remaining Time	C Elapsed Time
				July 06 2013 - 05:48PM Heated cover reached target temperature.



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

PurposeThe 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 384 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 following is an image of the Presence/Absence Plot for the example experiment:



Tips for viewing presence/absence plots in your own experiments

- The **IPC threshold** is calculated from the Negative Control- Blocked IPC reactions.
- **The Target Threshold** is calculated from the Negative Control- IPC reactions. If the 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 forThe Amplification Plot displays amplification of all samples in the selected wells.
There are three amplification plot views available:viewing $\Delta \mathbf{Rn vs Cycle} - \Delta \mathbf{Rn}$ is the difference in normalized fluorescence signal genera

- ΔRn vs Cycle ΔRn is the difference in normalized fluorescence signal generated by the reporter between the pre-PCR read and the post-PCR read. This plot displays ΔRn as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle** Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays Rn as a function of cycle number. You can use this plot to identify and examine irregular amplification.
- **C**_T **vs Well** C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. You can use this plot to locate outlying amplification (outliers).

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



Purpose

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

- Correct baseline and threshold values
- Irregular amplification
- Outliers

View the Amplification Plot

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

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

- **2.** Display all 384 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:



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

Your screen should look like this:



Tips for viewing amplification plots in your own experiments

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

- Outliers
- A typical amplification plot The QuantStudio[™] 6 and 7 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

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A following is an image of a typical amplification plot:

IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the QuantStudioTM 6 and 7 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.

View the Well Table

The well table displays results data for each well in the reaction plate, including:

- The well number, sample name, target name, task, and dyes
- The calculated values: ΔRn , ΔRn mean, and ΔRn SD

Note: ΔRn , ΔRn mean, and ΔRn SD are calculated only when the analysis call settings specify to analyze data from the pre-PCR read and the post-PCR read.

- Target and IPC thresholds, Call, Comments
- Flags

Purpose

In the Presence/Absence example experiment, you review the well table for:

- Call
- ΔRn
- Flag

To view the Well Table

b

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

Note: If no data are displayed, click Analyze.

2. Use the Group By drop-down menu to group wells by a specific category. For the example experiment, group the wells by flag, call, and ΔRn value.

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

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

뇐	Plat	e Layout	Wel	Ilable												
	Show	in Table	▼ Select	: Wells 🔻	Group Bv 🔻								a e	xoand All	🗖 Collaps	e All
	_														_	
Γ	#	Well	Omit	Flag	Samp Target Name	Task	Dyes	ΔRn	∆Rn Mean	∆Rn SD	Threshold	Call	Comments	NOAMP	EXPF	AJ
ľ			Flagged V	Vells												
	4	A4			Sampl IPC	IPC	VIC-NFQ-MGB	1.376	1.404	0.012	1.09	IPC Succe			1 17	
	4	A4		2	Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	0.38	0.381	0.006	0.414	Absence				
- 1	5	A5		2	Sampl IPC	IPC	VIC-NFQ-MGB	1.395	1.404	0.012	1.09	IPC Succe				
	5	A5		2	Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	0.378	0.381	0.006	0.414	Absence				
	6	A6		2	Sampl IPC	IPC	VIC-NFQ-MGB	1.403	1.404	0.012	1.09	IPC Succe				
- 1	6	A6		2	Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	0.38	0.381	0.006	0.414	Absence				
- 1	7	A7		2	Sampl IPC	IPC	VIC-NFQ-MGB	1.41	1.404	0.012	1.09	IPC Succe				
	7	A7		2	Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	0.386	0.381	0.006	0.414	Absence				
	8	A8		2	Sampl IPC	IPC	VIC-NFQ-MGB	1.401	1.404	0.012	1.09	IPC Succe				
1	8	A8		2	Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	0.389	0.381	0.006	0.414	Absence				
- 1	9	A9		2	Sampl IPC	IPC	VIC-NFQ-MGB	1.395	1.404	0.012	1.09	IPC Succe				
	9	A9		2	Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	0.382	0.381	0.006	0.414	Absence		II ♠ .		
	10	A10		2	Sampl IPC	IPC	VIC-NFQ-MGB	1.405	1.404	0.012	1.09	IPC Succe		II ♠ .		
	10	A10		2	Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	0.382	0.381	0.006	0.414	Absence		II .▲		
	11	A11		2	Sampl IPC	IPC	VIC-NFQ-MGB	1.407	1.404	0.012	1.09	IPC Succe		II ♠ .		
	11	A11		2	Sampl TGFB		FAM-NFQ-MGB	0.387	0.381	0.006	0.414	Absence		II ♠ .		
	12	A12		2	Sampl IPC	IPC	VIC-NFQ-MGB	1.402	1.404	0.012	1.09	IPC Succe		I <u>↑</u>		4
	12	A12		2	Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	0.382	0.381	0.006	0.414	Absence		•		4
	28	84		2	Sampl IPC	IPC	VIC-NEQ-MGB	1.396	1.404	0.012	1.09	IPC SUCCE				
	28	84		2	Sampi TGFB		FAMI-NEQ-MGB	0.384	0.381	0.006	0.414	Absence				
	29	B2		2	Sampi IPC	IPC	VIC-NEQ-MGB	1.4	1.404	0.012	1.09	IPC SUCCE				
	29	BO DE		2	Sampl TGFB		FAMI-NEQ-MGB	0.384	0.381	0.000	0.414	ADSERILE				
	30	B0 D6		2	Sampl IPC		VIC-INFQ-MGB	1.408	1.404	0.012	1.09	Abcopco				-
1			•	-			Contraction raised and									*
We	ell Sur	mmary	:	In Plate:	384 Set Up: 384	Analyzed	l: 384 Fla	agged: 144	Omitted by Ar	alysis: ()	Omitted Mar	nually: ()	Samples Used	:4	Targets U	sed: 2

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

- Negative Control
- Presence

凶	Plate Layou	t Well Table	a Group By a								E Evos	od All 🗖 (Collanza All
		V Delect overla	• croop by •										conopse mil
	# Well	Omit	Flag Samp Target Na	ame Task	Dyes	∆Rn	∆Rn Mean	∆Rn SD	Threshold	Call	Comments	NOAMP	EXPI
		Absence											
		Blocked IPC	: Control										
		IPC Succeer	ded										
		Negative Co	ontrol										
		Presence											
	16 A16		Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	1.769	1.706	0.034	0.414	Presence			
	17 A17		Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	1.789	1.706	0.034	0.414	Presence			
	18 A18		Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	1.744	1.706	0.034	0.414	Presence			
	19 A19		Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	1.722	1.706	0.034	0.414	Presence			
	20 A20		Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	1.722	1.706	0.034	0.414	Presence			
	21 A21		Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	1.739	1.706	0.034	0.414	Presence			
	22 A22		Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	1.695	1.706	0.034	0.414	Presence			
	23 A23		Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	1.708	1.706	0.034	0.414	Presence			
	24 A24		Sampl TGFB	UNKNOWN	FAM-NFQ-MGB	1.716	1.706	0.034	0.414	Presence			
	40 816		Sampl TGFB	UNKNOWN	FAM-NEQ-MGB	1.773	1.706	0.034	0.414	Presence			
	41 817		Sampl TGFB	UNKNOWN	FAM-NEQ-MGB	1.//3	1.706	0.034	0.414	Presence			
	42 818		Sampi TGFB	UNKNOWN	FAMINEQ-MGB	1.745	1.706	0.034	0.414	Presence			
	43 B19 44 D20		Sampl TGFB	UNKNOWN	FAM-NEQ-MGB	1.737	1.706	0.034	0.414	Presence			
	44 D20 45 D01		Campl TGFD		FAMINEQ MGB	1.720	1.700	0.034	0.414	Droconco			
	45 D21 46 D22		Sampl TGEP		EAM NEO MGB	1,000	1.700	0.034	0.414	Presence			
	47 022		Sampl TGEP		EAM NEO MGP	1.711	1.700	0.034	0.414	Droconco			
	49 B24		Sampl TGFB		EAM-NEO-MGB	1,000	1,700	0.034	0.414	Drecence			
	64 (16		Sampl. TGFB	LINKNOWN	EAM-NEO-MGB	1.090	1,706	0.034	0.414	Presence			
	65 C17		Samplini TGER	LINKNOMN	EAM-NEO-MGR	1 743	1 706	0.004	0.414	Droconco			-
		4								S			Þ
W	ell Summary	: In Pla	ate: 384 Set Up: 384	Analyzed: (384 Flagge	d: 144	Omitted by Analysi	s: O	Omitted Manually	0	Samples Used: 4	Tar	gets Used: 2

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.

<u>ک</u>	Plate Layou	: Well Ta	able												
	Show in Table	▼ Select W	ells 🔻	Group By	Ŧ				,				≡ Ехр	and All 🛛 🗖	Collapse All
Γ	# Well	Omit	Flag	Samp	. Target Name	Task	Dyes	∆Rn ≜1	∆Rn Mean	∆Rn SD	Threshold	Call	Comments	NOAMP	EXPI
ľ	J1			NTC	TGFB	NTC	FAM-NFQ-MGB	0.33	0.371	0.016		Negative C			
- 1	H1			NTC	TGFB	NTC	FAM-NFQ-MGB	0.33	0.371	0.016		Negative C			
- 1	I1			NTC	TGFB	NTC	FAM-NFQ-MGB	0.337	0.371	0.016		Negative C			
- 1	H2			NTC	TGFB	NTC	FAM-NFQ-MGB	0.34	0.371	0.016		Negative C			
- 1	I2			NTC	TGFB	NTC	FAM-NFQ-MGB	0.341	0.371	0.016		Negative C			
- 1	J2			NTC	TGFB	NTC	FAM-NFQ-MGB	0.345	0.371	0.016		Negative C			
- 1	G1			NTC	TGFB	NTC	FAM-NFQ-MGB	0.349	0.371	0.016		Negative C			
- 1	F1			NTC	TGFB	NTC	FAM-NFQ-MGB	0.349	0.371	0.016		Negative C			
- 1	G2			NTC	TGFB	NTC	FAM-NFQ-MGB	0.354	0.371	0.016		Negative C			
	K1			NTC	TGFB	NTC	FAM-NFQ-MGB	0.356	0.371	0.016		Negative C			
	J11		2	Sampl	. TGFB	UNKNOWN	FAM-NFQ-MGB	0.363	0.381	0.006	0.414	Absence	l.		
- 1	H13			NAC	TGFB	NTC	FAM-NFQ-MGB	0.363	0.381	0.007		Negative C			
- 1	K2			NTC	TGFB	NTC	FAM-NFQ-MGB	0.364	0.371	0.016		Negative C			
- 1	F14			NAC	TGFB	NTC	FAM-NFQ-MGB	0.364	0.381	0.007		Negative C			
- 1	I3			NTC	TGFB	NTC	FAM-NFQ-MGB	0.367	0.371	0.016		Negative C			
- 1	49 C1			NTC	TGFB	NTC	FAM-NFQ-MGB	0.367	0.371	0.016		Negative C			
- 1	H11		2	Sampl	. TGFB	UNKNOWN	FAM-NFQ-MGB	0.368	0.381	0.006	0.414	Absence	E		
- 1	F2			NTC	TGFB	NTC	FAM-NFQ-MGB	0.369	0.371	0.016		Negative C			
- 1	L11		2	Sampl	. TGFB	UNKNOWN	FAM-NFQ-MGB	0.369	0.381	0.006	0.414	Absence	1		
- 1	НЗ			NTC	TGFB	NTC	FAM-NFQ-MGB	0.369	0.371	0.016		Negative C			
- 1	J14			NAC	TGFB	NTC	FAM-NFQ-MGB	0.369	0.381	0.007		Negative C			
- 1	73 D1			NTC	TGFB	NTC	FAM-NFQ-MGB	0.37	0.371	0.016		Negative C			
- 1	H14			NAC	TGFB	NTC	FAM-NFQ-MGB	0.37	0.381	0.007		Negative C			
	J10		2	Sampl	. TGFB	UNKNOWN	FAM-NFQ-MGB	0.371	0.381	0.006	0.414	Absence			4
Ľ	k/11	•	-	Samnl	TGER	LINKNOWN	EAM_NEO_MGR	0 372	0.381	0.006	N 414	Ahsonco		•	
We	ll Summary	: In	Plate: 38	34	Set Up: 384	Analyzed: (384 Flagged:	144 (Omitted by Analys	sis: O	Omitted Manual	ly: O	Samples Used: 4	Ta	rgets Used: 2

Tips for analyzing your own experiments	 When you analyze your own Presence/Absence experiment, group the wells by: Flag –The software groups the flagged and unflagged wells. A flag indicates that the software has found an error in the flagged well. For a description of the QuantStudio[™] 6 and 7 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 accura	te dye signal using the Multicomponent Plot
	The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.
Purpose	In the Presence/Absence example experiment, you review the Multicomponent Plot screen for:
	 ROX[™] dye (passive reference)
	• FAM [™] dye (reporter)
	• VIC [®] dye (reporter)
	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 E 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.

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



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



8. Select the negative control (NAC) 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

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

- **Passive reference (ROX)** The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye (FAM)** The reporter dye fluorescence level should display a flat region corresponding to the baseline. If target is present in the sample (a Presence call is made), the baseline will be followed by a rapid rise in fluorescence as the amplification proceeds.
- Irregularities in the signal There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative control wells** There should not be any amplification in the negative control wells.

Determine signal accuracy using the Raw Data Plot

	The Raw Data Plot screen displays the raw fluorescence signal (not normalized) for each optical filter for the selected wells during each cycle of the real-time PCR.
Purpose	In the Presence/Absence example experiment, review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.
View the Raw Data Plot	 From the Experiment Menu pane, select Analysis > Raw Data Plot. Note: If no data are displayed, click Analyze.

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- 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 384 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:
 - Sample (-) wells: From the Select Wells with drop-down menus, select Sample (-).
 - Sample (+) wells: From the Select Wells with drop-down menus, select Sample (+).
 - Negative control-IPC wells: Select wells A1 P1, A2 P2, and A3 P3.
 - Negative control-blocked IPC wells: Select wells A13 P13, A14 P14, and A15 P15.
- 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.



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The filters used for the example experiment are:

PCR I	ilter —								
				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)	×							
Filter	x2(520±10)		V						
ation	x3(550±11)			\checkmark					
Excit	x4(580±10)				\checkmark				
	x5(640±10)					\checkmark			
	x6(662±10)								
Melt	Melt Curve Filter								
				Load Save Revert to	Defaults				
				Load Save Revert to Emission Filter	Defaults				
		m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10)	Defaults m4(623±14)	m5(682±14)	m6(711±12)		
	x1(470±15)	m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10)	Defaults (623±14)	m5(682±14)	m6(711±12)		
Filter	x1(470±15) x2(520±10)	m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10)	Defaults m4(623±14)	m5(682±14)	m6(711±12)		
ation Filter	x1(470±15) x2(520±10) x3(550±11)	m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10) Image: I	Defaults m4(623±14)	m5(682±14)	m6(711±12)		
Excitation Filter	x1(470±15) x2(520±10) x3(550±11) x4(580±10)	m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10)	Defaults m4(623±14)	m5(682±14)	m6(711±12)		
Excitation Filter	x1(470±15) x2(520±10) x3(550±11) x4(580±10) x5(640±10)	m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10)	Defaults m4(623±14)	m5(682±14)	m6(711±12)		
Excitation Filter	x1(470±15) x2(520±10) x3(550±11) x4(580±10) x5(640±10) x6(662±10)	m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10)	Defaults m4(623±14)	m5(682±14)	m6(711±12)		
Excitation Filter	x1(470±15) x2(520±10) x3(550±11) x4(580±10) x5(640±10) x6(662±10)	m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10)	Defaults m4(623±14)	<pre>m5(682±14)</pre>	m6(711±12)		
Excitation Filter	x1(470±15) x2(520±10) x3(550±11) x4(580±10) x5(640±10) x6(662±10)	m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10)	Defaults m4(623±14)	m5(682±14)	m6(711±12)		
Excitation Filter	x1(470±15) x2(520±10) x3(550±11) x4(580±10) x5(640±10) x6(662±10)	m1(520±15)	m2(558±11)	Load Save Revert to Emission Filter m3(586±10)	Defaults m4(623±14)	m5(682±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 QuantStudioTM 6 and 7 Flex Software flags, including the flag frequency and location for the open experiment. In the example experiment, 144 flags have been triggered.

Note: The flags triggered in the example experiment are seen in the Sample (-) wells. The flags, NOAMP and EXPFAIL indicate that the wells containing the Sample (-) did not amplify and that the software could not identify the exponential region of the amplification plot (as amplification did not take place). The occurrence of these flags in the 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 144 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 144 times, in the wells A4 P4, A5 P5, A6 P6, A7 P7, A8 P8, A9 P9, A10 P10, A11 P11, and A12 P12.
 - The EXPFAIL flag appears 144 times, in the same wells as the NOAMP flag, that is, A4 P4, A5 P5, A6 P6, A7 P7, A8 P8, A9 P9, A10 P10, A11 P11, and A12 P12.
- **4.** (*Optional*) For those flags with frequency >0, click the troubleshooting link to view information on correcting the flag.

	Flag:	Description	Frequency		Wells	
IADROX		Bad passive reference signal	0			
IOSIGNAL		No signal in well	0			
OFFSCALE		Fluorescence is offscale	0			-
MPNC		Amplification in negative control	0			
IOAMP		No amplification	144	A4,	A5, A6, A7, A8, A9, A10, A11, A1	2, B
IOISE		Noise higher than others in plate	0			
PIKE		Noise spikes	0			
XPFAIL		Exponential algorithm failed	144	A4,	A5, A6, A7, A8, A9, A10, A11, A1	.2, B
LFAIL		Baseline algorithm failed	0			
HOLDFAIL		Thresholding algorithm failed	0			
TFAIL		Ct algorithm failed	0			
	Flag Criteria: # Flagged Wells: # [k k	Amplification algorithm result < 0.1 M4, A5, A6, A7, A8, A9, A10, A11, A12, B4, D7, D8, D9, D10, D11, D12, E4, E5, E6, E S10, G11, G12, H4, H5, H6, H7, H8, H9, H1 (5, K6, K7, K8, K9, K10, K11, K12, L4, L5, J 48, N9, N10, N11, N12, O4, O5, O6, O7, O View NOAMP Troubleshooting Information	B5, B6, B7, B8, B9, B10, B11, B12, C4, 7, E8, E9, E10, E11, E12, F4, F5, F6, F7 10, H11, H12, I4, I5, I6, I7, I8, I9, I10, I: L6, L7, L8, L9, L10, L11, L12, M4, M5, N 8, O9, O10, O11, O12, P4, P5, P6, P7, I	C5, C6, C7, C8, , F8, F9, F10, F1 L1, I12, J4, J5, J 46, M7, M8, M9, P8, P9, P10, P1	C9, C10, C11, C12, D4, D5, D6 1, F12, G4, G5, G6, G7, G8, G9 6, J7, J8, J9, J10, J11, J12, K4, M10, M11, M12, N4, N5, N6, N7 1, P12	ŝ, , ,
otal Wells: Vells Set Lin:		384 Processed Wells: 384 Elanged Wells:	384 Manually Omitted Wells: 144 Analysis Omitted Wells:	0	Targets Used: Samples Used:	2

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

Flag	Description
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well
EXPFAIL	Exponential algorithm failed
BLFAIL	Baseline algorithm failed
THOLDFAIL	Thresholding algorithm failed
CTFAIL	C _T algorithm failed
	1

Secondary analysis flags					
AMPNC	Amplification in negative control				

Note: If the experiment does not include amplification, then the only flags are BADROX, NOSIGNAL, and OFFSCALE.

For more information

For more information on	Refer to	Publication number
Publishing data	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio[™] 6 and 7</i> Flex Real-Time PCR System Software Experimentss	4489822

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[™] 6 and 7 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 An

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 following is an image of the Analysis Settings dialog box for a Presence/ Absence experiment:

Analysis Settings for QuantStudio_3	384-Well_Presence-Absence_Exan	nple				
Call Settings CT Settings Flag S	Settings Advanced Settings					
Review the default settings for analys Settings, then change the settings.	sis of targets in this experiment. To use diff	erent settings for a target, select the target	t from the table, deselect Use Default			
Data Analysis Settings						
Analyze Data from Post-PCR Read	l Only 💿 Analyze Data from Pre-PCR	Read and Post-PCR Read				
Default Call Settings Default call settings are used to make pre	sence/absence calls for targets without cus	stom settings. To edit the default settings, c	lick Edit Default Settings.			
Confidence Value: 99% Edit De	efault Settings					
Select a Target			Call Settings for TGFB			
Target	Analysis Type	Confidence	Call Settings to Use: 🗹 Default Settings			
TGFB	Default	99%	Confidence Value: 99% V			
IPC	Default	99%				
Save to Library Load from Libr	rary	Revert to Default Analysis Settin	ngs Apply Analysis Settings Cancel			

5

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*[™] 6 and 7 Flex Real-Time PCR System Software Experiments.

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

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 check box.
 - 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

Use the Baseline Threshold algorithm to determine the C_T values.

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

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:
	 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[™] 6 and 7 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 following is an image of the Flag Settings tab:

🕲 Ai	nalysis Settings for	r QuantStudio_384-We	II_Presence-Absence	_Example			\mathbf{X}
С	all Settings CT Se	ettings Flag Settings	Advanced Settings)			
	Configure the flag	s and filtering. In this panel	you can enable, disable, an	d configure flags, and indica	ate if a well is to be rejected	d when a flag is raised.	
							<u> </u>
F	lag	Description	Use	Attribute	Condition	Value	Reject Well
BA	DROX	Bad passive referenc		Fluorescence	< 🗸	500.000	
NC	SIGNAL	No signal in well	\checkmark				
OF	FSCALE	Fluorescence is offscale					
AM	IPNC	Amplification in negat	V	Ст	< 🗸	35.000	
NC	AMP	No amplification	V	Amplification algorith	< 🗸	0.100	
NC	DISE	Noise higher than ot	V	Relative noise	> 🗸	4.000	
SP	IKE	Noise spikes	V	Spike algorithm result	> 🗸	1.000	
EX	PFAIL	Exponential algorithm	V				
BLI	FAIL	Baseline algorithm failed	V				
тн	IOLDFAIL	Thresholding algorith	V				
ст	FAIL	Ст algorithm failed	V				
						·	
Sa	we to Library	Load from Library		Revert to Def	ault Analysis Settings	Apply Analysis	Settings Cancel

Advanced Settings

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

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	Publication number
Amplification efficiency	Amplification Efficiency of TaqMan [®] Gene Expression Assays Application Note.	127AP05-03

Exporting 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**[™] **6 and 7** 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	QuantStudio_384-Well_Presence-Absence_Example_data
File Type	*.txt
Export File Location	C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\Export

Your Export screen should look like this:

port File Location: ms\Quai	ntstudio 6 and 7	FIEX SOTTW	are User Files Export	Browse Export File	Name: Quantstu	alo_384-well_Pres	ence-Absence_E	File Type: 📑 (*	txt) 🚩
🗸 Sample Setup 📃 Ray	v Data 🔽 Amj	olification	Multicomponent	Results					
Select Content		Well	Well Position	Sample Name	Sample Color	Target Name	Target Color	Task	B
All Fields	~		1 A1	NTC	RGB(255.0.0)	IPC	RGB(0.255.0)	IPC	VIC
			1 A1	NTC	RGB(255,0,0)	TGFB	RGB(0,0,255)	NTC	FA
V Well			2 A2	NTC	RGB(255,0,0)	IPC	RGB(0,255,0)	IPC	VIC
Wall Position			2 A2	NTC	RGB(255,0,0)	TGFB	RGB(0,0,255)	NTC	FA
Viel Posicion			3 A3	NTC	RGB(255,0,0)	IPC	RGB(0,255.0)	IPC	VIC
Sample Name			3 A3	NTC	RGB(255,0,0)	TGFB	RGB(0,0,255)	NTC	FA
			4 A4	Sample (-)	RGB(0,0,255)	IPC	RGB(0,255,0)	IPC	VIC
 Sample Color 			4 A4	Sample (-)	RGB(0,0,255)	TGFB	RGB(0,0,255)	UNKNOWN	FA
			5 A5	Sample (-)	RGB(0,0,255)	IPC	RGB(0,255,0)	IPC	VIC
Iarget Name	=		5 A5	Sample (-)	RGB(0,0,255)	TGFB	RGB(0,0,255)	UNKNOWN	FA
Target Color			6 A6	Sample (-)	RGB(0,0,255)	IPC	RGB(0,255,0)	IPC	VIC
Target color			6 A6	Sample (-)	RGB(0,0,255)	TGFB	RGB(0,0,255)	UNKNOWN	FA
✓ Task			7 A7	Sample (-)	RGB(0,0,255)	IPC	RGB(0,255,0)	IPC	VIC
			7 A7	Sample (-)	RGB(0,0,255)	TGFB	RGB(0,0,255)	UNKNOWN	FA
 Reporter 			8 A8	Sample (-)	RGB(0,0,255)	IPC	RGB(0,255,0)	IPC	VIC
Current and			8 A8	Sample (-)	RGB(0,0,255)	TGFB	RGB(0,0,255)	UNKNOWN	FA
V Quencher			9 A 9	Sample (-)	RGB(0,0,255)	IPC	RGB(0,255,0)	IPC	VI
Comments			9 A 9	Sample (-)	RGB(0,0,255)	TGFB	RGB(0,0,255)	UNKNOWN	FA
	20	1.0							

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Presence/Absence Experiments

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QuantStudio_384-Well_Presence-Absence_Example_data.txt - Notepad	
File Edit Format View Help	
<pre>P Block Type = 384-well Block calibration Background is expired = Yes calibration Background performed on = 12-09-2009 calibration HRM MELTDOCTOR performed on = 01-14-2010 calibration Normalization FAM-ROX is expired = Yes calibration Normalization TAM-ROX performed on = 12-09-2009 calibration Normalization VIC-ROX performed on = 12-09-2009 calibration Normalization VIC-ROX performed on = 12-09-2009 calibration Pure Dye FAM is expired = Yes calibration Pure Dye FAM sperformed on = 12-09-2009 calibration Pure Dye HELTDOCTOR performed on = 01-14-2010 calibration Pure Dye HELTDOCTOR performed on = 01-14-2010 calibration Pure Dye KOX performed on = 12-09-2009 calibration Pure Dye ROX beformed on = 12-09-2009 calibration Pure Dye ROX performed on = 12-09-2009 calibration Pure Dye SYBR performed on = 12-09-2009 calibration Pure Dye SYBR performed on = 12-09-2009 calibration Pure Dye SYBR performed on = 12-09-2009 calibration Pure Dye SYBR performed on = 12-09-2009 calibration Pure Dye VIC sepformed on = 12-09-2009 calibration Pure Dye VIC sepformed on = 12-09-2009 calibration Pure Dye VIC sepformed on = 12-09-2009 calibration Pure Dye VIC performed on = 12-09-2009 calibration Pure Dye VIC performed on = 12-09-2009 calibration Pure Dye VIC performed on = 12-09-2009 calibration PUFORME DY Compared = Yes calibration PUFORME ON SEG calibration PUFORME SEG calibration PUFORME ON SEG calibration PUFORME ON SEG calibration PUFORME ON SEG calibration PUFORME ON SEG calibratio</pre>	
[Sample Setup] (Well well Position Sample Name Sample Color Target Name Target Color Task Reporter Quencher Comments 1 NTC "RGB(255,0,0)" IPC "RGB(0,255,0)" IPC VIC NFQ-MGB 1 A1 NTC "RGB(255,0,0)" TGFB "RGB(0,255,0)" IPC VIC NFQ-MGB 2 A2 NTC "RGB(255,0,0)" TGFB "RGB(0,255,0)" IPC VIC NFQ-MGB 2 A2 NTC "RGB(255,0,0)" TGFB "RGB(0,255,0)" IPC VIC NFQ-MGB 3 A3 NTC "RGB(255,0,0)" TGFB "RGB(0,0,255,0)" IPC VIC NFQ-MGB 3 A3 NTC "RGB(255,0,0)" TGFB "RGB(0,0,255,0)" IPC VIC NFQ-MGB 3 A3 NTC "RGB(255,0,0)" IPC NFQ-MGB 4 A4 Sample (-) "RGB(0,0,255)" TGFB "RGB(0,0,255,0)" IPC VIC NFQ-MGB 4 A4 Sample (-) "RGB(0,0,255)" TGFB "RGB(0,0,255,0)" IPC VIC NFQ-MGB	•

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

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USER GUIDE



QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Melt Curve Experiments

Booklet 6

Publication Number 4489822 **Revision** A



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About Melt Curve Experiments

This chapter covers:

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IMPORTANT! First-time users of the QuantStudioTM 6 and 7 Flex Real-Time PCR System Software, please read Booklet 1, *Getting Started with QuantStudioTM 6 and 7 Flex Real-Time PCR System Software Experiments* and Booklet 7, *QuantStudioTM 6 and 7 Flex Real-Time PCR System Software - 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[™] 6 and 7 Flex Real-Time PCR System Software by pressing F1, clicking ? in the toolbar, or selecting Help > QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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[™] 6 and 7 Flex Real-Time PCR System 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[™] 6 or 7 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. Negative controls 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 QuantStudioTM 6 and 7 Flex Real-Time PCR System Software.

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 QuantStudioTM 6 or 7 Instrument or on another thermal cycler.

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

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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 2 in Booklet 1, *Getting Started with QuantStudio*TM 6 and 7 Flex Real-Time PCR System Software Experiments.

Define the experiment properties

juantStudio ¹¹¹ 6 and 7 Flex Software. Enter:			
Field or Selection	Entry		
Experiment Name	QuantStudio_384-Well_SYBR_Green_Melt_Example		
Barcode	Leave field empty		
User Name	Example User		
Comments	Melt Curve example		
Instrument type	QuantStudio [™] 6 Flex System		
Block	384-Well Block		
Experiment Type	Melt Curve		
Reagents	SYBR [®] Green Reagents		
Ramp speed	Standard		
Include PCR	Unchecked		

Click **Experiment Setup** → **Experiment Properties** to create a new experiment in the QuantStudio[™] 6 and 7 Flex Software. Enter:

Save the experiment.

Reagent information

NA

2

Your Experiment Properties screen should look like this:

How do you want to identify this expe	riment?		
Experiment Name: QuantStudio_384-Well_State Barcode: User Name: Example User	/BR_Green_Melt_Example	Comments: Melt Curve Experiment	*
Which instrument type are you using) to run the experiment?		
✓ QuantStudio [™] 6 Flex System	QuantStudio [™] 7 Flex System		
Which block are you using to run the	experiment?		
✓ 384-Well	96-Well (0.2mL)	Fast 96-Well (0.1mL)]
What type of experiment do you war	nt to set up?		
Standard Curve	Relative Standard Curve	Comparative Cτ (ΔΔCτ)	✓ Melt Curve
Genotyping	Presence/Absence		
* Which reagents do you want to use t	o detect the target sequence?		
✓ SYBR® Green Reagents	Other		
What properties do you want for the	instrument run?		
✓ Standard	Fast		
Include PCR			
What is the reagent information?			
New Delete			
Type Name	Part Number	Lot Number	Expiration Date

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
- 4. Custom Task Name Not applicable

Targets	Samples								
New Save to Library Import from Library De	lete			New	Save to Library	Import from L	brary Delete	import from File	
Target Name	Reporter	Quencher	Color	Sample	e Name	С	olor		
Target 1	SYBR 🗸	None 🗸	► ✓	Sample 1			~		
Passive Reference	Passive Reference				Custom Task Name				
ROX				New	Delete				
				Name			Color		Icon Char

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 - P24 (Columns 1 -24)	Unknown

Your Assign screen should look like this:

Targets	<	Plate Layout	Well Table										
Name Task	×												
Target 1		🛄 Show in W	ells 🔻 🔽 Select V	Wells 🔻 🖺	View Legend						٠	o 🛒	
		1	2	3	4	5	6	7	8	9	10	11	
	A	U Target 1	U Target 1	U Target 1	U Target 1	U Target 1	Target 1	U Target 1	Target 1	Target 1	U Target 1	U Target 1	<u>^</u>
	в	U Target 1	U Target 1	U Target 1	U Target 1	U Target 1	Target 1	U Target 1	Target 1	U Target 1	U Target 1	U Target 1	
Samples 💌	c	U Target 1	U Target 1	Target 1	Target 1	U Target 1	U Target 1	U Target 1	Target 1	U Target 1	U Target 1	U Target 1	μ
Name Sample 1	D	Target 1	Target 1	U Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	U Target 1	Target 1	
	E	Target 1	Target 1	U Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	U Target 1	Target 1	
	F	Target 1	U Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	U Target 1	U Target 1	
		•											▼ ▶
	ν	Vells: 🚺 384	ł N O									O Em	ipty

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Melt Curve Experiments

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.

Run Method										
Reaction \	Reaction Volume per Well: 20 µL									
Graphica	Graphical View Optical Filters									
Add Sh	ne 💌 Add Sten 💌 Delete Selecte	d Jada Rada Callect Da	ta 🕊 Onen Run Methad - Caue Ru	Malhad Bauetta Dafadh						
Add bla	ge y Hud Diep y Delete Deletite	d ondo nedo collectida	a y Opennanimenioa Saveria							
		Melt Curve Stage								
		C Step and Hold 0:05								
		Continuous								
100 °C	95 9 9C		05.0.00							
	00:15		00:15							
75 ℃		1.6 °C/s	0.05 °C/s							
	he sets	60.0 °C								
50 ℃	1.0 *C/S	01:00								
25 %										
20 0-										
0 °C	01-14	010	Ohan 2 (Diana sisting)							
	Step1	Step2	Step3 (Dissociation)	E Contractor a contractor a contractor a contractor a contractor a contractor a contractor a contractor a contra						
Legen	d									
🔞 Data	a Collection On 👘 Data Collectio	on Off 🔺 AutoDelta On 🔺 Aut	toDelta Off							

Your Run Method screen should look like this:

For more information

For more information on	Refer to	Publication number
Consumables	Chapter 1 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7</i> Flex Real-Time PCR System Software Experiments	4489822
	Appendix A in Booklet 7, <i>QuantStudio™ 6 and 7 Flex Real-Time PCR</i> System Software Experiments - Appendixes	
Using Alternative Setup	Chapter 3 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7</i> Flex Real-Time PCR System Software Experiments	4489822

Chapter 2 Design the Experiment *For more information*

2

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 QuantStudioTM 6 or 7 Instrument or on another thermal cycler.

This chapter covers:

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

Assemble required materials

- Items listed in Booklet 1, *Getting Started with QuantStudio*[™] 6 and 7 Flex Real-Time PCR System Software Experiments
- Sample 1
- Example experiment reaction mix components:
 - Power SYBR[®] Master Mix
 - Target Assay Mix Forward primer (10 μM)
 - Target Assay Mix Reverse primer (10 μM)

Prepare the sample dilutions

The stock concentration of each sample is 100 ng/ 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	Stock concentration (ng/µL)	Sample volume (µL)	Diluent volume (µL)	Total volume of diluted sample (µL)
Sample 1	100.0	2	18	20

3

Prepare the reaction mix ("cocktail mix")

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

Reaction Component	Volume for 1 reaction (µL)	Volume for 384 reactions + 10% excess (µL) = 424 reactions
Power SYBR [®] Green PCR Master Mix (2X)	10	4240
Forward primer (10 µM)	0.1	42.4
Reverse primer (10 µM)	0.1	42.4
Water	7.8	3307.2
Total reaction mix volume	18	7632

Procedure 1. Label an appropriately sized tube for the reaction mix: Power SYBR Reaction Mix.

- 2. Add the required volume of each cocktail mix component to the tube.
- **3.** Mix the cocktail thoroughly by gently pipetting up and down several times, then cap the tube.
- 4. Centrifuge the tube briefly to remove air bubbles.
- 5. Place the cocktail mix on ice until you prepare the reaction plate.

Calculations Determine the quantity of primer to be added to the reaction mix by performing the following calculation:

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

 $(10 \ \mu\text{M}) \times (V1) = (0.05 \ \mu\text{M}) (20 \ \mu\text{L})$

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

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	7632	Sample 1	848

3

- **b.** Mix the reactions by gently pipetting up and down, then cap the tubes.
- **c.** Centrifuge the tubes briefly to remove air bubbles.
- 2. Pipet 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	Publication number
Assigning the reaction plate components	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7</i> Flex Real-Time PCR System Software Experiments	4489822
Sealing the reaction plate	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7</i> Flex Real-Time PCR System Software Experiments	4489822

Chapter 3 Prepare the Reactions *For more information*

3
This chapter explains how run the example experiment on the QuantStudio $^{\rm TM}$ 6 or 7 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 QuantStudioTM 6 or 7 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 the QuantStudioTM 6 or 7 Instrument, 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[™] 6 and 7 Flex Real-Time PCR System Software using the Run screen, while the experiment is in progress.
- From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software (to monitor an experiment started from another computer or from the QuantStudio[™] 6 or 7 Instrument touchscreen).
- From the QuantStudio[™] 6 or 7 Instrument touchscreen.

From the Instrument Console of the QuantStudio[™] 6 and 7 Flex Real-Time PCR System 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[™] 6 and 7 Flex Real-Time PCR System Software for potential problems.

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

The following is an image of 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 following is an image of 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 following is an image of the Run Method screen as it appears in the example experiment.



View run data

Click View Run Data from the Run Experiment Menu.

The following is an image of the View Run Data screen as it appears in the example experiment.

Run Data Report	
Experiment Name:	QuantStudio_384-Well_SYBR_Green_Melt_Example
Start Time:	04-30-2010 14:41:31 SGT
Stop Time:	04-30-2010 14:56:52 SGT
Run Duration:	15 minutes 20 seconds
User Name:	DEFAULT
Instrument Name:	278880026
Firmware Version:	1.0.0
Software Version:	N/A
Instrument Serial Number:	278880026
Sample Volume:	20.0
Cover Temperature:	105.0
Block Type:	384-Well Block
Errors Encountered:	X X

From the QuantStudio[™] 6 or 7 Instrument touchscreen You can also view the progress of the run from the QuantStudio ${}^{^{\rm TM}}6$ or 7 Instrument touchscreen.

The following are images of the Run Method screen on the QuantStudioTM 6 or 7 Instrument touchscreen:

Experiment View



4

Time View

S Time View TH Experiment View	
Run Started: July 06 2013 - 05:48PM Reaction Volume: 20 µL	Sample: 95.0 °C Heated Cover (Set Point): 105.0 °C (105.0 °C) Stage / Step / Cycle: 1 / 2 / 1
01.3	1.52
	1.72
Remaining Time	Uly 06 2013 - 05-48PM
	Heated cover reached target temperature.



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	27
	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	33
Sect	tion 5.2 Adjust parameters for re-analysis of your own experiments	35
	Adjust analysis settings	35
	For more information	38



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.

PurposeThe purpose of viewing the Melt Curve Plot for the example experiment is to review
the melting temperature of the target.

- To view and assess1. From the Experiment menu pane, select Analysis > Melt Curve Plot.the Melt CurveNote: If no data are displayed, click Analyze.
 - **2.** Enter the Plot Settings:

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



The following is an image of the Melt Curve for the example experiment:

Tips for viewing melt curves in your own experiments

5

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 F experiment values C and flags

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

View the well table 1.

- le 1. Select the Well Table tab.
 - **2.** Click the **Flag** column header to sort the data so that the wells that triggered flags appear at the top of the table.
 - **3.** Confirm the integrity of the controls:
 - **a.** From the Group By menu, select **Task** to organize the table rows by their function on the reaction plate.
 - **b.** Confirm that each of the controls do not display flags (\mathbf{A}).

The following is an image of the well table of the example Melt Curve experiment.

<u>کا</u>	Plate	Layout	Well Tab	ole									
	Show i	in Table 🔻	Select We	ls γ Gr	oup By 🔻							Expand All	Collapse All
Ì	#	Well	Omit	Flag	Samp Target Name	Task	Dyes	Tm1	Tm2	Tm3	Comments		
			UNKNC	WN			,						
	1	A1			Sampl SYBR	UNKNOWN	SYBR-None	79.894					
	2	A2			Sampl SYBR	UNKNOWN	SYBR-None	79.894					
	3	A3			Sampl SYBR	UNKNOWN	SYBR-None	79.894					
	4	A4			Sampl SYBR	UNKNOWN	SYBR-None	79.894					
	5	A5			Sampl SYBR	UNKNOWN	SYBR-None	79.894					
	6	A6			Sampl SYBR	UNKNOWN	SYBR-None	80.06					
	7	A7			Sampl SYBR	UNKNOWN	SYBR-None	79.894					
	8	A8			Sampl SYBR	UNKNOWN	SYBR-None	79.894					
	9	A9			Sampl SYBR	UNKNOWN	SYBR-None	79.894					
	10	A10			Sampl SYBR	UNKNOWN	SYBR-None	80.06					
	11	A11			Sampl SYBR	UNKNOWN	SYBR-None	80.06					
	12	A12			Sampl SYBR	UNKNOWN	SYBR-None	80.06					
	13	A13			Sampl SYBR	UNKNOWN	SYBR-None	80.06					
	14	A14			Sampl SYBR	UNKNOWN	SYBR-None	80.06					
	15	A15			Sampl SYBR	UNKNOWN	SYBR-None	80.06					
	16	A16			Sampl SYBR	UNKNOWN	SYBR-None	80.06					
	17	A17			Sampl SYBR	UNKNOWN	SYBR-None	80.06					
	18	A18			Sampl SYBR	UNKNOWN	SYBR-None	79.894					
	19	A19			Sampl SYBR	UNKNOWN	SYBR-None	79.894					
	20	A20			Sampl SYBR	UNKNOWN	SYBR-None	79.894					
	21	A21			Sampi SYBR	UNKNOWN	SYBR-None	79.894					
	22	A22			Sampi SYBR		SYBR-None	79.894					
	23	A23			Sampi SYBK		SYBK-NONE	79.894					
	24	A24			Sampl STBR		STER-NORE	79.894					•
w	ell Sum	mary:	In	Plate: 384	Set Up: 384	Analyzed: 38	34 Flag	gged: ()	Omitted by Analysi	is: O	Omitted Manually:	0 Samples Used: 1	Targets Used: 1

The following table 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.

Column	Description
Flag	A 1 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).

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.
- 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.

5

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 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. 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. Check the SYBR® dye signal. In the example experiment, because the PCR run has already been completed, the SYBR® dye signal shows gradual decrease throughout the run and a sudden dip in the fluorescence at one point; the sudden drop in the SYBR® dye signal indicates the melting temperature of the target.

The following is an image of the Multicomponent Plot screen for the example experiment:



Tips for confirming dye accuracy in your own experiment

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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.

5

- **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 130. 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 following is an image of the Raw Data plot for the example experiment:



The filters used for the example experiment are:

Emission Filter						
	m1(520±15)	m2(558±11)	m3(586±10)	m4(623±14)	m5(682±14)	m6(711±12)
×1(470±15)						
x2(520±10)						
x3(550±11)						
x4(580±10)						
x5(640±10)						
x6(662±10)			Load Save Revert & Emission Filter	Defaults		
x6(662±10)	m1(520±15)	m2(558±11)	Load Save Revert & Emission Filter m3(586±10)	Default m4(623±14)	m5(682±14)	m6(711±12)
x6(662±10) urve Filter x1(470±15)	m1(520±15)	m2(558±11)	Load Save Revert & Emission Filter m3(586±10)	Сибр.Из m4(623±14)	m5(682±14)	m6(711±12)
x6(662±10) urve Filter x1(470±15) x2(520±10)	m1(520±15)	m2(558±11)	Lord Sive Revert Emission Filter m3(586±10)	CofeeAr m4(623±14)	m5(682±14)	m6(711±12)
x6(662±10) urve Filter x1(470±15) x2(520±10) x3(550±11)	m1(520±15)	m2(558±11)	Lost Sm Rewrit Emission Filter m3(596±10)	Cofects m4(623±14)	m5(682±14)	m6(711±12)
x6(662±10) urve Filter x1(470±15) x2(520±10) x3(550±11) x4(580±10)	m1(520±15)	m2(558±11)	Last Son Rewith Emission Filter m3(586±10)	Cefeuits m4(623±14) 	m5(682±14)	m6(711±12)
x6(662±10) xrve Filter x1(470±15) x2(520±10) x3(550±11) x4(580±10) x5(640±10)	m1(520±15)	m2(558±11)	Lost Sox Root Emission Filter m3(586±10)	Edfult: m4(623±14) 	m5(682±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[™] 6 and 7 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.

The following is an image of the QC Summary for the example experiment:

QC Summary									
Flag Details									
	Flag:		Description		Fred	quency		Wells	
NOSIGNAL		No signal in well			0				
OFFSCALE		Fluorescence is offscal	Э		0				
MTP		Multiple Tm peaks			0				
Fi Flag De Flagged We	ag: NOSIGNAL—No s ail: The well produce Ils: None <u>View NOSIGNAL T</u>	ignal in well d very low or no fluc Troubleshooting Infor	rescence.						
Total Wells: Wells Set Up:		384 Processed Wells 384 Flagged Wells:	:	384 N 0 A	Ianually Omitted Wells: nalysis Omitted Wells:) Targets U) Samples U	lsed: Jsed:	1
Well Summary:	In Plate: 384	Set Up: 384	Analyzed: 384	Flagged: ()	Omitted by Analysis: I	0 Omitted Mar	ually: ()	Samples Used: 1	Targets Used:

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
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
Se	econdary analysis flags
MTP	Multiple Tm peaks
OUTLIERRG	Outlier in replicate group
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group

For more information

For more information on	Refer to	Publication number
Publishing data	Chapter 2 in Booklet 1, <i>Getting Started with QuantStudio™ 6 and 7 Flex</i> <i>Real-Time PCR System Software Experiments</i>	4489822



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[™] 6 and 7 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**.

ings 2.

- **2.** Click **Analysis** → **Analysis Settings** to open the Analysis Settings dialog box. In the example experiment, the default analysis settings are used for each tab:
 - Melt Curve Settings
 - C_T Settings
 - Flag Settings
 - Advanced Settings

Note: The C_T Settings and Advanced Settings tabs appear in the Analysis Settings dialog box only if the Melt Curve experiment you are performing includes the PCR process.

Note: Select the **Include PCR** check box on the Experiment Properties screen to include amplification in your Melt Curve experiment.

The following is an image of the Analysis Settings dialog box for a Melt Curve experiment:

4 Analysis Settings fo	or QuantStudio_384-Well_SYBR_G	reen_Melt_Example	nple	
Melt Curve Settings	Cr Settings Flag Settings A	dvanced Settings		
Review the defai	ult settings for analysis of targets in this exp lling, then change the settings.	eriment. To use differer	erent settings for a target, select the target from the table, deselect Enable	2
- Coloct a Target			Multi Daak Satting for Target 1	
Target	Multi-Peak Calling	Peak Level (%)	6)	
Target 1	Enabled	10.00	Enable Multi-Peak Calling	
			Peak level relative to the dominant peak (%): 10.00	
			V	
Save to Library	Load from Library	F	Revert to Default Analysis Settings	Cancel

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Melt Curve Experiments

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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*[™] 6 and 7 Flex Real-Time PCR System Software 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 the 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 the 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

Use the Baseline Threshold algorithm to determine the C_T values.

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

• 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**.

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide for Melt Curve Experiments

• 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 blackground. 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[™] 6 and 7 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 following is an image of the Flag Settings tab:

🕮 Analysis Settings for	QuantStudio_384-Well_	_SYBR_Green_Melt_Ex				Þ
Melt Curve Settings	CT Settings Flag Settin	gs Advanced Settings				
Configure the flags	and filtering. In this panel you	ı can enable, disable, and cor	nfigure flags, and indicate	if a well is to be rejected when	a flag is raised.	?
Flag	Description	Use	Attribute	Condition	Value	Reject Well
МТР	Multiple Tm peaks		2			
NOSIGNAL	No signal in well					
OFFSCALE	Fluorescence is offscale					
Save to Library	Load from Library		Revert	to Default Analysis Set	tings	sis Settings Cancel

Advanced Settings

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

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	Publication number
Amplification efficiency	Amplification Efficiency of TaqMan [®] Gene Expression Assays Application Note.	127AP05-03

Export Experiment 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[™] 6 and 7** 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	QuantStudio_384-Well_SYBR_Green_Melt_Example_data
File Type	*.txt
Export File Location	C:\Applied Biosystems\QuantStudio 6 and 7 Flex Software\User Files\Export

Your Export screen should look like this:

port File Location: ms\Q	uantStudio 6 an	d 7 Flex Soft	tware\User Files\Export	Browse Export File	Name: 2384-Well	_SYBR_Green_Melt_	Example_data F	ile Type: 📋 (*.tx	t) 🗸
✓ Sample Setup	Raw Data 🛛 🔲 ,	Amplification	Multicomponent	Results					
Select Content				Course Norma		Dia anna Marcal	Disease October	1 -	
All Fields		vveli	Well Position	Sample Name	Sample Color	Biogroup Name	Biogroup Color	Target Name	1.
			1 A1	Sample 1	RGB(0,255,0)			Target 1	RG /
🗹 Well			2 42	Sample 1	RGB(0,255,0)			Target 1	RG
			4 44	Sample 1	RGB(0,255,0)			Target 1	RG
Well Position			5.45	Sample 1	RGB(0,255.0)			Target 1	RG
Sample Name			6 46	Sample 1	RGB(0,255.0)			Target 1	RG
C Sample Marrie			7 A7	Sample 1	RGB(0.255.0)			Target 1	RG
Sample Color			8 A8	Sample 1	RGB(0,255,0)			Target 1	RG
			9 A 9	Sample 1	RGB(0,255,0)			Target 1	RG
Biogroup Name			10 A10	Sample 1	RGB(0,255,0)			Target 1	RG
Ringroup Color			11 A11	Sample 1	RGB(0,255,0)			Target 1	RG
			12 A12	Sample 1	RGB(0,255,0)			Target 1	RG
Target Name			13 A13	Sample 1	RGB(0,255,0)			Target 1	RG
			14 A14	Sample 1	RGB(0,255,0)			Target 1	RG
Target Color	-	_	15 A15	Sample 1	RGB(0,255,0)			Target 1	RG
W Tock			16 A16	Sample 1	RGB(0,255,0)			Target 1	RG
T I DON			17 A17	Sample 1	RGB(0,255,0)			Target 1	RG
Reporter			18 A18	Sample 1	RGB(0,255,0)			Target 1	RG
	•	V 2							>

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QuantStudio_384-Well_SYBR_Green_Melt_Example_data.txt - Notepad	×
Fle Edit Format View Help	
<pre>helick Type = 384.well Block calibration Background is expired = Yes calibration Background performed on = 04-28-2010 (calibration Normalization FAM-ROX is expired = Yes calibration Normalization VIC-ROX is expired = Yes calibration Normalization VIC-ROX is expired = Yes calibration Pure Dye FAM is expired = Yes calibration Pure Dye FAM is expired = Yes calibration Pure Dye FAM is expired = Yes calibration Pure Dye ROX is expired = Yes calibration Pure Dye ROX is expired = Yes calibration Pure Dye ROX is expired = Yes calibration Pure Dye ROX is expired = Yes calibration Pure Dye ROX is expired = Yes calibration Pure Dye ROX is expired = Yes calibration Pure Dye ROX is expired = Yes calibration Pure Dye ROX is expired = Yes calibration Pure Dye SWB reformed on = 04-28-2010 calibration Pure Dye SWB reformed on = 04-28-2010 calibration Pure Dye SWB reformed on = 04-28-2010 calibration Pure Dye TAMRA is expired = Yes calibration Pure Dye VIC is expired = Yes calibration Pure Dye VIC is expired = Yes calibration RUPE of Dye TAMRA is expired = Yes calibration RUPE of the Sepired = Yes calibration RUPE of the Sepired = Yes calibration RUPE of Stized = Yes calibration RUPE of the Sepired = Yes calibration RUPE of Stized = Yes calibration UNIformity is expired = Yes calibration UNIformity is expired = Yes calibration UNIformity Stized = Yes store and the come = NA experiment Comment = NA experiment Run End Time 2.78880026 instrument Run End Time 2.7</pre>	
[Sample Setup] Well well position Sample Name Sample Color Biogroup Name Biogroup Color Target Name Target Color Task Reporter Quencher Quantity Comments Comments Comments None 1 A1 Sample 1 "RcB(0, 255, 0)" Target 1 "RcB(0, 255)" UNKNOWN SYBR None 2 A2 Sample 1 "RcB(0, 255, 0)" Target 1 "RcB(0, 0, 255)" UNKNOWN SYBR None 3 A3 Sample 1 "RcB(0, 255, 0)" Target 1 "RcB(0, 0, 255)" UNKNOWN SYBR None 4 A4 Sample 1 "RcB(0, 255, 0)" Target 1 "RcB(0, 0, 255)" UNKNOWN SYBR None 5 A5 Sample 1 "RcB(0, 255, 0)" Target 1 "RcB(0, 0, 255)" UNKNOWN SYBR None 6 A6 Sample 1 "RcB(0, 255, 0)" Target 1 "RcB(0, 0, 255)" UNKNOWN SYBR None 7 A7 Sample 1 "RcB(0, 255, 0)" Target 1 "RcB(0, 0, 255)"	X

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

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USER GUIDE



QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Experiments - Appendixes Booklet 7

Publication Number 4489822 Revision A



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Ordering Information

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How to order from the Life Technologies website

You can order materials accessories directly from the Life Technologies store over the internet.

Note: Product availability and pricing may vary according to your region or country. Online ordering through the Life Technologies store is not available in all countries. Contact your local Life Technologies representative for help.

To order through the website:

- Confirm that your computer has an Internet connection.
- We recommend the following browsers and Adobe[®] Acrobat[®] Reader[®] Software versions to use the Life Technologies website:

Operating system Internet Explorer		Apple® Safari®	Mozilla® Firefox®	Adobe [®] Acrobat [®] Reader [®]
Microsoft [®] Windows [®]	v6.x or later	None [†]	v2.x or later	v4.0 or later
Macintosh [®]	None [†]	v2.0.4 or later		

† Browser not available for this platform.

Note: Confirm that cookies and Javascript[®] are turned on for the website to function correctly.

To purchase reagents, accessories, and calibration kits:

- 1. Go to www.lifetechnologies.com
- 2. Under "I Want to Buy," select the product of interest.

Reagents and consumables

The reagents and consumables listed below are required for calibrating and for performing experiments with the QuantStudioTM 6 and 7 Flex Software.

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

CalibrationThe following table shows the reagents and consumables required to calibrate andreagents andverify the performance of the QuantStudioTM 6 and 7 Instruments when run with the
QuantStudioTM 6 and 7 Flex Software.

384-well	samp	le b	loc	<
----------	------	------	-----	---

Consumable	Part number	Shelf-life at environmental temperature	Storage conditions (°C)
384-Well Spectral Calibration Plate with FAM [™] Dye	4432271	Use the consumable by the expiration date mentioned	–15°C to –25°C
384-Well Spectral Calibration Plate with VIC [®] Dye	4432278		
384-Well Spectral Calibration Plate with ROX [™] Dye	4432284		
384-Well Spectral Calibration Plate with SYBR® Green Dye	4432290	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		

96-well (0.2 mL) sample block

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

Consumable	Part number	Shelf-life at environmental temperature	Storage conditions (°C)
Fast 96-Well Spectral Calibration Plate with FAM [™] Dye	4432389	Use the	–15°C 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 ${\sf SYBR}^{\circledast}$ 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		

Fast 96-well (0.1 mL) sample block

Array card sample block

Consumable	Part number	Shelf-life at environmental temperature	Storage conditions (°C)
Array Card Spectral Dye Calibration Kit	4432376	Use the	–15°C to –25°C
Array Card RNase P Instrument Verification Kit	4432464	consumable by the expiration date mentioned on the package	

Note: To prepare calibration plates, refer to the instrument user guide.

Note: The Array card sample block is applicable only to the QuantStudioTM 7 Flex System.

QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Experiments - Appendixes
Experiment consumables

The following table shows the consumables required to perform experiments with the QuantStudio TM 6 and 7 Flex Software.

96-well (0.2 mL) sample block

Consumable	Part number	
MicroAmp [®] Optical 8-Cap Strip	300 strips	4323032
MicroAmp [®] Optical 8-Tube Strip (0.2 mL)	125 strips	4316567
MicroAmp [®] Optical Tube without cap (0.2 mL)	2000 tubes	N8010933
MicroAmp [®] 96-Well Tray/Retainer Set (Blue) (for 0.2 mL)	10 pairs	4381850
MicroAmp [®] Optical 96-Well Reaction Plate (0.2 mL)	10 plates	N8010560
MicroAmp [®] Optical 96-Well Reaction Plate with Barcode (0.2 mL)	10 plates	4306737

Fast 96-well (0.1 mL) sample block

Consumable	Part number	
MicroAmp [®] Fast 8-Tube Strip (0.1 mL)	125 strips	4358293
MicroAmp [®] Fast Reaction Tube with cap (0.1 mL)	1000 tubes	4358297
MicroAmp [®] 96-Well Tray (Black) (for 0.1 mL)	10 plates	4379983
MicroAmp [®] Fast Optical 96-Well Reaction Plate (0.1 mL)	10 plates	4346907
MicroAmp [®] Fast Optical 96-Well Reaction Plate with Barcode (0.1 mL)	10 plates	4346906

Array card sample block

Consumable	Part number	
Array Card	4-pack	4334812
	1-pack	4351471

Note: The Array card sample block is applicable only to the QuantStudioTM 7 Flex System.

Miscellaneous

Consumable	Part number	
MicroAmp [®] Multi-Removal Tool	1 tool	4313950
MicroAmp [®] Cap Installing Tool (Handle)	1 tool	4330015

Consumable			
MicroAmp [®] Optical Adhesive Film	25 films	4481193	
MicroAmp [®] Adhesive Film Applicator	5 applicators	4481196	
RT-PCR Grade Water	-	AM9935	

QuantStudio[™] 6 or 7 Instrument accessories

The accessories listed in the following table are for the QuantStudioTM 6 or 7 Instrument when run with the QuantStudioTM 6 and 7 Flex Software.

Accessory		Part number
384-Well Plate/Array Card Heated Cover		4453555
384-Well Plate Sample Block		4453553
96-Well Plate Heated Cover		4453560
96-Well Plate Sample Block	4453556	
Fast 96-Well Plate Heated Cover	4459838	
Fast 96-Well Plate Sample Block		4453559
Array Card Buckets/Clip Set	1st Generation	4337762
	2nd Generation	4442571
Array Card Sample Block	·	4453554
Array Card Staker/ Sealer		4331770
Handheld Barcode Scanner		4453271

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ExperimentThe following table lists the reagents that can be ordered for performing experimentsreagentswith the QuantStudioTM 6 and 7 Flex Software.

To perform	Recommended reagent kits	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	444432
	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



The following general-use materials and consumables are required to calibrate, maintain, and perform experiments with the QuantStudio[™] 6 and 7 Flex Software. 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
Optical clear adhesive film for PCR	MLS
Deionized water	MLS

Supplemental Information

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(Optional) Libraries for designing your own experiments

The QuantStudioTM 6 and 7 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:

- Dye Library
- Targets Library
- Samples Library
- Control Library
- SNP Assay Library (only available for Genotyping experiments)
- Run Method Library
- Analysis settings Library

Dye library You can access the Dye library from the Tools menu to add new custom dyes, edit existing dyes, and delete dyes.

To add a new dye to the Dye Library:

- **1.** Go to **Tools** ▶ **Dye Library...**.
- 2. Click New at the bottom of the Dye Library dialog box.

3. In the New Dye dialog box, enter the name and wavelength (optional) of the custom dye in the respective fields.

IMPORTANT! Ensure that you perform a custom dye calibration with the new dye before you run an experiment using this dye.

4. Select the Type of dye: Reporter, Quencher, or Both.

The new dye gets added to the Dye Library.

📣 Dye Library				×
Add new dyes	, edit existing dye	es, delete dyes.		2
Name	Creation	Туре	Wavelength (
CY3	Default	Reporter		~
CY5	Default	Reporter		
FAM	Default	Reporter		
JOE	Default	Reporter		
MELTDOCTOR	Default	Reporter		=
NED	Default	Reporter		
NFQ-MGB	Default	Quencher		
OTHER	Default	Reporter		
ROX	Default	Reporter		
SYBR	Default	Reporter		
TAMRA	Default	Both		~
New	Delete	Edit	Close	

5. Click OK.

📣 New Dye				
Perform a custom dye calibration with this dye before you run an experiment using this dye. For HRM experiments, also perform an HRM calibration before running and analyzing an HRM experiment using this dye.				
Name: ND-06				
Wavelength (Optional):				
Туре				
○ Reporter				
Ouencher				
○ Both				
OK Cancel				

- **6.** To edit or delete any of the dyes in the Dye Library, select the dye and click **Edit** or **Delete** respectively.
- 7. Click **Close** to exit the Dye Library.

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

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

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

To add a run method to the Run Method Library:

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

To select a run method from the Run Method Library:

- 1. Click Open Run Method on the Run Method screen.
- **2.** Select one from the saved run methods.
- 3. Click OK.

Analysis Settings Library Analysis Settings are different for each experiment type. If the default analysis settings in the QuantStudio[™] 6 and 7 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.

To access the Analysis Settings Library, go to **Tools** Analysis Settings Library. The Analysis Settings Library dialog box looks like this:

😃 Analysis Settings Library 🛛 🔀					
Select analysis settings or delete analysis settings. Apply a filter to reduce the number of analysis settings displayed.					
Enter a filter query, then	click "Apply Filter."				
IF Analysis Settings 🗸			Apply	Filter Remove Filter	
Delete Delete All					
Analysis Settings	Experiment Type	Comments	Created On	Last Modified	
RNaseP	Standard Curve		07-04-2013 16:27:29 SGT	07-04-2013 16:27:48 SGT	
GT-7	Genotyping		07-04-2013 16:28:15 SGT	07-04-2013 16:28:30 SGT	
MC-90	Melt Curve		07-04-2013 16:28:50 SGT	07-04-2013 16:29:01 SGT	
E <u>s</u> it Analysis Settings Library					

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

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 according to your requirement.

B

4. Click **Save to Library** to save the changes you have made to the Analysis Settings Library.

Note: 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.

Changing default analysis settings in Preferences

(*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

To change the default analysis settings:

- **1.** Go to **Tools > Preferences**.
- Click the Experiment tab. Select the Auto Analysis and Auto Save check boxes for the QuantStudio[™] 6 and 7 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.

В

Instrument Console

The Instrument Console displays all the QuantStudio $^{\rm \tiny TM}\,$ 6 and 7 Instruments discovered on a network.

Refresh Add to Manage Instrument Open Door Close Door Create Group Rename Group Delete Group Assign to Group y	QS7_0009	?
Display Groups V Filtered by: All States V	Instrument Type: Instrument Status: Run Status:	
My Instruments (1)	Calibration Status Type Last Run Maintenance Info	On the Network By Status Value
On the Network (1)	Lamp Life Total Cycles for Block	208 hours
	Total Degrees for Block Instrument Properties	
057_0009 ONLINE	Property Instrument Label Serial Number Instrument Firmware Ve	Value ruo 287880009 . 1.0.3
	IP Address Block Type Controller Firmware Version Optics Firmware Version Thermana Block Firmware	172.28.1.122
	Heated Cover Firmware	•
Image: A Home Image: A		



Left panel

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

- Instrument access: Open and close the QuantStudio[™] 6 or 7 Instrument door from the QuantStudio[™] 6 and 7 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[™] 6 or 7 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, in Chapter 1, Booklet 1 *Getting Started with QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Experiments.*
- 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, in Chapter 1, Booklet 1 Getting Started with QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Experiments.
 - Maintain instruments (check the calibration status of instruments and perform different calibrations). For more information on instrument maintenance, refer to the instrument user 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 \triangle 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.

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.

QuantStudio[™] 6 or 7 Instrument status icon

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
QS6_0008 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
28	No longer connected to the network
	Error occurred during run



About the reagents

TaqMan[®] Reagents Description

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

Advantages

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

Limitations

Require synthesis of a unique fluorogenic probe.

TaqMan[®] Reagents detection process



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.

B

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.



Appendix B Supplemental Information *About the reagents*

Documentation and Support

Related documentation

The following related documents are shipped with the instrument:

Document	Pub. no.	Description
QuantStudio [™] 6 and 7 flex Real-Time PCR System Software Getting Started Guide	4489822	Contains seven individual booklets that explain how to perform the six different experiments on the QuantStudio [™] 6 and 7 flex Real-Time PCR System Software.
		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[™] 6 and 7 flex Real-Time PCR System Software.
		 A guide for your own experiments.
		Intended for laboratory staff and principal investigators who perform experiments using the QuantStudio [™] 6 and 7 flex Real-Time PCR System Software.
QuantStudio [™] 6 and 7 Flex Real-Time PCR Systems Maintenance and Administration Guide	4489821	Explains how to use and maintain the QuantStudio [™] 6 and 7 Flex Real-Time PCR Systems
		Intended for laboratory staff responsible for the use and maintenance of the QuantStudio [™] 6 and 7 Instruments.
QuantStudio [™] 6 and 7 Flex Real-Time PCR Systems Site Preparation Guide	4489824	Explains how to prepare your site to receive and install the QuantStudio [™] 6 and 7 Instruments Intended for personnel who schedule, manage, and perform the tasks required to prepare your site for installation of the QuantStudio [™] 6 and 7 Instruments.
QuantStudio [™] 6 and 7 Flex Real-Time PCR System Software Programming Supplement	4489825	Provides IT administrative personnel with sufficient information to integrate the instrument and software with a LIS/LIMS.
		Intended to be used with the $QuantStudio^{M}$ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide.

Document	Pub. no.	Description
Applied Biosystems [®] Twister [®] Robot Automation Accessory Quick Reference	4468146	Provides abbreviated instructions for operating an Applied Biosystems [®] Twister [®] Robot Automation Accessory that has been installed with the QuantStudio [™] 6 or 7 Flex Real-Time PCR System.
		Intended for laboratory staff and principal investigators who perform experiments using the QuantStudio [™] 6 or 7 Flex Real-Time PCR System.
QuantStudio [™] 6 and 7 flex Real-Time PCR System Software Help	NA	Explains how to use the QuantStudio [™] 6 and 7 flex Real-Time PCR System Software to:
		 Set up, run, analyze, audit, sign, export, and print experiments.
		 Monitor networked QuantStudio[™] 6 and 7 Instruments.
		 Calibrate and verify the performance of the QuantStudio[™] 6 and 7 Instruments.
		 Intended for laboratory staff and principal investigators who perform experiments using the QuantStudio[™] 6 and 7 flex Real-Time PCR System Software.

Note: For additional documentation, see "How to obtain support" on page 25.

Other related documents

Documents related		
to Standard Curve experiments	Document	Pub. no.
	Amplification Efficiency of TaqMan [®] Gene Expression Assays Application Note	127AP05
	Custom TaqMan [®] Gene Expression Assays Protocol	4334429
	Primer Express $^{\textcircled{8}}$ 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 Relative	Document	Pub. no.
Standard Curve and Comparative	Amplification Efficiency of TaqMan [®] Gene Expression Assays Application Note	127AP05
	Applied Biosystems [®] High-Capacity cDNA Reverse Transcription Kits Protocol	4375575
of experiments	Custom TagMan [®] Gene Expression Assays Protocol	4334429

Primer Express[®] Software Version 3.0 Getting Started Guide

User Bulletin #2: Relative Quantitation of Gene Expression

TaqMan[®] Gene Expression Assays Protocol

4362460

4333458

4303859

Documents related to Genotyping experiments

Document	Pub. no.
Allelic Discrimination Pre-Developed TaqMan $^{ extsf{B}}$ Assay Reagents Quick Reference Card	4312212
Custom TaqMan [®] Genomic Assays Protocol	4367671
Custom TaqMan [®] SNP Genotyping Assays Protocol	4334431
Ordering TaqMan [®] SNP Genotyping Assays Quick Reference Card	4374204
Pre-Developed TaqMan [®] Assay Reagents Allelic Discrimination Protocol	4312214
TaqMan [®] Drug Metabolism Genotyping Assays Protocol	4362038
TaqMan [®] SNP Genotyping Assays Protocol	4332856

Documents related to Presence/ Absence experiments

Document	Pub. no.
DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol	4343586
NucPrep $^{\textcircled{B}}$ Chemistry: Isolation of Genomic DNA from Animal and Plant Tissue Protocol	4333959
PrepMan [®] Ultra Sample Preparation Reagent Protocol	4318925

How to obtain support

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

www.lifetechnologies.com/support

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 Life Technologies 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[™] 6 and 7 flex Real-Time PCR System 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 ② in the toolbar of the QuantStudio[™] 6 and 7 flex Real-Time PCR System Software window.
- Select Help ▶ QuantStudio[™] 6 and 7 flex Real-Time PCR System 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

Limited Product Warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

Glossary

AIF	See assay information file (AIF).
AIX	XML version of the assay information file.
	See also assay information file (AIF).
allele	In a diploid organism, one of two DNA sequences found at the same locus (for example, a particular gene), but located on homologous chromosomes. Two corresponding alleles may have the identical sequence, or they may differ somewhat, often at one or more single-base sites (SNPs).
aligned melt curve plot	Plot of the re-scaled melt curve.
allelic discrimination plot	Display of genotyping data collected during the post-PCR read. The allelic discrimination plot is a graph of the normalized reporter signal from the allele 1 probe, plotted against the normalized reporter signal from the allele 2 probe.
amplicon	A segment of DNA amplified during PCR.
amplification	Part of the instrument run in which PCR amplifies the target. Fluorescence data collected during amplification are displayed in an amplification plot, and the data are used to calculate results.
	Note: Only quantitative real-time PCR experiments, not end-point experiments, take amplification data into account.
amplification efficiency (EFF%)	Calculation of the efficiency of the PCR amplification in an experiment. EFF% is calculated using the slope of the regression line in the standard curve. A slope close to –3.32 indicates optimal, 100% PCR amplification efficiency.
amplification plot	Display of data collected during the cycling stage of PCR amplification. The amplification plot can be viewed as:
	• Baseline-corrected normalized reporter (ΔRn) vs. cycle
	• Normalized reporter (Rn) vs. cycle
	• Threshold cycle (C_T) vs. well
amplification stage	Part of the instrument run in which PCR amplifies the target. The amplification stage, called a cycling stage in the thermal profile, consists of denaturing, primer annealing, and extension steps that are repeated. Fluorescence data collected during the extension stage are displayed in an amplification plot, and the data are used to calculate results. With TaqMan [®] chemistry, the last two steps of a PCR stage are typically combined.

See also cycling stage.

Analysis Settings Library	In the software, a collection of analysis settings to use in experiments. You can save settings and reuse them. You cannot edit or import settings into the library.
assay	In a PCR reaction mix, two target-specific primers or two primers and a probe used to amplify a target.
Assay ID	Identifier assigned by Life Technologies to TaqMan [®] assays.
assay information file (AIF)	Tab-delimited data file on a CD shipped with each assay order. The AIF contains technical details about all assays in the shipment. It includes information about assay concentrations; reporters and quenchers used; part and lot numbers; and assay, vial, and plate ID numbers. The file name includes the number from the barcode on the plate.
assay mix	PCR reaction component in Applied Biosystems [®] TaqMan [®] assays. The assay mix contains primers designed to amplify a target and a TaqMan [®] probe designed to detect amplification of the target.
AutoDelta	 In the run method, a setting to increase or decrease the temperature and/or time for a step with each subsequent cycle in a cycling stage. When AutoDelta is enabled for a cycling stage, the settings are indicated by an icon in the thermal profile: AutoDelta on: ▲
	• AutoDelta off: 🔺
automatic baseline	An analysis setting in which the software calculates the baseline start and end cycles for the amplification plot.
	See also baseline.
automatic threshold	An analysis setting in which the software calculates the baseline start and end cycles and the threshold in the amplification plot. The software uses the baseline and threshold to calculate the threshold cycle (C_T).
	See also threshold cycle (C _T).
background calibration	Type of calibration in which the instrument performs reads of a background plate, averages the spectra recorded during the run, and extracts the resulting spectral component to a calibration file. The software then uses the calibration file during subsequent runs to remove the background fluorescence from the run data.
baseline	In the amplification plot, a cycle-to-cycle range that defines background fluorescence. This range can be set manually on an assay-by-assay basis, or automatically to set each individual well.
baseline-corrected normalized reporter (ΔRn)	The magnitude of normalized fluorescence signal generated by the reporter. In experiments that contain data from real-time PCR, the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. In the Δ Rn vs Cycle amplification plot, Δ Rn is calculated at each cycle as:
	Δ Kn (cycle) = Kn (cycle) –Kn (baseline), where Kn = normalized reporter

	In genotyping experiments and presence/absence experiments, the difference in normalized fluorescence signal generated by the reporter between the pre-PCR read and the post-PCR read. In the allelic discrimination plot (genotyping experiments) and the presence/absence plot (presence/absence experiments), Δ Rn is calculated as:
	Δ Rn = Rn (post-PCR read) –Rn (pre-PCR read), where Rn = normalized reporter
	See also normalized reporter (Rn).
baseline threshold algorithm	Expression estimation algorithm (C _T) which subtracts a baseline component and sets a fluorescent threshold in the exponential region for gene quantification.
biological replicates	Reactions that contain identical components and volumes, but evaluate separate samples of the same biological source (for example, samples from three different mice of the same strain, or separate extractions of the same cell line or tissue sample).
	When an experiments uses biological replicate groups in a gene expression study, the values displayed in the Biological Replicates tab are calculated by combining the results of the separate biological samples and treating this collection as a single population (that is, as one sample). For ΔC_T computations (normalizing by the endogenous control) in a singleplex experiment, the software treats separate biological samples as unpaired data when computing variability estimates of the single biological replicate. Individual contributions of the separate biological samples to the single biological replicate results are observed in the Technical Replicates tab.
	See also technical replicates.
blocked IPC	In presence/absence experiments, a reaction that contains IPC blocking agent, which blocks amplification of the internal positive control (IPC). In QuantStudio [™] 6 and 7 Flex Real-Time PCR System Software, also the name of the task for the IPC target in wells that contain IPC blocking agent. See also negative control-blocked IPC wells.
calibrator	See reference sample.
chemistry	See reagents.
comparative C_T ($\Delta\Delta C_T$) method	Method for determining relative target quantity in samples. The 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 target quantity in each sample to normalized target quantity in the reference sample.
C _T	See threshold cycle (C_T).
C _T algorithm	Algorithm used to determine the threshold cycle. The software provides the Baseline Threshold $C_{\rm T}$ algorithm.
cycle threshold	See threshold cycle (C_T).
cycling stage	In the thermal profile, a stage that is repeated. A cycling stage is also called an amplification stage. See also amplification stage.
Cq	See quantification cycle (C _q).

data collection	 During the instrument run, a process in which an instrument detects fluorescence data from each well of the reaction plate. The instrument transforms the signal to electronic data and saves the data in the experiment file. In the QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software, a data collection point is indicated by an icon in the thermal profile: Data collection on: [™] Data collection off: [™]
delta $R_n (\Delta R_n)$	See baseline-corrected normalized reporter (ΔRn).
derivative reporter (-Rn')	The rate of change in fluorescence as a function of temperature. Rn' is used to plot a melting curve. Significant decrease of the fluorescent signal generates a positive peak on the derivative view of the melting curve.
diluent	A reagent used to dilute a sample or standard before it is added to the PCR reaction.
dilution factor	See serial factor.
dye calibration	Type of calibration in which the software collects spectral data from a series of dye standards and stores the spectral information for the dye standards in a pure spectra calibration file. This file is used during experiment runs to characterize and distinguish the individual contribution of each dye in the total fluorescence collected by the instrument.
EFF%	See amplification efficiency (EFF%).
efficiency correction	In Comparative C_T experiments, a feature that allows you to manually enter previously-determined amplification efficiencies for each experiment, following the experimental run. The real-time software mathematically compensates for differences in efficiency between each target assay and the endogenous control when calculating sample-to-sample relative quantities. This method can be employed as a substitute for the Relative Standard Curve Method.
endogenous control	A gene that is used to normalize template differences and sample-to-sample or run-to- run variation.
endpoint read	See post-PCR read.
error	The standard error of the slope of the regression line in the standard curve.
	The error can be used to calculate a confidence interval (CI) for the slope. Because the amplification efficiency (EFF%) is calculated from the slope, knowing the error allows a CI for the amplification efficiency to be calculated.
experiment	Refers to the entire process of performing a run, including setup, run, and analysis. You can perform the following types of experiments:
	Quantification - Standard curve
	Quantification - Relative standard curve
	• Quantification - Comparative $C_T (\Delta \Delta C_T)$

	Melt Curve
	Genotyping
	Presence/Absence
experiment name	Entered during experiment setup, the name that is used to identify the experiment.
Experiment Setup	A software feature that allows you to set up an experiment according to your experiment design. Experiment Setup provides you with maximum flexibility in the design and setup of your experiment.
experiment type	The type of experiment to perform:
	Standard curve
	• Comparative $C_T (\Delta \Delta C_T)$
	Relative standard curve
	Genotyping
	Presence/Absence
	Melt curve
	The experiment type that you select affects setup, run, and analysis.
export	A software feature that allows you to export experiment setup files, experiment results, instrument information, and security and auditing settings to spreadsheet, presentation, or text files. You can edit the default location of the exported file.
filter	Dye excitation and emission filter combination that you select for an experiment. The QuantStudio TM 6 and 7 Instruments include a five-color filter set and six-color filter set, respectively, that support FAM TM , SYBR [®] Green, VIC [®] , JOE TM , NED TM , Cy [®] 3, TAMRA TM , ROX TM , and Texas Red [®] dyes.
flag	A quality control (QC) indicator which, when applied by the software to a well during analysis, indicates a possible issue with that reaction. For example, a flag may be issued if no amplification is detected in a well. Flags indicating potential problems are displayed in the Quality Control tab of the plate layout, well table, and QC Summary screens.
forward primer	Oligonucleotide that flanks the 5' end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.
genotyping experiment	An experiment used to identify known mutations in a DNA sample. With this experiment type, you can determine if a DNA sample is:
	Homozygous (samples having only allele 1). Also called wild type homozygote.Homozygous (samples having only allele 2). Also called variant homozygote.
	• Heterozygous (samples having both allele 1 and allele 2).
heterozygote	Samples having both allele 1 and allele 2.
	See also genotyping experiment.

holding stage	In the thermal profile, the stage that holds the temperature constant for a defined period of time. A stage that includes one or more steps. You can add a holding stage to the thermal profile to activate enzymes, to inactivate enzymes, or to incubate a reaction.
homozygote	Samples having only allele 1 or only allele 2. See also genotyping experiment.
housekeeping gene	A gene that is involved in basic cellular functions and that may be constitutively expressed. Housekeeping genes may be candidates for use as endogenous controls; however, their constancy should always be validated experimentally.
	See also endogenous control.
import	A software feature that allows you to import plate setup information or security settings before an experiment run. You can also import information into some libraries in the software.
Instrument Console	A software feature that allows you to view information about instruments on the network. In the Instrument Console, you can monitor the status of any instrument on the network; view calibration, maintenance, and instrument properties for a selected instrument; and open and close the instrument drawer.
Instrument Manager	A software feature that allows you to view information about instrument available on the network. In the Instrument Manager, you can monitor the status of an instrument; monitor amplification plots and temperature plots in real time; view the calibration status, perform calibrations and manage files on the instrument, including downloading completed experiments to your computer.
internal positive control (IPC)	In presence/absence experiments, a short synthetic DNA template that is added to PCR reactions. The IPC can be used to distinguish between true negative results (the target is absent in the samples) and negative results caused by PCR inhibitors, incorrect assay setup, or reagent or instrument failure.
inventoried assays	TaqMan [®] Gene Expression Assays and TaqMan [®] SNP Genotyping Assays that have been previously manufactured, passed quality control specifications, and stored in inventory.
IPC	See internal positive control (IPC).
IPC blocking agent	Reagent added to PCR reactions to block amplification of the internal positive control (IPC).
IPC+	See negative control-IPC wells.
made-to-order assays	TaqMan [®] Gene Expression Assays that are manufactured at the time of order. Only assays that pass manufacturing quality control specifications are shipped.
manual baseline	An analysis setting for the Baseline Threshold algorithm. You enter the baseline start and end cycles for the amplification plot.
	See also baseline.

manual threshold	An analysis setting for the Baseline Threshold algorithm. You enter the threshold value and select whether to use automatic baseline or manual baseline values. The software uses the baseline and the threshold values to calculate the threshold cycle (C_T).
melt curve	A plot of data collected during the melt curve stage. Peaks in the melt curve can indicate the melting temperature (T_m) of the target, or they can identify nonspecific PCR amplification. In the software, you can view the melt curve as normalized reporter (R_n) vs. temperature or as derivative reporter $(-R_n')$ vs. temperature. In a high resolution melting experiment, you can view the melt curve as fluorescence vs. temperature. Also called dissociation curve.
melt curve characteristics	The melt curve shape and the difference in melting temperature (T_m) values.
melt curve stage	In the thermal profile, a stage with a temperature increment to generate a melt curve.
melt curve plot	The default view of the melting curve. It plots the negative derivative data (–Rn') vs. temperature.
melting temperature (T _m)	The temperature at which 50% of the DNA is double-stranded and 50% of the DNA is dissociated into single-stranded DNA. In a melt curve experiment, the melt curve plot displays the melting temperature.
multicomponent plot	A plot of the complete spectral contribution of each dye for the selected well(s) over the duration of the PCR run.
negative control (NC)	The task for targets or SNP assays in wells that contain water or buffer instead of sample. No amplification of the target should occur in negative control wells. Previously called no template control (NTC).
negative control- blocked IPC wells	In presence/absence experiments, wells that contain IPC blocking agent instead of sample in the PCR reaction. No amplification should occur in negative control-blocked IPC wells because the reaction contains no sample and amplification of the IPC is blocked. Previously called no amplification control (NAC).
negative control-IPC wells	In presence/absence experiments, wells that contain IPC template and buffer or water instead of sample. Only the IPC template should amplify in negative control-IPC wells because the reaction contains no sample. Previously called IPC+.
no amplification control (NAC)	See negative control-blocked IPC wells.
no template control (NTC)	See negative control (NC).
nonfluorescent quencher-minor groove binder (NFQ- MGB)	Molecules that are attached to the 3' end of TaqMan [®] probes. When the probe is intact, the nonfluorescent quencher (NFQ) prevents the reporter dye from emitting fluorescence signal. Because the NFQ does not fluoresce, it produces lower background signals, resulting in improved precision in quantification. The minor groove binder (MGB) increases the melting temperature (T _m) of the probe without increasing its length, allowing for the design of shorter probes.

normalization calibration	Type of calibration in which the software collects data from the normalization standards, then stores it in a normalization calibration file. This file is used in comparisons of data from multiple instruments within a study.
normalized quantity	Either the C_T Avg. of the target gene minus the C_T Avg. of the endogenous control (Comparative C_T experiments), or the Q Avg. of the target divided by the Q Avg. of the endogenous control (Relative Standard Curve experiments).
normalized quantity mean	The relative standard curve equivalent of the ΔC_T mean value found in Comparative C_T experiments (computed as the geometric mean).
normalized quantity SE	The relative standard curve equivalent of the ΔC_T SE value found in Comparative C_T experiments (computed as the geometric standard error of the mean).
normalized reporter (Rn)	Fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference dye (usually ROX^{TM} dye on Life Technologies instruments).
omit well	An action that you perform before reanalysis to omit one or more wells from analysis. Because no algorithms are applied to omitted wells, omitted wells contain no results. You can add wells back in to the analysis; no information is permanently discarded.
outlier	A measurement (such as a C_T) that deviates significantly from the measurement of the other replicates for that same sample.
passive reference	A dye that produces fluorescence signal independent of PCR amplification, and that is added to each reaction at a constant concentration. Because the passive reference signal should be consistent across all wells, it is used to normalize the reporter dye signal to account for non-PCR related fluorescence fluctuations caused by minor well- to-well differences in volume. Normalization to the passive reference signal generally results in data with noticeably high precision among technical replicates.
plate layout	An illustration of the grid of wells and assigned content in the reaction plate. The number of rows and columns in the grid depends on the sample block that you use.
	In the software, you can use the plate layout as a selection tool to assign well contents, to view well assignments, and to view results. The plate layout can be printed, included in a report, exported, and saved as a slide for a presentation.
plate setup file	A file (.txt, .csv, .xml, or .sds) that contains setup information such as the well number, sample name, sample color, target name, dyes, and other reaction plate contents.
point	One standard in a standard curve. The standard quantity for each point in a standard curve is calculated based on the starting quantity and serial factor.
positive control	In genotyping and presence/absence experiments, a DNA sample with a known genotype, homozygous or heterozygous.
	In the software, the task for the SNP assay in wells that contain a sample with a known genotype.

post-PCR read	In genotyping and presence/absence experiments, the part of the instrument run that occurs after amplification. In genotyping experiments, fluorescence data collected during the post-PCR read are displayed in the allelic discrimination plot and used to make allele calls. In presence/absence experiments, fluorescence data collected during the post-PCR read are displayed in the presence/absence plot and used to make detection calls. Also called endpoint read.
pre-PCR read	In genotyping and presence/absence experiments, the part of the instrument run that occurs before amplification. The pre-PCR read is optional but recommended. Fluorescence data collected during the pre-PCR read can be used to normalize fluorescence data collected during the post-PCR read.
primer mix	PCR reaction component that contains the forward primer and reverse primer designed to amplify the target.
primer/probe mix	PCR reaction component that contains the primers designed to amplify the target and a TaqMan [®] probe designed to detect amplification of the target.
pure dye	Fluorescent compound used to calibrate the instrument.
	See system dye.
quantification cycle (C _q)	The fractional PCR cycle used for quantification, according to the Real-time PCR Data Markup Language (RDML) data standard. C_T is the algorithm-specific calculations of C_q .
quantification method	In quantification experiments, the method used to determine the quantity of target in the samples.
quantity	In quantification experiments, the amount of target in the samples. Absolute quantity can refer to copy number, mass, molarity, or viral load. Relative quantity refers to the fold-difference between normalized quantity of target in the sample and normalized quantity of target in the reference sample.
quencher	A molecule attached to the 3' end of TaqMan [®] probes to prevent the reporter from emitting fluorescence signal while the probe is intact. With TaqMan [®] reagents, a nonfluorescent quencher-minor groove binder (NFQ-MGB) can be used as the quencher.
QuickStart	A feature that allows you to run an experiment without entering plate setup information, if your instrument and computer are in the same network. QuickStart requires an experiment template file.
R ² value	Regression coefficient calculated from the regression line in the standard curve. An important quality value, the R^2 value indicates the closeness of fit between the standard curve regression line and the individual C_T data points from the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points.
ramp	The step at which the temperature changes during the instrument run. The ramp rate is defined as °C per second. In the graphical view of the thermal profile, the ramp rate is indicated by a diagonal line.

ramp speed	Speed at which the temperature ramp occurs during the instrument run. Available ramp speeds include fast and standard.
Raw data plot	A plot of raw fluorescent signal as detected through each emission filter, used to view raw data for individual wells and at individual cycles.
reaction mix	A solution that contains all components to run the PCR reaction, except for the template (sample, standard, or control). Also called a "PCR cocktail".
reagents	The PCR reaction components used to amplify the target and to detect amplification.
real-time PCR	Process of collecting fluorescence data during PCR. Data from the real-time PCR are used to calculate results for quantification experiments or to troubleshoot results for genotyping or presence/absence experiments.
Real-time PCR Data Markup Language (RDML)	A reporting format that is compliant with the Minimum Information for Publication for Quantitative Real Time Experiments (MIQE) guidelines.
reference sample	In relative standard curve and Comparative $C_T (\Delta \Delta C_T)$ experiments, the sample used as the basis for relative quantification results. Also called the calibrator.
refSNP ID	The reference SNP (refSNP) cluster ID. Generated by the Single Nucleotide Polymorphism Database of Nucleotide Sequence Variation (dbSNP) at the National Center for Biotechnology Information (NCBI). The refSNP ID can be used to search the Life Technologies Store for an Applied Biosystems [®] SNP Genotyping Assay. Also called an rs number.
region of interest (ROI) calibration	Type of calibration in which the software maps the positions of the wells on the sample block of the instrument. The software uses the ROI calibration data to associate increases in fluorescence during a run with specific wells of the plate. A calibration image for each individual filter must be generated to account for minor differences in the optical path.
regression coefficients	Values calculated from the regression line in standard curves, including the R ² value, slope, and y-intercept. You can use the regression coefficients to evaluate the quality of results from the standards. See also standard curve.
regression line	In standard curve and relative standard curve experiments, the best-fit line from the standard curve. Regression line formula:
	$C_T = m [log (Qty)] + b$
	where m is the slope, b is the y-intercept, and Qty is the standard quantity.
	See also regression coefficients.
reject well	An action that the software performs during analysis to remove one or more wells from further analysis if a specific flag is applied to the well.

relative standard curve method	An experimental method to determine relative quantities. This method compensates for target and endogenous control efficiency differences within each run. In all experiments, unknown samples and dilution series of template (such as cDNA) are amplified. Following a run, the instrument software interpolates relative quantities for each unknown sample from the appropriate dilution curve, then normalizes the data for each sample (or set of replicates) as follows: target Q_{Avg} . \div endogenous control Q_{Avg} .
replicate group	A user-defined biological grouping. A replicate group may be a set of identical reactions in an experiment.
replicates	Total number of identical reactions containing identical components and identical volumes.
reporter	A fluorescent dye used to detect amplification. With TaqMan [®] reagents, the reporter dye is attached to the 5' end. With SYBR [®] Green reagents, the reporter dye is SYBR [®] Green dye. SYBR [®] dyes are DNA-binding dyes.
reverse primer	An oligonucleotide that flanks the 3' end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.
reverse transcriptase	An enzyme that converts RNA to cDNA.
R _n	See normalized reporter (Rn).
ROX [™] dye	A dye supplied by Life Technologies, which is used as a passive reference in some experiments.
rs number	See refSNP ID.
run method	Definition of the reaction volume and the thermal profile for the instrument run. The run method specifies the temperature, time, ramp, and data collection points for all steps and stages of the instrument run.
sample	The biological tissue or specimen that you are testing for a target gene.
sample definition file	A tab-delimited file (*.txt or *.csv) that contains the following setup information: well number, sample name, and custom sample properties.
Sample Library	In the software, an editable collection of sample names to use in experiments. The samples in the library contain the sample name and the sample color. The samples in the library may also contain comments about the sample.
sample/SNP assay reaction	In genotyping experiments, the combination of the sample to test and the SNP assay to perform in one PCR reaction. Each PCR reaction can contain only one sample and one SNP assay.
sample/target reaction	In quantification experiments, the combination of the sample to test and the target to detect and quantify in one PCR reaction.

security, auditing	An optional software module that provides:
and eSignature	 System Security – Controls user access to the software. Provides a default Administrator user account. You can define additional user accounts and permissions.
	• Auditing – Tracks changes made to library items, actions performed by users, and changes to the Security and Audit settings. The software automatically audits some actions silently. You can select other items for auditing and specify the audit mode. Provides reports for audited library items, Security and Audit changes, and actions.
	• Electronic Signature (eSignature) – Controls whether users are permitted, prompted, or required to provide a user name and password when accessing certain software features. You can select which features are controlled and the number of signatures required for access. When authorized persons use this feature, they are creating a legally binding signature.
serial factor	In the software, a numeric value that defines the sequence of quantities in the standard curve. The serial factor and the starting quantity are used to calculate the standard quantity for each point in the standard curve. For example, if the standard curve is defined with a serial factor of 1:10 or 10*, the difference between any 2 adjacent points in the curve is 10-fold.
slope	Regression coefficient calculated from the regression line in the standard curve. The slope indicates the PCR amplification efficiency for the assay. A slope of –3.32 indicates 100% amplification efficiency.
	See also amplification efficiency (EFF%) and regression line.
SNP	Single nucleotide polymorphism. The SNP can consist of a base difference or an insertion or deletion of one base.
SNP assay	Used in genotyping experiments, a PCR reaction that contains primers to amplify the SNP and two probes to detect different alleles.
SNP Assay Library	In the software, an editable collection of SNP assays to add to genotyping experiments. The SNP assays in the library contain the SNP assay name; SNP assay color; and for each allele, the allele name or base(s), reporter, quencher, and allele colors. The SNP assays in the library may also contain the assay ID and comments about the SNP assay.
stage	In the thermal profile, a group of one or more steps. Examples: PCR stage, cycling stage (also called amplification stage), and hold stage.
standard	A sample that you dilute and amplify along with unknown samples. This dilution series can contain known starting quantities of the target of interest (absolute standard curve) or it can be of known dilution factor (relative standard curve). Following the run, the software interpolates the C_T values of the unknowns to this curve, yielding either specific quantities of the target (for absolute curves) or relative quantities (for relative dilution curves).
	See also standard curve.

standard curve	In standard curve and relative standard curve experiments:
	 The best-fit line in a plot of the C_T values from the standard reactions plotted against standard quantities. See also regression line.
	• A set of standards containing a range of known quantities. Results from the standard curve reactions are used to generate the standard curve. The standard curve is defined by the number of points in the dilution series, the number of standard replicates, the starting quantity, and the serial factor.
standard curve method	Method 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.
	See also standard and standard curve.
standard dilution series	In standard curve and relative standard curve experiments, a set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards. For example, the standard stock is used to prepare the first dilution point, the first dilution point is used to prepare the second dilution point, and so on. In the software, the volumes needed to prepare a standard dilution series are calculated by the number of dilution points, the number of standard replicates, the starting quantity, the serial factor, and the standard concentration in the stock. See also standard curve.
standard quantity	In the PCR reaction, a known quantity. In standard curve experiments, the quantity of target in the standard. In the software, the units for standard quantity can be for mass, copy number, viral load, or other units for measuring the quantity of target. Standard quantity can also refer to dilution factor.
starting quantity	When defining a standard curve in the software, the highest quantity.
step	A component of the thermal profile. For each step in the thermal profile, you can set the ramp rate (ramp increment for melt curve steps), hold temperature, and hold time (duration).You can turn data collection on or off for the ramp or the hold parts of the step. For cycling stages, a step is also defined by the AutoDelta status.
SYBR [®] Green reagents	PCR reaction components that consist of two primers designed to amplify the target and SYBR [®] Green dye to facilitate detection of the PCR product.
system dye	Dye supplied by Life Technologies. Before you use system dyes in your experiments, make sure the system dye calibration is current in the Instrument Console.
	The system dyes are: • FAM [™] dye • JOE [™] dye • ROX [™] dye • NED [™] dye • SYBR [®] Green dye • TAMRA [™] dye

	• VIC [®] dve
	• Cy [®] 3 dye
	• Texas Red [®] dye
TaqMan [®] reagents	PCR reaction components that consist of primers designed to amplify the target and a TaqMan [®] probe designed to detect amplification of the target.
target	The nucleic acid sequence to amplify and detect.
target color	In the software, a color assigned to a target to identify the target in the plate layout and analysis plots.
Target Library	In the software, an editable collection of targets to use in experiments. Targets in the library contain the target name, reporter, quencher, and target color. The targets in the library may also contain comments about the target.
task	In the software, the type of reaction performed in the well for the target or SNP assay. Available tasks:
	• Unknown
	Negative Control
	Standard (standard curve and relative standard curve experiments)
	Positive control (genotyping experiments)
	IPC (presence/absence experiments)
	Blocked IPC (presence/absence experiments)
technical replicates	Wells containing identical reaction components, including sample; important for evaluating precision.
temperature plot	In the software, a display of temperatures for the instrument cover and instrument block during the instrument run.
template	The type of nucleic acid to add to the PCR reaction.
template file	A user-created file that contains experiment setup information (experiment type, sample names, target name, and thermal conditions) to be used as a starting point for new experiment setup. Template files have an .edt extension.
thermal profile	Part of the run method that specifies the temperature, time, ramp, and data collection points for all steps and stages of the instrument run.
threshold	• In amplification plots, the level of fluorescence above the baseline and within the exponential growth region. For the Baseline Threshold algorithm, the threshold can be determined automatically (see automatic threshold) or can be set manually (see manual threshold).
	 In presence/absence experiments, the level of fluorescence above which the software assigns a presence call.

threshold cycle (C _T)	The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.
T _m	See melting temperature (T _m).
touchscreen	Instrument display that you touch to control the instrument.
uniformity calibration	Type of calibration in which the software measures sample block uniformity. The calibration generates data that compensate for the physical effects of the QuantStudio [™] 6 and 7 Instruments' filters on data collected during an experiment.
unknown	In the software, the task for the target or SNP assay in wells that contain the sample being tested. In quantification experiments, the task for the target in wells that contain a sample with unknown target quantities. In genotyping experiments, the task for the SNP assay in wells that contain a sample with an unknown genotype. In presence/ absence experiments, the task for the target in wells that contain a sample in which the presence of the target is not known. In melt curve experiments, the task for the target in wells that contain a sample with an unknown melt curve profile.
unknown-IPC wells	In presence/absence experiments, wells that contain a sample and internal positive control (IPC).
variant	A sample (or group of samples) with a unique melt curve (that is, the melt curve is different from the melt curves of other samples or controls used in the experiment). The software determines melt curve differences by the melting temperature (Tm) and the shape of the melt curve.
y-intercept	In the standard curve, the value of y where the regression line crosses the y-axis. The y-intercept indicates the expected threshold cycle (C_T) for a sample with quantity equal to 1.

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