

Applied Biosystems™ Standard Curve Analysis Module

USER GUIDE

Publication Number MAN0014819

Revision B.0

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

DISCLAIMER

TO THE EXTENT ALLOWED BY LAW, LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

REVISION HISTORY: History of Pub. no. MAN0010504

Revision	Date	Description
B.0	March 2015	Software user interface updates
A.0	September 2015	Document release

NOTICE TO PURCHASER: DISCLAIMER OF LICENSE: Purchase of this software product alone does not imply any license under any process, instrument or other apparatus, system, composition, reagent or kit rights under patent claims owned or otherwise controlled by Life Technologies Corporation, either expressly, or by estoppel.

Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

TRADEMARKS: All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified.

©2016 Thermo Fisher Scientific Inc. All rights reserved.

Contents

■	CHAPTER 1	Getting Started	6
		About the software	6
		Analysis workflow	7
		Compatible Real-Time PCR System Data	8
		System requirements	9
		About the software interface	10
		Best practices and tips for using the software	10
■	CHAPTER 2	Manage experiment data	11
		Create a project and add experiment data	11
		Manage projects and experiment data	12
		Share experiments, folders, and projects	13
		About experiment data/files	15
■	CHAPTER 3	Set up the project	16
		Manage samples and targets	16
		Import sample information from design files	17
		Import target information from AIF files	17
■	CHAPTER 4	Edit experiment properties	18
		Review and edit the plate setups	19
		Apply samples and targets	19
		Specify and assign tasks	20
		Specify and assign standards	21
		Apply plate setup information using a template file	23
		Template files	24

■	CHAPTER 5	Review the quality data and results	26
		Specify a master standard curve and/or inter-plate controls	26
		Configuring the analysis settings	30
		Reviewing amplification and quality data	32
		About the quality data summary	34
		Omit wells from the analysis	35
■	CHAPTER 6	Export the results	36
		Export the analyzed data from a project	36
		Export project data as a slide presentation	37
		Export plots for presentation and publication	38
		Export data for use in other projects	39
■	CHAPTER 7	Screens and plots	41
		Amplification Plot	42
		Multicomponent Plot	43
		Standard Curve Plot	44
		Well Table	46
■	CHAPTER 8	Quality flags	48
		AMPNC (Amplification in negative control) quality flag	49
		AMPSCORE (Low signal in linear phase) quality flag	49
		BADROX (Bad passive reference signal) quality flag	50
		BLFAIL (Baseline algorithm failed) quality flag	50
		CQCONF (Calculated confidence in the C _q value is low) quality flag	51
		CRTAMPLITUDE (Broad C _q Amplitude) quality flag	51
		CRTNOISE (C _q Noise) quality flag	51
		CTFAIL (C _q algorithm failed) quality flag	51
		DRNMIN (Detection of minimum ΔR_n due to abnormal baseline) quality flag	52
		EXPFAIL (Exponential algorithm failed) quality flag	52
		HIGHSD (High standard deviation in replicate group) quality flag	53
		LOWROX (Low ROX™ Intensity) quality flag	54
		MTP (Melt curve analysis shows more than one peak) quality flag	54
		NOAMP (No amplification) quality flag	54
		NOISE (Noise higher than others in plate) quality flag	55
		NOSAMPLE (No sample assigned to well) quality flag	55
		NOSIGNAL (No signal in well) quality flag	55
		OFFSCALE (Fluorescence is offscale) quality flag	56
		OUTLIERRG (Outlier in replicate group) quality flag	57

PRFDROP (Passive reference signal changes significantly near the Cq/Ct) quality flag . .	57
PRFLOW (Average passive reference signal is below the threshold) quality flag	58
SPIKE (Noise spikes) quality flag	59
THOLDFAIL (Thresholding algorithm failed) quality flag	59
■ APPENDIX A Documentation and support	61
Customer and technical support	61
Limited product warranty	61
Glossary	62



Getting Started

■ About the software	6
■ Analysis workflow	7
■ Compatible Real-Time PCR System Data	8
■ System requirements	9
■ About the software interface	10
■ Best practices and tips for using the software	10

The Applied Biosystems™ Analysis Software is a secure web application for analysis of data generated on Thermo Fisher Scientific real-time PCR instruments. The software provides project-based analysis of real-time and end-point data for a variety of quantitative and qualitative PCR applications.

About the software

The Standard Curve (SC) module of the Applied Biosystems™ Analysis Software performs absolute quantification analysis of standard curve experiments run on Thermo Fisher Scientific real-time qPCR instruments. The standard curve method is used to determine the absolute target quantity in samples. With the standard curve method, the Applied Biosystems™ Analysis 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.

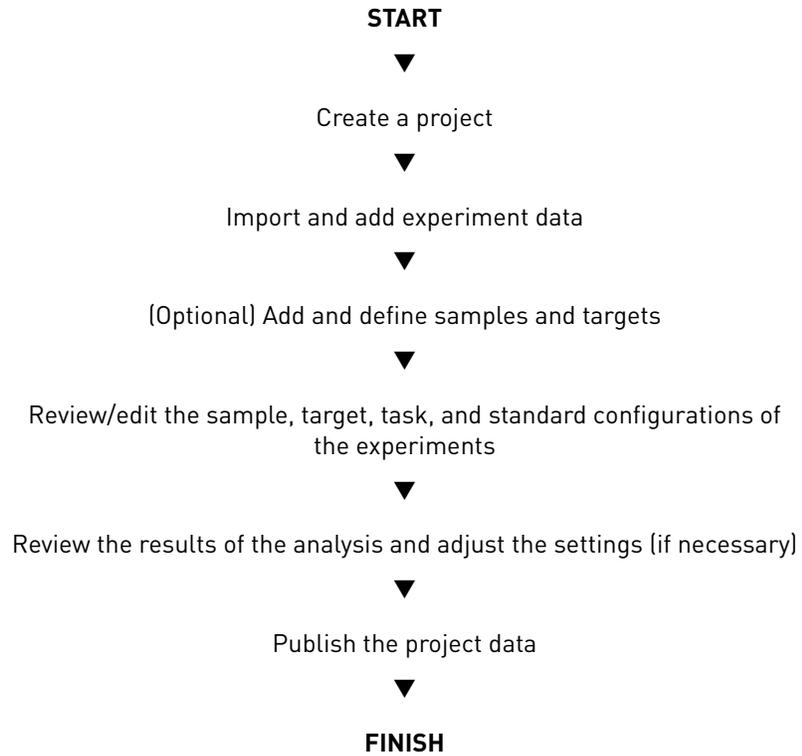
Required experiment components

The following components are required to perform a standard curve analysis and must be present on all experiments added to the project:

- **Samples** – The sample in which the quantity of the target is unknown.
- **Standard** – A sample that contains known standard quantities; used in quantitation 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.

Analysis workflow

The following figure shows the general workflow for analyzing standard curve projects using the Applied Biosystems™ Analysis Software.



Compatible Real-Time PCR System Data

The Applied Biosystems™ Analysis Software can import and analyze data generated by any of the supported instruments listed in the following table. The software versions listed in the table represent only those tested for use with the Applied Biosystems™ Software. Data generated by versions other than those listed can be imported and analyzed by the software, but are not supported by Thermo Fisher Scientific.

IMPORTANT! The Applied Biosystems™ Analysis Software can import and analyze data from unsupported versions of the instrument software; however, we cannot guarantee the performance of the software or provide technical support for the analyses.

Real-Time PCR System	Supported software version(s)	File extension
Applied Biosystems™ 7900 HT Fast Real-Time PCR System	v2.4 or later	.sds
Applied Biosystems™ 7500 and 7500 Fast Real-Time PCR System	v1.4.1 or later	
Applied Biosystems™ 7500 and 7500 Fast Real-Time PCR System	v2.0.5 or later	.eds
Applied Biosystems™ StepOne™ and StepOnePlus™ Real-Time PCR System	v2.0.1, v2.1, or later	
Applied Biosystems™ ViiA™ 7 Real-Time PCR System	v1.1 or later	
Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System	v1.1.1 or later	
Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System	v1.0 or later	
Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System		
Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System	v1.0 or later	
Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System		

System requirements

The following table summarizes the system requirements for the user environment. Applied Biosystems™ Analysis Software performance may vary based on your system configuration.

Category	Requirement
Web Browser	<ul style="list-style-type: none">• Apple™ Safari™ 8 Browser• Google™ Chrome™ Browser Version 21 or later• Microsoft™ Internet Explorer™ Browser Version 10 or later• Mozilla™ Firefox™ Browser Version v10.0.12 or later
Operating System	<ul style="list-style-type: none">• Windows™ XP, Vista, 7, or 8• Macintosh™ OS 8 or later
Network Connectivity	An internet connection capable of 300kbps/300kbps (upload/download) or better. If your network employs a firewall that restricts outbound traffic, it must be configured to allow outbound access to apps.lifetechnologies.com on HTTPS-443.

About the software interface

The Applied Biosystems™ Software features a simple interface for analyzing experiment data and includes the following buttons/icons in many of the screens and plots:



- ① **Analysis Modules** – Click to analyze the current project using the selected module.
- ② **DATA** [Data Manager] – Click to view the Data Manager, which can be used to view, add, or remove data from the current project.
- ③ **Home** [Project Manager] – Click to view the Project Manager, which can be used to modify the current project or open another.
- ④ **Account Management Menu** – Click to manage your application licenses or storage.
- ⑤ **Project name** – The name of the current project.
Note: Click to close the project.
- ⑥ **Project tabs** – Click to view the settings, data, or plot(s) for the current project.
- ⑦ **Notifications** – Click to view important information and notifications for the current project. The digit within the icon indicates the number of messages.
- ⑧ **Help** – Click to access help topics relevant to the current settings, data, or plot that you are viewing.
- ⑨ **Profile Menu** – Click to change your profile settings or to log out of the Applied Biosystems™ Software.
- ⑩ **Analyze** – Click to analyze the project after you have made a change.
- ⑪ **Zoom** – Click to magnify the related table or plot to fill the screen.
Note: Once expanded, click [Close] to collapse the plot or table to its original size.
- ⑫ **Actions** – Click to select from a list of actions that pertain to the related table or plot.

Best practices and tips for using the software

The Applied Biosystems™ Analysis Software provides a variety of useful user interface elements that will enable you to better organize your data for analysis and presentation. This topic describes the essentials of the user interface and how to best use them.

Perform the following actions to help ensure optimal performance of the Applied Biosystems™ Software:

- Refresh your browser regularly
- Clear your browser cache



Manage experiment data

- Create a project and add experiment data 11
- Manage projects and experiment data 12
- Share experiments, folders, and projects 13
- About experiment data/files 15

Use the Data Manager screen to add and remove experiments to and from your project. The screen displays all experiments associated with the current project. You can also use the Data Manager to upload new .eds and .sds files or view the details of individual experiments already added to the project.

Create a project and add experiment data

1. Click  (Manage Projects) to view the Dashboard.
 2. Create the project:
 - a. Click  **New Project**.
 - b. In the Create Project dialog box, enter a name for the project, select the folder within which you want to place the project, then click **OK**.
- Note:** The project name cannot exceed 50 characters and cannot include any of the following characters: / \ < > * ? " | : ; & % \$ @ ^ () !

3. From the Manage Data screen, add any additional experiment data to the project.

To import experiment data stored on...	Action
Your computer	<ol style="list-style-type: none"> 1. Click Import from local. 2. From the Open dialog box, select one or more experiment files (.sds or .eds), then click Open. Note: Ctrl- or Shift-click to select multiple files. Wait for the Applied Biosystems™ Software to upload the selected data. 3. Click Close prompted that the import is complete.
Thermo Fisher Cloud	<ol style="list-style-type: none"> 1. Click Import from Thermo Fisher Cloud. 2. Select one or more experiment files (.sds or .eds) from the table, then click Add. 3. When you are done adding files to the queue, click OK. 4. Click Close prompted that the import is complete.

4. Repeat step 3 until your project contains all of the desired experiment data.
5. Click the appropriate analysis module on the left side of the screen to begin the analysis.

Manage projects and experiment data

Use the Manage Data screen to add and remove experiment data to/from your project:

- **Add** experiment data to your project:
 - a. While viewing your project, click  (Manage Data) from the bar on the left side of the screen.
 - b. From the Manage Data screen, add any additional experiment data to the project.

To import experiment data stored on...	Action
Your computer	<ol style="list-style-type: none"> 1. Click Import from local. 2. From the Open dialog box, select one or more experiment files (.sds or .eds), then click Open. Note: Ctrl- or Shift-click to select multiple files.
Thermo Fisher Cloud	<ol style="list-style-type: none"> 1. Click Import from Thermo Fisher Cloud. 2. Select one or more experiment files (.sds or .eds) from the table, then click Add. 3. When you are done adding files to the queue, click OK.

- c. Wait for the Applied Biosystems™ Software to import the selected data. When you are prompted that the upload is complete, click **Close**.
- **Delete** projects, experiments, or folders:
 - a. Select the experiments from the Files in this project table that you want to remove.
 - b. From the Manage Data screen, select **Actions ▶ Delete**.
 - c. When prompted, click **OK** to remove the experiment(s) from your project.

Note: Click the appropriate analysis module on the left side of the screen to return to the analysis.

Share experiments, folders, and projects

The Applied Biosystems™ Analysis Software allows you to share any data (experiments, folders, and projects) with other users that have access to the software. Sharing data with other users grants them different access to the data depending on the type of object shared:

- **Projects** – Sharing a project with other users grants them read/write access to the unlocked project.

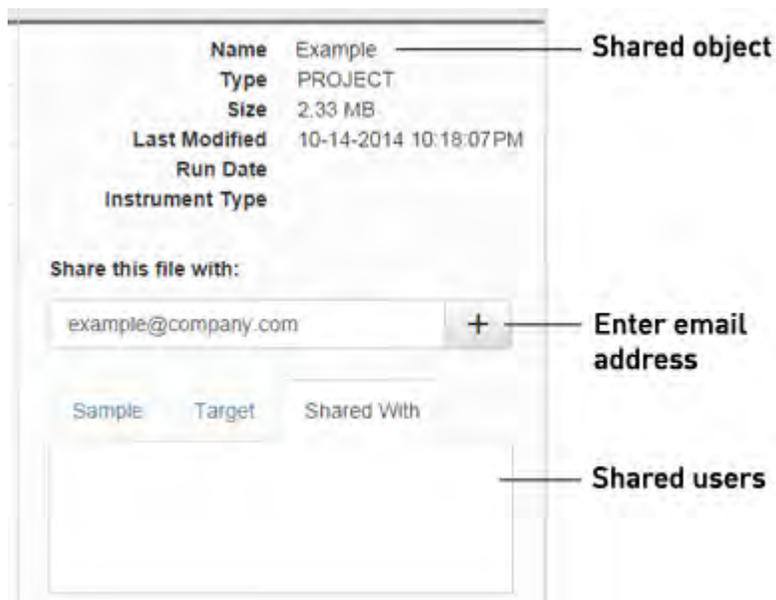
IMPORTANT! A project is locked (preventing access) when it is open (in use) by any user with shared access to the project. For example, User A shares a project with two colleagues (User B and User C), User B opens the project and begins data analysis (the project is locked and unavailable to Users A and C) until User B closes the project at which time it is available again to all three users.

- **Experiments** – Sharing experiment files with other users grants them full access to the data, allowing them to import the data to their own projects or download the experiment data file.
- **Folders** – Sharing a folder with another user grants access to the contents of the folder (projects, experiments, and subfolders).

To share projects, experiments, and subfolders with another user:

- **Share** an experiment, folder, or project:
 - a. Click  (Home), then click  **All Files** to view your data.
 - b. From the Home Folder screen, select the check box to the left of the object (project, experiment, or folder) that you want to share, then click  (display details).

- c. Enter the email address of the user with whom you want to share the selected object, then click **+**.



The user is notified via email that you have shared with them and the shared item will appear in their Home Folder.

IMPORTANT! To share multiple files:

1. Select the desired objects (projects, experiments, and subfolders) from the Home Folder screen, then click **Actions ▶ Share**.
2. In the Share Files dialog box, enter the email address of the user with whom you want to share the selected objects, then click **Share**.

- **Un-share** a file, folder, or project:
 - a. Click (Home), then click **All Files** to view your data.
 - b. Select the shared object, then click the display details icon.
 - c. In the details pane, select the **Shared With** tab, then click **un-share** adjacent to the email address of the user from which you want to remove sharing privileges.

The selected users are notified via email that you are no longer sharing the specified file with them and the shared file(s) will no longer appear in their Home Folder.

About experiment data/files

The Applied Biosystems™ Analysis Software can import and analyze experiment files (.eds and .sds) that are generated by a variety of Thermo Fisher Scientific real-time qPCR instruments. Every consumable run on a Thermo Fisher Scientific real-time qPCR instrument requires the creation of one or more experiment files that store the associated data. Each experiment file is a virtual representation of a specific consumable (plate, array, or chip) that contains data for all aspects of the qPCR experiment.

Experiment files contain the following information:

- Target information and arrangement on the plate
- Sample information and arrangement on the plate
- Method parameters for the run

File compatibility

The Applied Biosystems™ Software can import data the following experiment file formats generated by Applied Biosystems™ real-time qPCR instruments:

IMPORTANT! The Applied Biosystems™ Analysis Software can import and analyze data from unsupported versions of the instrument software; however, we cannot guarantee the performance of the software or provide technical support for the analyses.

Real-Time PCR System	Supported software version(s)	File extension
Applied Biosystems™ 7900 HT Fast Real-Time PCR System	v2.4 or later	.sds
Applied Biosystems™ 7500 and 7500 Fast Real-Time PCR System	v1.4.1 or later v2.0.5 or later	
Applied Biosystems™ StepOne™ and StepOnePlus™ Real-Time PCR System	v2.0.1, v2.1, or later	.eds
Applied Biosystems™ ViiA™ 7 Real-Time PCR System	v1.1 or later	
Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System	v1.1.1 or later	
Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System	v1.0 or later	
Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System		
Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System	v1.0 or later	
Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System		



Set up the project

- Manage samples and targets 16
- Import sample information from design files 17
- Import target information from AIF files 17

After importing one or more experiments (.eds or .sds files) into your HRM project, use the Overview screen to set up the project.

Manage samples and targets

The Applied Biosystems™ Analysis Software populates the Overview screen with the samples and targets present in the experiments added to the project. If necessary, you can add, edit, or remove the samples and targets as needed before the analysis.

- **Create** a new sample or target:
 - a. From the Samples or Targets table in the Overview screen, click **Actions ▶ Add**.
 - b. In the New Sample/Target dialog box, enter a name for the new sample or target (up to 256 characters), then edit the properties of the new sample/target.
 - c. Click **OK**.
- **Update** an existing sample or target by editing the entry directly in the table.
Note: Alternately, select a sample or target from the table, then select **Actions ▶ Assign/Update**.
- **Delete** a sample or target:
 - a. From the Samples or Targets table in the Overview screen, select the sample or target of interest, then click **Actions ▶ Delete**.
 - b. In the confirmation dialog box, click **OK** to delete the sample or target.

Import sample information from design files

This Applied Biosystems™ Analysis Software Analysis Module does not support sample import by design file. To populate your project with samples, enter them directly into the Samples list in the Overview screen. For more information on entering sample information, see “Manage samples and targets” on page 16.

Import target information from AIF files

For convenience, the Applied Biosystems™ Software can import target information directly from assay information files (.aif), which are supplied with assays manufactured by Thermo Fisher Scientific. AIF are tab-delimited data files provided on a CD shipped with each assay order. The file name includes the number from the barcode on the plate.

1. From the Targets table in the Overview screen, click **Actions ▶ Import AIF File**.
2. Locate the .aif file with the target information, then click **Open**.

If the import is successful, the target is populated to the appropriate table. If a target of the same target name is already present in the project, it is overwritten with the information from the AIF.

Note: Assay/target name matching is not case sensitive.



Edit experiment properties

- Review and edit the plate setups 19
- Apply samples and targets 19
- Specify and assign tasks 20
- Specify and assign standards 21
- Apply plate setup information using a template file 23
- Template files 24

After populating your project with samples, targets, and controls, use the Plate Setup screen to make changes to the plate setups of the experiments added to your project. The editor can be used to edit sample, target, task, and control assignments to correct missing or incorrect settings.

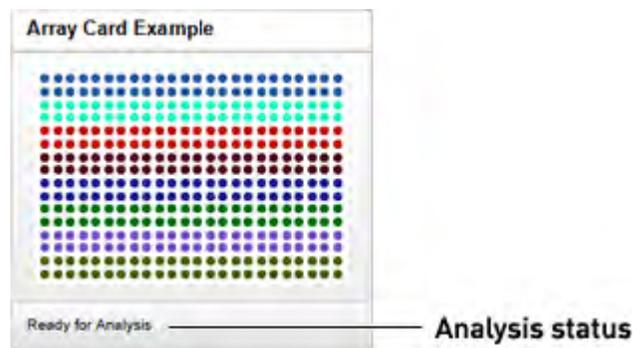
Review and edit the plate setups

After configuring your project with all necessary samples and targets, use the Plate Setup screen to review the experiments for problems that can prevent the analysis of the project. The Applied Biosystems™ Analysis Software displays plate configuration errors that can prohibit analysis in the margin beneath each image of the related experiment. Before you can analyze your project, you must use the Plate Setup screen to address them.

To review the plate setup information for your project:

1. Select **Plate Setup** to display Plate Setup screen.
2. From the Plate Setup screen, review the experiment records for errors.
3. If errors are present, click the experiment record of interest and address the problem that is preventing the analysis of the file.

Note: The software displays plate configuration problems that will prevent analysis of an experiment beneath the image of the related plate.



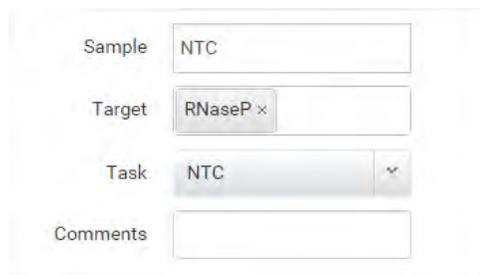
Apply samples and targets

If the sample or target assignments of one or more of your experiments contain errors or are missing, you can use the Applied Biosystems™ Analysis Software to correct the problem prior to analysis.

Note: When reviewing a plate layout, click **Actions** ▶ **Clear Well Setup** to remove the well information (sample, task, and target assignments) from the selected wells in the plate grid.

1. From the Plate Setup screen, select the experiment that you want to modify.
2. (Optional) From the Edit Plate screen, click **View** , then select **Target** and **Sample** to color the plate setup according to the element that you intend to modify.
3. Select the wells of the plate layout to which you want to apply the target or sample.

4. When the wells are selected, click the appropriate field to the right of the plate grid, then select the appropriate item from the list.



Sample	NTC
Target	RNaseP ×
Task	NTC ▼
Comments	

Note: If you have not yet created a sample or target, enter the name in the appropriate field and press **Enter** to create the new sample or target.

5. Once you are finished making changes to the plate layout, click **Analyze** to reanalyze your project.

Specify and assign tasks

If the task assignments of one or more of your experiments contain errors or are missing, you can use the Applied Biosystems™ Analysis Software to correct the problem prior to analysis.

Note: When reviewing a plate layout, click **Actions ▶ Clear Well Setup** to remove the well information (sample, task, and target assignments) from the selected wells in the plate grid.

1. From the Plate Setup screen, select the experiment record that you want to modify.
2. From the Edit Plate screen, click **View** , then select **Task** to color the plate setup according to task assignment.
3. Select the wells of the plate layout to which you want to apply a task.

- When the wells are selected, click the **Task** menu, then select the appropriate task from the list.

Available tasks include:

- **Unknown** – The task for wells that contain a sample with unknown target quantities.
- **Standard** – The task for wells that contain a sample of known standard quantity. Standard reactions are used in quantitation experiments to generate standard curves.
- **NTC** – The task for wells that contain water or buffer instead of sample (no template controls). No amplification of the target should occur in negative control wells.

- Repeat steps 3 and 4 as needed.
- Once you have completed making changes to the plate layout, click **Analyze** to reanalyze your project.

Specify and assign standards

If the standard assignments of one or more of your experiments contain errors or are missing, you can use the Applied Biosystems™ Analysis Software to correct the problem prior to analysis.

Note: When reviewing a plate layout, click **Actions ▶ Clear Well Setup** to remove the well information (sample, task, target, and standard assignments) from the selected wells in the plate grid.

- From the Plate Setup screen, select the experiment that you want to modify.
- From the Edit Plate screen, click **View Options**, then select **Task** to color the plate setup according task assignment.
- In the plate layout, click **Actions ▶ Define Standards**.
- Define the standard curve:
 - Select the target to use to define the standard curve.

b. Use the standard definition wizard to enter the data for the standard curve:

- **# of Points** – Enter the number of points for each standard curve in the reaction plate. Thermo Fisher Scientific recommends at least five dilution points for each standard curve.
- **# of Replicates** – Enter the number of replicates (identical reactions) for each point in the standard curve. Thermo Fisher Scientific recommends three replicates for each point.
- **Starting Quantity** – Enter the quantity or concentration (smallest or largest) for the standards from which the software will construct the curve.

Because the range of standard quantities affects the amplification efficiency calculations, carefully consider the appropriate range of standard quantities for your assay:

- For more accurate measurements of amplification efficiency, use a broad range of standard quantities, spanning between 5 and 6 logs. If you specify a broad range of quantities for the standards, you need to 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.
- **Serial Factor** – Enter the serial factor used to calculate the quantities in all points of the standard curve. If your starting quantity is the highest quantity, select a dilution factor such as 1:2, 1:3, and so on. If your starting quantity is the smallest quantity, select a concentration factor such as 2X, 3X, and so on.

c. Select the wells that contain the standards.

- To specify the standards automatically, select **Automatically select wells**, then select **Row** or **Column** to highlight the appropriate wells in the plate layout.
- To specify the standards manually, select **Let me select wells**, then select the appropriate wells in the plate layout.

d. Click **Apply Standard Curve**.

Note: Alternatively, you can specify or edit the standards manually as follows:

1. Select the wells of the plate layout that contain the standards that you want to edit.
2. When the wells are selected, click the Task menu to the right of the plate grid, then select **Standard**.
3. In the Quantity field to the right of the plate grid, enter the appropriate quantity or concentration for the well(s).

5. Once you have completed making changes to the plate layout, click **Analyze** to reanalyze your project.

IMPORTANT! The data format for the quantity or concentration values that you enter must match those applied to the other standards in the experiments added to your project.

Apply plate setup information using a template file

The Applied Biosystems™ Software can import plate layout information directly from design files that you can create using a text editor or spreadsheet application.

Note: For detailed information on the structure of template files, see “Template files” on page 24.

From the Plate Setup screen, you can perform the following actions:

- **Download** the plate setup information from an existing experiment as a template file:
 - a. Open the project that includes the experiment with the desired plate layout, then select **Plate Setup**.
 - b. From the Plate Setup screen, select the experiment record that contains the desired plate setup.
 - c. From the Edit Plate screen, click **Actions ▶ Apply Template**, then save the file to the desired location.
- **Apply** plate setup information using a template file.
 - a. Create a template file that contains the desired plate setup information.

Note: See “Template files” on page 24 for detailed information on constructing template files.
 - b. Open the project that includes the experiment to which you want to apply the template, then click **Plate Setup**.
 - c. From the Plate Setup screen, select the experiment record that you want to modify.
 - d. From the Edit Plate screen, click **Actions ▶ Download Template**.
 - e. Select the template file that contains the desired plate setup, then click **Open**.

If the import is successful, the sample, assay/target, and task assignments of the current plate layout are overwritten with the imported settings.

IMPORTANT! The imported plate layout overrides the existing plate setup and cannot be undone once imported.

Template files

The Applied Biosystems™ Analysis Software allows you to apply plate layout information (such as the target, sample, and task configurations) from template files that you can create using a text editor or spreadsheet application. Template files are comma-separated value (.csv) files that contain the target, sample, and task configurations for a single reaction plate. You can create a template file using a spreadsheet application or a text editor, then import it using the Applied Biosystems™ Software to apply target, sample, and/or task information to experiments added to a project.

If you have already added an experiment to your project, you can download a template file that you can use as a starting point to create your own template files. The following figure illustrates the general structure of the exported file.

		A	B	C	D	E	F
Experiment data (do not edit):	1	* Block Type = Array Card Block					
	2	* Experiment Type = Absolute Quantitation					
	3	* Instrument Type = QuantStudio 12K Flex Real-Time PCR System					
	4	* No. Of Wells = 384					
Column headings (do not edit):	5	Set Up Well Section Info					
	6	Well	Well Position	Sample Name	Task	Target Name	Quantity
Plate setup content (add well data in any order):	7	14	A15	NTC	NTC	RNaseP	
	8	80	D9	Ua	UNKNOWN	RNaseP	
	9	195	I4	400	STANDARD	RNaseP	1600

Use the following guidelines when editing the file:

- Rows 1 to 6 contain file header information that describes the experiment. In general, you should not edit this information as it will be identical for all files that you use. Enter the headings exactly as shown, including upper- and lowercase letters:
 - * Block Type =
 - * Experiment Type =
 - * Instrument Type =
 - * No. Of Wells =
 - * Set Up Well Section Info =

- Well
- Well Position
- Sample Name
- Task
- Target Name
- Quantity
- Rows 7 and below contain the plate setup information for the experiment, where each row contains the information for the contents of a single well on the reaction plate. As shown in the example above, the rows can occur in any order, but the location information (in columns 1 and 2) must be accurate.

For each well the file contains the following information:

- **Column A (Well)** – The numerical position of the well on the plate, where wells are numbered left to right and top to bottom. For example, on a 96-well plate, the number of well A1 is "0" and the number of well G12 is "95".
 - **Column B (Well Position)** – The coordinates of the well on the plate.
 - **Column C (Sample Name)** – The name of the sample within the well (up to 256-characters).
 - **Column D (Task)** – The task of the sample within the well, where acceptable values include **UNKNOWN**, **NTC**, and **STANDARD**.
 - **Column E (Target Name)** – The name of the assay added to the well, or the identity of the target sequence (up to 256-characters).
 - **Column F (Quantity)** – The quantity of the standard contained in the well.
- Note:** Do not enter standard values for wells that contain unknowns or no template controls.
- If the samples and/or targets that you include in the template file are present in other experiments included in the project, the names in the file must match those in the other experiments exactly (including case) in order for the software to associate the data.
 - When importing plate setup information from a template file, the Applied Biosystems™ Software overwrites all existing settings with the information in the file.

5

Review the quality data and results

- Specify a master standard curve and/or inter-plate controls 26
- Configuring the analysis settings 30
- Reviewing amplification and quality data 32
- About the quality data summary 34
- Omit wells from the analysis 35

After adding experiments to your project, use the Quality Check & Results screen to make a first pass of your analyzed project data and to view the results of the analysis. The plots and features of the screen can help you review your project for irregular amplification and other common problems.

Specify a master standard curve and/or inter-plate controls

If you are using an external master standard curve and/or inter-plate controls, you can use the Standard Curve Options dialog box to configure them for use in your project.

The Applied Biosystems™ Software supports the use of inter-plate controls to identify variance/difference in multi-plate standard curve analyses in the performance of the external standard curve. For each control, the software allows you to specify a tolerance parameter that defines the acceptable performance for the control. During the analysis, the software uses these settings to alert you to controls that fail to perform within the defined ranges.

Note: The use of inter-plate controls is optional.

If you are using a master standard curve to analyze your project, you can either create the standard curve data file using a text editor, or export the data from an existing experiment in your project. If you create the file manually, save the standard curve data to a comma separated value (.csv) text file in the following format:

```
#Instrument:QuantStudio 12K Flex Real-Time PCR System
#Block:Array Card
Reporter,Target,Slope,Y-Intercept,R2,Efficiency
(%),Quantities,Std Dev,Std Error
FAM,RNaseP,-4.6577,39.9765,0.5632,63.9450,"800.0, 1600.0,
3200.0",0.8608,0.2658
```

Alternatively, you can export the standard curve data file from an existing experiment in your project as follows:

1. Select the **Quality Control & Result** tab, then click the experiment containing the desired standard curve data.
2. While viewing the experiment, click **Analysis Options**, then select **Standard Curve Settings**.
3. In the Standard Curve Settings, select the desired standard curve, then click **Export** to save the data to a .csv file.

To specify a master standard curve and/or inter-plate controls for your project:

1. From the Quality Control & Results screen, click **Standard Curve Options**.
2. From the General tab of the Standard Curve Options dialog box, select the option to determine how the software will use the inter-plate control data present in the experiments added to your project.

Option	Settings
Use standard curves configured for individual plate	Select this option to use the standard curves on each individual plate to analyze your project data.
Share any on-plate standard curves for all plates	<p>Select this option to analyze your project data by sharing the standard curve data present on the experiments in the project. If selected, the software computes the quantities of unknown samples using the relevant standard curve(s) present on the plates added to your project.</p> <p>To configure the software to share on-plate standard curve data:</p> <ol style="list-style-type: none"> 1. Select Share any on-plate standard curves for all plates in the General tab of the Standard Curve Options dialog box. If you are not using inter-plate controls, skip to step 3. 2. Select Inter-plate Controls tab to view the control settings. <p>Note: The use of inter-plate controls is optional.</p> 3. If the option is not selected, select Use Inter-plate Controls. 4. Select the method (standard deviation or calculated quantity) that the software will use to monitor inter-plate control performance: <ul style="list-style-type: none"> • Use Standard Deviation – If selected, the software will alert you if the standard deviation (σ_{Cq}) of the Cq (Ct) values for an inter-plate control on any plate is greater than the specified Cq Standard Deviation Threshold. For example, if the Cq Standard Deviation Threshold for an inter-plate control is set to 0.5, then the software will flag any plate where the inter-plate control generates a $\sigma_{Cq} > 0.5$. • Use Quantity – If selected, the software will alert you to if the calculated quantity of a specified inter-plate control on any plate deviates from the specified Quantity Mean by the specified margin. For example, if the Quantity Mean for an inter-plate control is set to 1000 copies and the Margin is set to 15%, then the software will flag any plate where the calculated quantity of the inter-plate control is not between 850 to 1150 copies. 5. Add the inter-plate controls to use in your analysis. For each control: <ol style="list-style-type: none"> a. Click Add to add an inter-plate control. b. Select the Target and Sample combination that identifies the desired sample. c. Specify the thresholds that you want the software to apply to the inter-plate controls in your analysis: <ul style="list-style-type: none"> • If using standard deviation, specify the Cq Standard Deviation Threshold that defines the limit beyond which the software will alert you to the performance of the inter-plate control. • If using quantity, specify the Quantity Mean and the Margin (%) that define the upper and lower quantities beyond which the software will alert you to the performance of the inter-plate control.

Option	Settings
<p>Use master external standard curves for all plates</p>	<p>Select this option to analyze all plates in your project using data from a single "master" standard curve. If selected, the software computes the quantities of all unknown samples in your project using imported master standard curve data, relying on inter-plate controls to perform quality control across the experiments in the project.</p> <p>To set the external standard curve:</p> <ol style="list-style-type: none"> 1. Select Use master external standard curves for all plates in the General tab of the Standard Curve Options dialog box. 2. Select Master External Curves to view the master curve settings. 3. Click Import and select the comma-separated file that contains the desired master standard curve data. If you are not using inter-plate controls, skip to step 3. 4. Select Inter-plate Controls to view the inter-plate calibration settings. Note: The use of inter-plate controls is optional. 5. Select the method (standard deviation or calculated quantity) that the software will use to monitor inter-plate control performance: <ul style="list-style-type: none"> • Use Standard Deviation – If selected, the software will alert you if the standard deviation (σ_{Cq}) of the Cq (Ct) values for an inter-plate control on any plate is greater than the specified Cq Standard Deviation Threshold. For example, if the Cq Standard Deviation Threshold for an inter-plate control is set to 0.5, then the software will flag any plate where the inter-plate control generates a $\sigma_{Cq} > 0.5$. • Use Quantity – If selected, the software will alert you to if the calculated quantity of a specified inter-plate control on any plate deviates from the specified Quantity Mean by the specified margin. For example, if the Quantity Mean for an inter-plate control is set to 1000 copies and the Margin is set to 15%, then the software will flag any plate where the calculated quantity of the inter-plate control is not between 850 to 1150 copies. 6. Add the inter-plate controls to use in your analysis. For each control: <ol style="list-style-type: none"> a. Click Add to add an inter-plate control. b. Select the Target and Sample combination that identifies the desired sample. c. Specify the thresholds that you want the software to apply to the inter-plate controls in your analysis: <ul style="list-style-type: none"> • If using standard deviation, specify the Cq Standard Deviation Threshold that defines the limit beyond which the software will alert you to the performance of the inter-plate control. • If using quantity, specify the Quantity Mean and the Margin (%) that define the upper and lower quantities beyond which the software will alert you to the performance of the inter-plate control.

3. When done modifying the options, click **Finish**.

Configuring the analysis settings

When a project is created, the Applied Biosystems™ Analysis Software processes the project data using the default analysis settings of the experiments added to the project. If desired, you can modify the analysis settings from the Quality Control & Results screen (for example, manual versus automatic thresholding or stringent versus relaxed quality thresholds).

1. From the Quality Control & Results screen, select an experiment of interest.
2. From the Review Result screen, click **Analysis Settings**.

3. From the Edit Analysis Setting dialog box, modify the analysis settings as desired.

Group	Settings
Cq Settings	<p>Select the method (C_T or C_{RT}) that the Applied Biosystems™ Software will use to compute the Cq (C_T) values for the analysis:</p> <ul style="list-style-type: none"> • CT method – Define whether each target will use automatic thresholding and/or baselining. If you are using manual settings, enter the manual threshold and baseline values for the appropriate targets. • CRT method – Specify the cycle number that the software will use as the minimum parameter in defining the relative threshold for the C_{RT} calculation. <p>Note: The Applied Biosystems™ Software allows you to import the Cq settings from a file exported from another project (click Import CT Settings to import the file).</p>
Advanced C _T Settings	<p>Define whether individual wells will use automatic baselining. If you choose to use manual settings for one or more wells, enter the manual start and end baseline values for the appropriate targets.</p>
Flag Settings	<p>Specify the quality measures that the Applied Biosystems™ Software will compute during the analysis.</p> <ol style="list-style-type: none"> 1. In the Use column, select the check boxes for flags you want to apply during analysis. 2. If an attribute, condition, and value are listed for a flag, you can specify the setting for applying the flag. For example, with the default setting for the no amplification flag (NOAMP), wells are flagged if the amplification algorithm result is less than 0.1. <p>Note: If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.</p> <ol style="list-style-type: none"> 3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag. Rejected wells are not considered for data analysis.
Standard Curve Settings	<p>Select the method that the Applied Biosystems™ Software will use to compute the standard curve used for the analysis:</p> <ul style="list-style-type: none"> • On-Plate Standard Curves – Select to use the standard curve generated from the standards data present on the experiments added to the project. <p>Note: You can generate the standard curve data either manually or by exporting the data set from an existing experiment using the Export function in the Standard Curve Settings of the analysis settings.</p> <ul style="list-style-type: none"> • External Standard Curves – Select to use a standard curve generated from an imported data set. To specify the data for the external standard curve, click Import and select the comma-separated file that contains the desired data. The imported file must be formatted as follows, where the first line of text contains the required column headings and the second line contains the specifications for the standard curve: <pre>Reporter, Target, Slope, Y-Intercept, R2, Efficiency (%) , Quantities FAM, RNaseP, -4.6577, 39.9765, 0.5632, 63.945024741716864, "800.0, 1600.0, 3200.0"</pre> <p>IMPORTANT! The specified standard curve quantities for the imported file must be separated by commas (,) and enclosed within double quotations (").</p>

4. When done modifying the analysis settings, click **Finish**.

Reviewing amplification and quality data

After the Applied Biosystems™ Analysis Software processes your project, you can use the Quality Control & Results screen to review the quality data generated by the analysis. The software provides a variety of options to review the quality data; however, the strategy that you employ will depend on the type of quantitation you are performing and the samples/targets that you are evaluating. The following procedure describes a general approach to data review and provides an overview of the software features.

1. If you have not already done so, click **Analyze** to analyze your project.
2. In the Applied Biosystems™ Software, click **Quality Control & Results** to view the Quality Control & Results screen.
3. Review the amplification plots for irregularities and quality flags.
Note: The Applied Biosystems™ Software displays summaries of the quality data in the margin beneath each amplification plot. You can view the identity of any flag by hovering the mouse over the flag of interest.
4. If flags or irregularities are present, or you would like to review the amplification data for a specific target, sample, or experiment, click the amplification plot of interest to zoom the display.
5. View and modify the data in the Well table:

Tool	Use this tool to...
Mouse/cursor	Select wells. To select: <ul style="list-style-type: none"> • An individual well, select the well in the Well table. • More than one well at a time, press the Ctrl key or Shift key when you select the wells in the Well table. When you select wells in the Well table, the corresponding data points are selected in the amplification plot.
Actions menu	Omit/Un-Omit well from the analysis. After you omit or un-omit a well, click Analyze to reanalyze the project. For omitted wells, the software: <ul style="list-style-type: none"> • Does not display data or tasks in the Well table (the Task column is empty/blank). • Does not include the omitted wells in the analysis. For un-omitted wells, the software reassigns the tasks based on the settings in the Analysis Settings dialog box.
◀ or ▶	Expand or collapse the Well table.

6. Review the data in the Well table data.

Column	Use this column to...
Omit	View the omission status of the related well.
Well	View the location of the well in the reaction plate. For example, P18 indicates that the sample is located in row P, column 18.
Target	View the ID (a unique name or number) of the nucleic acid sequence targeted by the assay added to the well.
Sample	View the ID (a unique name or number) of the sample.
Amp Status	View the amplification status for the well: amplification, no amplification, reviewed, and undetermined.
Amp Score	View the amplification score calculated for the well.
Cq Conf	View the Cq confidence score calculated for the well.
Task	View the task assigned to the well. A task is the function that a sample performs: <ul style="list-style-type: none"> • Unknown • Standard • No template control (NTC) (control identifier)
Quantity	View the calculated quantity for the sample at the related well.
Quantity Mean	View the arithmetic mean generated from the quantities calculated for the technical replicates of the well.
Quantity SD	View the standard deviation generated from the quantities calculated for the technical replicates of the well.
Ct	View the C _T calculated for the related well.
Ct Mean	View the arithmetic mean generated from the C _T s calculated for the technical replicates of the well.
Ct SD	View the standard deviation generated from the C _T s calculated for the technical replicates of the well.
Flags	View the number of flags generated for the well.
Quality data	View any quality flags generated by the associated well.

7. Review the amplification plots as needed (see About the Amplification Data on page 44 for more information on the plot and typical signal characteristics).

When reviewing the amplification data, look for:

- Regular, characteristic amplification of all samples. If irregular amplification is present, consider omitting the individual wells from the analysis.
- Correct baseline and threshold values. If not, consider manually adjusting the baseline and/or threshold values in the analysis settings.

8. When ready, click **Multicomponent** to review the multicomponent plot as needed (see “Multicomponent Plot” on page 43 for more information on the plot).

When reviewing the multicomponent plot, look for:

- Consistent fluorescence of the passive reference. The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
 - Consistent fluorescence of the 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.
 - Irregular fluorescence. There should not be any spikes, dips, and/or sudden changes in the fluorescence.
 - No amplification in negative control wells. There should not be any amplification in the negative control wells.
9. When ready, click **Standard Curve** to review the standard curve plot as needed (see “Standard Curve Plot” on page 44 for more information).
 10. When ready, click  to return to the thumbnails view.
 11. When ready, click  to review the quality summary.

Review the Quality Summary for any flags generated by the project data. For each quality flag, the table displays the number of times the flag was triggered by the project data. To examine the data that triggered the flag, click the link in the Name column to view the amplification data for the related target, sample, or plate.

Note: You can view the identity of any flag by hovering the mouse over the flag of interest.

In response to the presence of quality flags, consider the following resolutions:

- Change the quality settings in the analysis group:
 - Adjust the sensitivity of the quality flags so that more wells or fewer wells are flagged.
 - Deactivate the quality flags that triggered by the data.
- Omit individual wells from the analysis.

About the quality data summary

The quality summary displays a table of the experiments included in the current analysis and the number of quality flags generated by the associated data. To examine the data that triggered the flags, click the link in the Name column to view the amplification data for the related plate.

In response to the presence of quality flags, consider the following resolutions:

- Change the quality settings in the analysis group:
 - Adjust the sensitivity of the quality flags so that more wells or fewer wells are flagged.
 - Deactivate the quality flags that triggered by the data.
- Omit individual wells from the analysis.

Omit wells from the analysis

To omit the data from one or more wells that you do not want included in the analysis:

- Select one or more wells in a plot or table, then click **Actions** ▶ **Omit**. After the wells are omitted, click **Analyze** to reanalyze the project without the omitted well(s).

6

Export the results

■ Export the analyzed data from a project	36
■ Export project data as a slide presentation	37
■ Export plots for presentation and publication	38
■ Export data for use in other projects	39

After you are finished analyzing your project, you can use the Applied Biosystems™ Analysis Software to publish the project data.

Export the analyzed data from a project

The Applied Biosystems™ Analysis Software allows you to export project data as comma-separated or tab-delimited text, which can be imported by most spreadsheet applications for further analysis or presentation.

1. From the main menu of the project that contains data to export, click **Export**.
2. From the Export screen, click , then enter the following information:
 - a. Enter a name for the exported report in the Name field.

Note: Naming the report will allow you to repeat the export if you need to do so again.
 - b. Select the file type for the exported data:
 - **.txt** - To export data to a tab-delimited text file.
 - **.csv** - To export data to a comma-separated text file.
 - c. (*CSV and TXT exports only*) Select the check boxes for the data that you want to export.
 - **Results Data** - Exports the results from the standard curve analysis.
 - **Amplification Data** - Exports amplification results for each well in the project, such as cycle numbers, and Rn or ΔRn values.
 - **QC Summary** - Exports a summary of the quality metrics (flags) generated by the data analysis.
 - **Melt Data** - Exports the results from a melt analysis, including calculated Tm and call data, if one was included in the thermal cycling protocol.
3. If you want to customize the export to include specific data, click **Actions ▶ Customize**, then select the data columns that you want to export from each selected tables.

4. From the Export Details screen, select the fields from the data tables to include in the exported file, then click **Start Export**.
After starting the export, wait for the Applied Biosystems™ Analysis Software to generate the report. The export is complete when the Status column of the exported report displays "Download".
After generating the data export, the Applied Biosystems™ Software displays the package as a row in the Export History table.
5. (Optional) Click the entry in the Comments column, then enter any additional information for the exported report.
6. Click **Download**, select the location for the exported data file, then click **Save**.

Once generated, a data export package remains in the Export History indefinitely or until you remove it. To delete a package, select an export package from the table, then click **Actions** and select **Delete File(s)**.

Export project data as a slide presentation

The Applied Biosystems™ Analysis Software allows you to export your project data as a Microsoft™ PowerPoint® slide presentation. The exported file summarizes the project data and saves the exported file in a generic template that you can override by importing a Microsoft™ PowerPoint® template file.

1. From the main menu of the project that contains data to export, click **Export**.
2. From the Export screen, click , then enter the following information:
 - a. Enter a name for the exported report in the Name field.
Note: Naming the report will allow you to repeat the export if you need to do so again.
 - b. From the File type menu, select **.pptx**.
3. From the Export Details screen, select the fields from the data tables to include in the exported file, then click **Start Export**.
After starting the export, wait for the Applied Biosystems™ Analysis Software to generate the report. The export is complete when the Status column of the exported report displays "Download".
After generating the data export, the Applied Biosystems™ Software displays the package as a row in the Export History table.
4. (Optional) Click the entry in the Comments column, then enter any additional information for the exported report.
5. Click **Download**, select the location for the exported data file, then click **Save**.

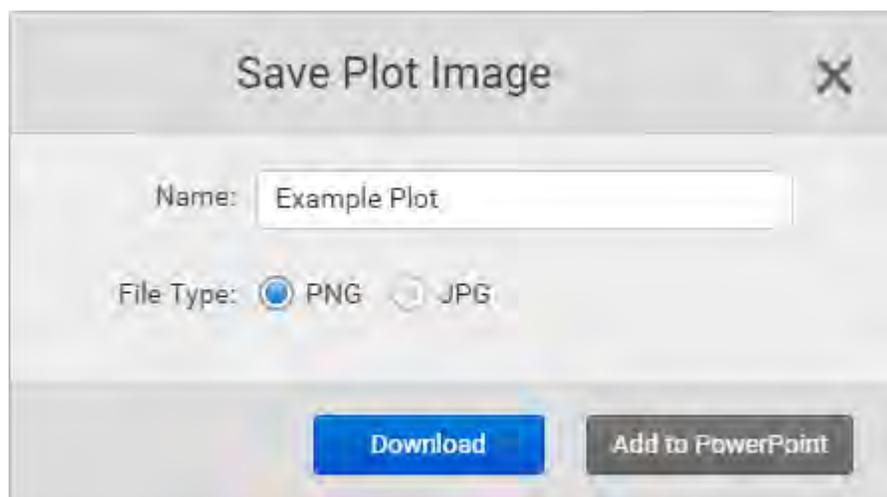
Once generated, a data export package remains in the Export History indefinitely or until you remove it. To delete a package, select an export package from the table, then click **Actions** and select **Delete File(s)**.

You can use the Microsoft™ PowerPoint® Application to reformat the exported slide presentation. For more information on applying a theme or template to your presentation, refer to the Microsoft™ PowerPoint® Help.

Export plots for presentation and publication

The Applied Biosystems™ Analysis Software allows you to export any plot as a Portable Network Graphics (.png) or Joint Photographic Expert Group (.jpg) file, which can be imported by most spreadsheet and desktop publishing software for presentation.

1. When viewing a plot, click  (Save as image) or select **Actions** ▶ **Save as Image**.
2. Save the image.
 - a. Click the File Name field, then enter a name for the exported graphics file.
 - b. Select the appropriate file format (.png or .jpg).
 - c. Click **Download** to download the plot image file, *or* click **Add to PowerPoint** to add the plot to an exported PowerPoint presentation (see “Export project data as a slide presentation” on page 37).



3. In the Save As dialog box, select the location for the exported data file, then click **Save**.

Export data for use in other projects

The Applied Biosystems™ Analysis Software allows you to export the following data from a project for use in other analyses.

- Export a template file

Template files contain plate layout information (target, sample, and task configurations) that you can use to easily set up experiments added to your projects. The Applied Biosystems™ Software allows you to export template files from existing experiments or to create them using a text editor or spreadsheet application.

- a. Open the project that includes the desired experiment, then select **Plate Setup**.
- b. From the Plate Setup screen, select the experiment record that contains the plate setup information of interest.
- c. From the Edit Plate screen, click **Actions ▶ Download Template**, then save the file to the desired location.

- Export the C_T settings

The C_T settings file associates targets with specific methods for calculating baseline and threshold values (manual or automatic). Once exported, you can use the file to import the settings to other projects (especially useful for projects involving large numbers of targets).

- a. Open the project that includes the analysis settings with the desired C_T settings, then select **Quality Control & Results**.
- b. From the Review Result screen, select the experiment that contains the C_T settings of interest.
- c. While viewing the experiment results, click **Analysis Setting**.

- d. From the Analysis Settings dialog box, click **Cq Settings** to view the Cq settings, then click **Export CT Settings**.

Target	Auto Threshold	Threshold	Auto Baseline	Baseline Start	Baseline End
18S-Hs99999901_s1	<input checked="" type="checkbox"/>	0.571	<input checked="" type="checkbox"/>	N/A	N/A
GAPD-Hs99999905_m1	<input checked="" type="checkbox"/>	0.317	<input checked="" type="checkbox"/>	N/A	N/A
GUSB-Hs99999908_m1	<input checked="" type="checkbox"/>	0.569	<input checked="" type="checkbox"/>	N/A	N/A
HPRT1-Hs99999909_m1	<input checked="" type="checkbox"/>	0.398	<input checked="" type="checkbox"/>	N/A	N/A
Hs00153809_m1	<input checked="" type="checkbox"/>	0.571	<input checked="" type="checkbox"/>	N/A	N/A

- e. In the Export CT Settings dialog box, select the format for the exported file (.txt or .csv), then click **OK**.
- f. Select the location and name for the exported file, then click **Save**.



Screens and plots

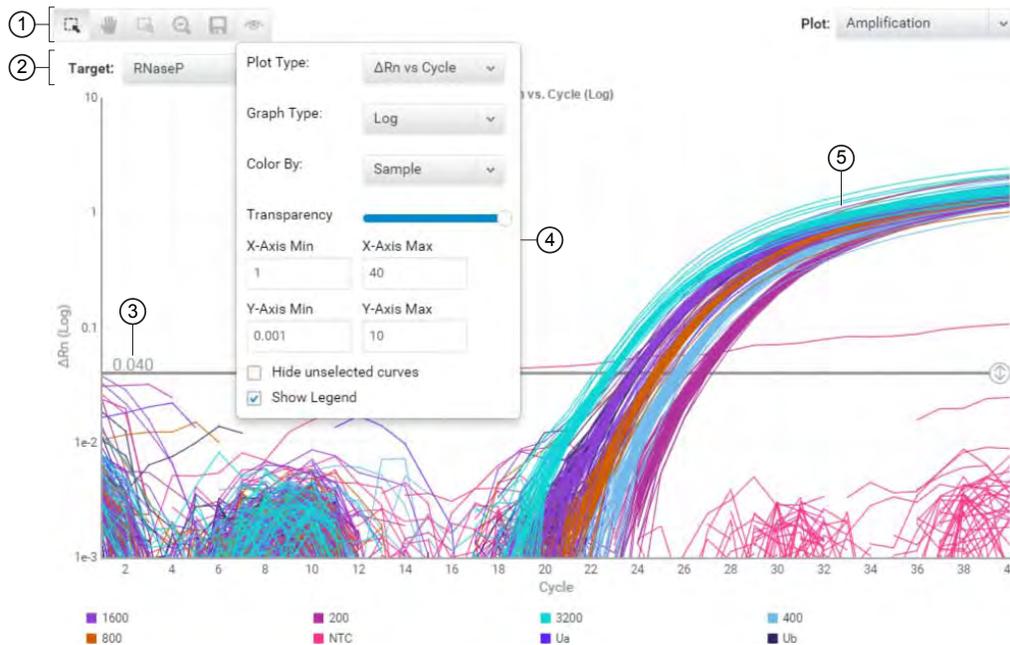
- Amplification Plot 42
- Multicomponent Plot 43
- Standard Curve Plot 44
- Well Table 46

The Applied Biosystems™ Analysis Software provides the following screens and plots that can be used to edit and visualize experiment setups and results that have been added to your project.

Amplification Plot

The Amplification Plot screen displays post-run amplification of the samples of each experiment added to your project. Three plots are available:

- **ΔRn vs Cycle** – ΔRn is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification ($\Delta Rn = Rn - \text{baseline}$). 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 (C_Q) 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).

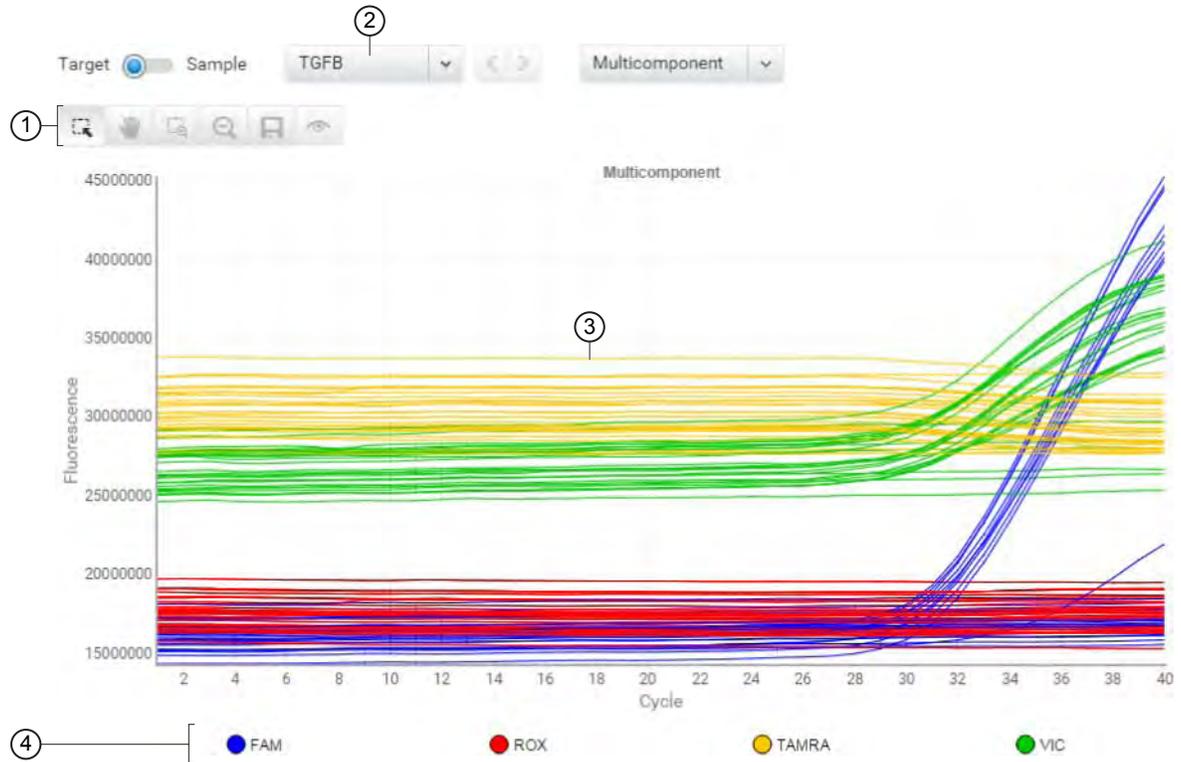


- 1 **Toolbar** – Contains the following tools for controlling the plot:
 - Select individual data points from the plot.
 - Allows you to click and manually move the position of the plot.
 - Zoom the plot to the selected area.
 - Zooms out the plot to show all data points.
 - Saves the plot as an image (.png or .jpg).
 - Allows you to adjust the display options for the plot.
- 2 **Target** drop-down list – Selects the target data displayed by the plot.

- 3 **Threshold** – The threshold (calculated or manual) that is currently applied to the project data.
- 4 **View Options** – The view options for the Amplification Plot. Use the drop-down lists to display the type of plot displayed by the software (**ΔRn vs Cycle**, **Rn vs Cycle**, or **C_T vs Well**), the scale of the y-axis (**log** or **linear**), and the color scheme for the plot.
- 5 **Amplification curves** – Normalized fluorescence for individual wells throughout the course of the thermal cycling protocol.

Multicomponent Plot

The Multicomponent Plot is a plot of the complete spectral contribution of each dye for the selected well(s) over the duration of the PCR run.



① **Toolbar** – Contains the following tools for controlling the plot:

- 🖱️ – Select individual data points from the plot.
- 👤 – Allows you to click and manually move the position of the plot.
- 🔍 – Zoom the plot to the selected area.
- 🔍 – Zooms out the plot to show all data points.
- 💾 – Saves the plot as an image (.png or .jpg).

👁️ – Allows you to adjust the display options for the plot.

② **Target/Sample** drop-down list – Selects the data from the target or sample data displayed by the plot.

③ **Normalized fluorescence** – Displays the normalized fluorescence for all wells throughout the duration of the thermal cycling protocol.

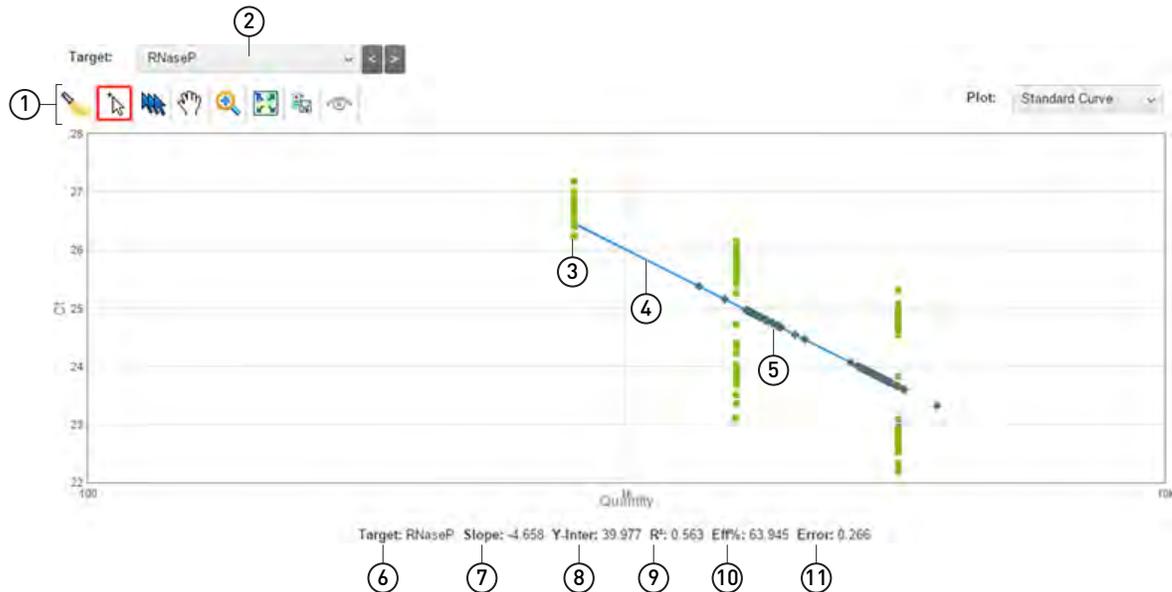
④ **Legend** – Fluorescent dyes present in the analyzed data.

When you analyze your own experiment, confirm the following:

- The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- 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.
- There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- There should not be any amplification in negative control wells.

Standard Curve Plot

The standard curve plot displays the best-fit line generated from the C_T values from the standards containing a range of known quantities. 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.



- ① **Toolbar** – Contains the following tools for controlling the plot:
 - Shows/hides the presence of the legend.
 - Select individual data points from the plot.
 - Selects all data points in the plot.
 - Allows you to click and manually move the position of the plot.
 - Zoom the plot to the selected area.
 - Zooms out the plot to show all data points.
 - Saves the plot as an image (.png or .jpg).
 - Allows you to adjust the display options for the plot.
- ② **Target** drop-down list – Selects the data from the target sequence displayed by the plot.
- ③ **Unknowns** – Data points for wells that contain samples with unknown quantities of the target sequence.
- ④ **Regression line** – The best-fit line from the standard curve, calculated as:

$$C_T = m [\log (Qty)] + b$$
 where m is the slope, b is the y-intercept, and Qty is the standard quantity.
- ⑤ **Standards** – Data points for wells that contain samples with known quantities of the target sequence.
- ⑥ **Target** – Name of the target sequence currently displayed in the plot.
- ⑦ **Slope** – The amplification efficiency is calculated using the slope of the regression line in the standard curve. A slope close to -3.3 indicates optimal, 100% PCR amplification efficiency. Factors that affect amplification efficiency:
 - **Range of standard quantities** – For more accurate and precise efficiency measurements, use a broad range of standard quantities, 5 to 6 logs (10^5 to 10^6 fold).
 - **Number of standard replicates** – For more accurate efficiency measurements, include replicates to decrease the effects of pipetting inaccuracies.
 - **PCR inhibitors** – PCR inhibitors in the reaction can reduce amplification efficiency.
- ⑧ **Y-Inter** – 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.
- ⑨ **R^2** – The R^2 (correlation coefficient) value is a measure of the closeness of fit between the regression line and the individual C_T data points of the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points. An R^2 value >0.99 is desirable.
- ⑩ **Eff%** – The calculation of the efficiency of the PCR amplification in a standard curve experiment. Eff% is calculated using the slope of the regression line in the standard curve, where a slope close to -3.32 indicates optimal, 100% PCR amplification efficiency.
- ⑪ **Error** – The calculated error for the

When you analyze your own standard curve experiment, review the C_T s of the :

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.

Column	Definition
Omit	The omission status of the related well.
Well	The location of the well in the reaction plate. For example, P18 indicates that the sample is located in row P, column 18.
Target	The ID (a unique name or number) of the nucleic acid sequence targeted by the assay added to the well.
Sample	The ID (a unique name or number) of the sample.
Amp Status	The amplification status for the well: <ul style="list-style-type: none"> • NOAMP – No significant amplification in the well. • AMP – Well exhibited a significant increase in fluorescent signal.
Amp Score	The amplification score calculated for the well.
Cq Conf	The calculated Cq (C_T) confidence score for the well.
Task	The task assigned to the well. A task is the function that a sample performs: <ul style="list-style-type: none"> • Unknown • Standard • No template control (NTC) (control identifier)
Quantity	The calculated quantity for the sample at the related well.
Quantity Mean	The arithmetic mean generated from the quantities calculated for the technical replicates of the well.
Quantity SD	The standard deviation generated from the quantities calculated for the technical replicates of the well.
Ct	The C_T calculated for the related well.
Ct Mean	The arithmetic mean generated from the C_T s calculated for the technical replicates of the well.
Ct SD	The standard deviation generated from the C_T s calculated for the technical replicates of the well.
Flags	The number of flags generated for the well.
Quality data	The number of quality flags generated by the associated well.

Well Table

The Well Table summarizes the analyzed data for a single experiment from the project. To view the Well Table, select **Quality Control & Results**, select an experiment of interest, then select **Well Table** from the View By drop-down list.

You can organize the contents of the well table as follows:

- Use the "Group By" table setting to group the data displayed within the table by sample, plate, or task. When grouped, select rows to evaluate subsets of the amplification data in the plot, which can be useful when reviewing amplification across replicate wells.
- Click a table column heading to *sort* the contents (or click  in the header, then select  or ). The presence of an arrow ( or ) in the column header indicates the direction of the sort.
- Click  in a column header, then click  and select a parameter to *filter* the contents. When filtered, click **Clear** to remove the filter from the table.
- Click  in any column header, then click  and select the columns that you want to *show* or *hide*.
- Click  in a column header, then click  (or ) to *lock* (or *unlock*) the horizontal position of the column within the table. When a column is unlocked, you can click and drag the column header to reposition the column within the table.

Column	Definition
Omit	The omission status of the related well.
Well	The location of the well on the reaction plate. For example, P18 indicates that the sample is located in row P, column 18.
Target	The ID (a unique name or number) of the nucleic acid sequence targeted by the assay added to the well.
Sample	The ID (a unique name or number) of the sample.
Amp Status	The amplification status for the well: <ul style="list-style-type: none"> • NOAMP – No significant amplification in the well. • AMP – Well exhibited a significant increase in fluorescent signal.
Amp Score	The amplification score calculated for the well.
Cq Conf	The calculated Cq (C _T) confidence score for the well.
Task	The task assigned to the well. A task is the function that a sample performs: <ul style="list-style-type: none"> • Unknown • Standard • No template control (NTC) (control identifier)
Quantity	The calculated quantity for the sample at the related well.
Quantity Mean	The arithmetic mean generated from the quantities calculated for the technical replicates of the well.

Column	Definition
Quantity SD	The standard deviation generated from the quantities calculated for the technical replicates of the well.
Ct	The C_T calculated for the related well.
Ct Mean	The arithmetic mean generated from the C_T s calculated for the technical replicates of the well.
Ct SD	The standard deviation generated from the C_T s calculated for the technical replicates of the well.
Flags	The number of flags generated for the well.
Quality data	The number of quality flags generated by the associated well.



Quality flags

- AMPNC (Amplification in negative control) quality flag 49
- AMPSCORE (Low signal in linear phase) quality flag 49
- BADROX (Bad passive reference signal) quality flag 50
- BLFAIL (Baseline algorithm failed) quality flag 50
- CQCONF (Calculated confidence in the Cq value is low) quality flag 51
- CRTAMPLITUDE (Broad Cq Amplitude) quality flag 51
- CRTNOISE (Cq Noise) quality flag 51
- CTFAIL (Cq algorithm failed) quality flag 51
- DRNMIN (Detection of minimum DRn due to abnormal baseline)
quality flag 52
- EXPFAIL (Exponential algorithm failed) quality flag 52
- HIGHSD (High standard deviation in replicate group) quality flag 53
- LOWROX (Low ROX™ Intensity) quality flag 54
- MTP (Melt curve analysis shows more than one peak) quality flag 54
- NOAMP (No amplification) quality flag 54
- NOISE (Noise higher than others in plate) quality flag 55
- NOSAMPLE (No sample assigned to well) quality flag 55
- NOSIGNAL (No signal in well) quality flag 55
- OFFSCALE (Fluorescence is offscale) quality flag 56
- OUTLIERRG (Outlier in replicate group) quality flag 57
- PRFDROP (Passive reference signal changes significantly near the
Cq/Ct) quality flag 57
- PRFLOW (Average passive reference signal is below the threshold)
quality flag 58
- SPIKE (Noise spikes) quality flag 59
- THOLDFAIL (Thresholding algorithm failed) quality flag 59

AMPNC (Amplification in negative control) quality flag

The AMPNC () quality flag indicates that a sequence in a negative control reaction amplified.

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. Make sure that the well corresponds to a negative control well (Task = Negative Control or NTC).
3. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and confirm the fluorescence signal increased for the flagged negative control well. If the fluorescence signal did not increase, omit the well from analysis.

Possible Cause	Recommended Action
Contamination in one or more PCR reaction components	<ul style="list-style-type: none"> • Replace all PCR reaction components with new components, then repeat the experiment. Make sure to add water or buffer instead of sample to the well. • Decontaminate the work area and pipettors.
Unstable reaction mix	<ul style="list-style-type: none"> • Use a hot-start enzyme. • If you are not using a hot-start enzyme, run the reactions as soon as possible after you prepare them.
Poor primer and/or probe design	Redesign the primers and/or probe.

AMPSCORE (Low signal in linear phase) quality flag

The AMPSCORE () quality flag indicates that, for a given well, the amplification in the linear region is below a certain threshold, corresponding to the score set in the analysis settings.

Use the AMPSCORE flag to easily identify and, optionally, omit potentially poor results without manually inspecting every amplification curve. The numeric value for the amplification score is found in the Amp Score column of the well table for the amplification and multicomponent plots.

Note: For Quantitative or Genotyping applications, this flag is only appropriate when ROX™ dye is used as the passive reference or the data is from OpenArray™ plates. For Absolute Quantification applications, this flag is only appropriate when ROX™ dye is used as the passive reference.

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. Make sure that the well does not correspond to a negative-control (NTC) well.
3. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and check the shape of the curve. If the curve is atypical, consider omitting the flagged well(s) from analysis.

BADROX (Bad passive reference signal) quality flag

The BADROX  quality flag indicates that the passive reference (usually ROX™ dye) signal is abnormal. The passive reference signal may not be acceptable for normalization of the reporter dye signal.

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the multicomponent plot, and review the passive reference signal for abnormalities.
3. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and review the data in the C_q region for abnormalities.
4. Examine the reaction plate, and check for condensation and/or inconsistent reaction volumes.

Possible Cause	Recommended Action
Droplets on the sides of the wells.	Repeat the experiment, and make sure you centrifuge the plate briefly before loading it into the instrument.
Evaporation resulting from improper sealing or seal leaks.	Repeat the reactions, and make sure you seal the plate properly.
Condensation on the reaction plate.	
Inconsistent volumes across the plate.	Confirm that pipettes are calibrated and functioning properly.
Incorrect concentration of reference dye.	Confirm that you are using the appropriate master mix for your instrument.
Pipetting errors.	Calibrate your pipettors, then repeat the experiment.

BLFAIL (Baseline algorithm failed) quality flag

Note: The BLFAIL flag is only valid when you use the Baseline Threshold algorithm to analyze your experiments, though it is always shown in the QC Summary.

The BLFAIL  quality flag indicates that the automatic baseline algorithm failed, and the software cannot calculate the best-fit baseline for the data.

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and check for late amplification or no amplification.
3. If the amplification looks acceptable, set the baseline manually.
4. Click **Analyze** to reanalyze the data.
5. Evaluate the results and, if needed, make any additional changes to the baseline.

CQCONF (Calculated confidence in the C_q value is low) quality flag

The CQCONF (cc) quality flag indicates that the calculated confidence for the C_q/C_T value of the well is less than the minimum value defined in the analysis settings.

Use the CQCONF flag to easily identify and, optionally, omit potentially poor results without manually inspecting every amplification curve. The minimum limit is set in the Flag Settings tab of the Analysis Settings dialog box.

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and check the shape of the curve. If the curve is atypical, consider omitting the flagged well(s) from analysis.

CRTAMPLITUDE (Broad C_q Amplitude) quality flag

The CRTAMPLITUDE (ca) quality flag indicates that the amplitude of the relative standard curve, generated from the data set that includes the given well, is significantly lower than the other curves generated for the related target.

CRTNOISE (C_q Noise) quality flag

The CRTNOISE quality flag indicates that for the relative standard curve, generated from the data set that includes the given well, exhibited a significant amount of unexplained variability in comparison to the other curves generated for the related target.

CTFAIL (C_q algorithm failed) quality flag

Note: The CTFAIL flag is only valid when you use the Baseline Threshold algorithm to analyze your experiments, though it is always shown in the QC Summary.

The CTFAIL (f) quality flag indicates that the automatic C_q algorithm failed for the given well, and the software cannot calculate the threshold cycle (C_q).

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)] and check for:
 - Amplification too early
 - Amplification too late
 - Low amplification
 - No amplification
3. If the amplification looks acceptable, set the threshold and baseline manually.

4. Click **Analyze** to reanalyze the data.
5. Evaluate the results. If the adjustments do not produce a valid C_q , consider omitting the well from analysis.

DRNMIN (Detection of minimum ΔR_n due to abnormal baseline) quality flag

The DRNMIN () quality flag indicates that the normalized fluorescence (ΔR_n) for a given well dropped below the threshold defined in the analysis settings.

Use the DRNMIN flag to easily identify and, optionally, omit potentially poor results without manually inspecting every amplification curve. The ΔR_n threshold value is set in the Flag Settings tab of the Analysis Settings dialog box.

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification and multicomponent plots [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and check the shape of the curve. If the curve is atypical, consider omitting the flagged well(s) from analysis.

EXPFAIL (Exponential algorithm failed) quality flag

Note: The EXPFAIL flag is only valid when you use the Baseline Threshold algorithm to analyze your experiments, though it is always shown in the QC Summary.

The EXPFAIL () quality flag indicates that the automatic C_q algorithm failed for the given well, and the software cannot identify the exponential region of the amplification plot.

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and check for:
 - Amplification too early
 - Amplification too late
 - Low amplification
 - No amplification
3. If the amplification looks acceptable, set the threshold manually:
 - a. Click the threshold (the horizontal line across the plot) and drag it up or down to a location within the exponential region of the amplification.
 - b. Click **Analyze** to reanalyze the data.
 - c. Evaluate the results and, if needed, make any additional changes to the threshold.

HIGHSD (High standard deviation in replicate group) quality flag

The HIGHSD () quality flag indicates that the C_q standard deviation for the replicate group exceeds the current flag setting (all replicates in the group are flagged).

If a replicate group is flagged, confirm the results:

1. Select the flagged replicate group in the plate layout or well table.
2. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and evaluate whether the signal varies significantly from others in the replicate group. If so, omit the outlier well(s) or omit the entire replicate group from analysis.
3. Only for experiments analyzed with the Baseline Threshold algorithm, if the amplification looks acceptable, set the threshold manually and reanalyze the data:
 - a. Click the threshold (the horizontal line across the plot) and drag it up or down to a location within the exponential region of the amplification.
 - b. Click **Analyze** to reanalyze the data.
 - c. Evaluate the results, and if needed, make any additional changes to the threshold.

Possible Cause	Recommended Action
Droplets on the sides of the wells.	Repeat the experiment, and make sure you centrifuge the plate briefly before loading it into the instrument.
Improper sealing or seal leaks.	Repeat the reactions, and make sure you seal the plate properly.
Condensation on the reaction plate.	
Inconsistent volumes across the plate.	
Pipetting errors.	Calibrate your pipettors, then repeat the experiment.
Missing reaction component.	Repeat the experiment, and make sure to include all reaction components. Try not to pipet less than 5 μ L of sample when setting up the PCR.
Incorrect reaction setup.	Make sure you follow the manufacturer's instructions for setting up the reactions.
Poor DNA template.	Repeat the experiment with higher quality template.
Inadequate mixing	Mix the reaction thoroughly by pipetting or using a medium setting on a vortex mixer.

LOWROX (Low ROX™ Intensity) quality flag

A Low ROX™ Intensity () quality flag can be raised for any data point. If the ROX™ dye intensity determined by the software for a data point is below the threshold, a flag will be raised.

If a well is flagged, no action should be taken for the data point. If the ROX™ dye intensity is below the default threshold, the data point does not meet the minimum conditions for assigning a call.

MTP (Melt curve analysis shows more than one peak) quality flag

The MTP () quality flag indicates that the melt curve generated from the collected data exhibits multiple peaks, indicating possible PCR irregularities such as contamination or nonspecific amplification.

Note: The MTP flag is present only in experiments with PCR methods that include a melting curve stage (a temperature ramp configured for data collection).

If a replicate group is flagged, confirm the results in the Melt Curve Plot. Peaks in the melt curve can indicate the melting temperature (T_m) of a target nucleic acid or nonspecific PCR amplification.

NOAMP (No amplification) quality flag

The NOAMP () quality flag indicates that the sample did not amplify.

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. Make sure that the well does not correspond to a negative-control well.
3. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and make sure that the fluorescence signal did not increase in the well.
4. View the multicomponent plot, and look for fluorescence signal higher than the background.

Possible Cause	Recommended Action
Missing template.	Repeat the experiment, and make sure to include all reaction components.
Target is not expressed in the sample.	<ul style="list-style-type: none"> • If this occurs in just one sample, it may be correct. • If this occurs in all samples of a particular tissue, search the literature for evidence that the target is expressed in the tissue or sample type of interest.

NOISE (Noise higher than others in plate) quality flag

The NOISE (🔊) quality flag indicates that the well produced more noise in the amplification plot than the other wells on the same plate.

If a well is flagged, confirm the results:

1. Select the flagged well(s) and some unflagged unknown wells in the plate layout or well table.
2. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)] and check for a noisy amplification curve in the flagged wells.
3. In the multicomponent plot:
 - a. From the Color by drop-down list, select Dye to color the data according to the dye.
 - b. Check for a drop in ROX™ signal relative to the reporter dye and compare flagged wells with unflagged wells.
 - c. If there is a drop in the ROX™ signal compared to the reporter dye, consider omitting the flagged well(s) from analysis.

NOSAMPLE (No sample assigned to well) quality flag

The NOSAMPLE (🚫) quality flag indicates that no sample is assigned to the well.

In the Applied Biosystems™ Analysis Software, omit the well missing the sample, then click **Analyze** to reanalyze the project.

NOSIGNAL (No signal in well) quality flag

The NOSIGNAL (🚫) quality flag indicates that the well produced very low or no fluorescence signal.

If a well is flagged, confirm the results:

1. Select the flagged well(s) and a few unflagged wells in the plate layout or well table.
2. View the multicomponent plot and compare the flagged well(s) to the unflagged wells:
 - If the fluorescence signals for all dyes are low and similar to the instrument's background signal, the well is empty.
 - If the fluorescence signals are higher than the instrument's background signal and constant throughout the instrument run, no amplification occurred.
3. If the flagged well produced no fluorescence signal, omit the well from analysis.
4. If you still have the plate that was run, note the location for each flagged well, and check each corresponding well in the reaction plate for low reaction volume.

OFFSCALE (Fluorescence is offscale) quality flag

The OFFSCALE () quality flag indicates that the fluorescence signal for one or more dyes in the well exceeds the instrument's maximum detectable range for one or more cycles.

Confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)] or the well table, and note the threshold cycle.
3. View the multicomponent plot, and review the data for a plateau over one or more cycles. A plateau indicates saturation of the instrument's detectors. If the signal plateaus before the threshold cycle, omit the well(s).

Possible Cause	Recommended Action
Too much TaqMan™ probe or SYBR™ Green dye added to the reaction.	Reduce the concentration of reagent added to the reaction.
Fluorescent contaminant on the reaction plate, sample block, or adhesive cover.	Perform a background calibration. If you detect fluorescent contamination, clean the block.
Fluorescent contaminant in the reaction.	Replace the reagents.

OUTLIERRG (Outlier in replicate group) quality flag

The OUTLIERRG (RD) quality flag indicates that the C_q for the well deviates significantly from values in the associated replicate group (only the outlier is flagged).

Outlier removal is based on a modified Grubb's test. For a well to be considered an outlier, it must be identified as an outlier by Grubb's test and its C_q value must be a minimum of 0.25 cycles from the mean.

If a well is flagged, confirm the results:

1. Select the flagged well(s) and the associated replicate group in the plate layout or well table.
2. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and compare the data from the flagged well to the data from the unflagged replicates. If the C_q or the amplification curve for the flagged well vary significantly, carefully consider omitting the flagged well from analysis.

Possible Cause	Recommended Action
Pipetting errors.	Repeat the reactions, and follow these guidelines to reduce pipetting errors: <ul style="list-style-type: none"> • Prepare enough master reaction mix for the entire replicate group, then transfer aliquots to all appropriate wells in the reaction plate. • Calibrate and service your pipettors regularly. • Pipette larger volumes. • Reduce the number of pipetting steps.
Contamination in that well.	Replace all reagents, then repeat the experiment.
Decontaminate the work area and pipettors.	Repeat the reactions, and make sure you seal the reaction plate properly.
Improper sealing or seal leaks.	

PRFDROP (Passive reference signal changes significantly near the C_q/C_t) quality flag

The PRFDROP (PD) quality flag indicates that the florescent signal from the passive reference changes significantly within defined range around the calculated C_q/C_T for a given well.

Use the PRFDROP flag to easily identify and, optionally, omit potentially poor results without manually inspecting every amplification curve. The limits of the range are defined by a detection threshold that is set in the Flag Settings tab of the Analysis Settings dialog box. The flag is triggered when the passive reference signal for a well changes within the number of cycles (+/-) defined by the setting from the calculated C_q/C_T .

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and check the shape of the curve. If the curve is atypical, consider omitting the flagged well(s) from analysis.

PRFLOW (Average passive reference signal is below the threshold) quality flag

The PRFLOW (PL) quality flag indicates that, for the replicate group of a given well, the average passive reference signal is below the minimum allowed value.

Use the PRFLOW flag to easily identify and, optionally, omit potentially poor results without manually inspecting every amplification curve. The minimum allowed value is set in the Flag Settings tab of the Analysis Settings dialog box.

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification and multicomponent plots [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and check the shape of the curve. If the curve is atypical, consider omitting the flagged well(s) from analysis.

SPIKE (Noise spikes) quality flag

The SPIKE (S^{PK}) quality flag indicates that the amplification curve for the given well contains one or more data points inconsistent with the other points in the curve.

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and evaluate whether the noise spike adversely affects the baseline or C_q .
3. If the baseline is adversely affected, set the baseline and threshold values manually.
4. Click **Analyze** to reanalyze the data.
5. Evaluate the results. If the adjustments do not produce a valid C_q , consider omitting the well from analysis.

Possible Cause	Recommended Action
Bubbles in the reaction.	Repeat the reactions, and make sure you centrifuge the plate for 2 minutes at <1500 rpm and confirm that the liquid in each well of the plate is at the bottom of the well.
Overall low signal for all dyes in the reaction.	Repeat the reactions, pipetting a larger volume into all wells.
ROX™ dye not used as passive reference.	Repeat the reactions, using ROX™ dye as the passive reference.
Evaporation due to improper sealing or seal leaks.	Repeat the reactions, and make sure you seal the reaction plate properly.

THOLDFAIL (Thresholding algorithm failed) quality flag

Note: The THOLDFAIL flag is only valid when you use the Baseline Threshold algorithm to analyze your experiments, though it is always shown in the QC Summary.

The THOLDFAIL (T^{HF}) quality flag indicates that the automatic C_q algorithm failed, and the software cannot calculate the threshold for the given well.

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification plot [ΔR_n vs. Cycle (Linear) or ΔR_n vs. Cycle (Log)], and check for:
 - Amplification too early
 - Amplification too late
 - Low amplification
 - No amplification for all wells with this target
3. If the amplification looks acceptable, set the baseline and threshold manually.



Chapter 8 Quality flags

THOLDFAIL (Thresholding algorithm failed) quality flag

4. Click **Analyze** to reanalyze the data.
5. Evaluate the results and, if needed, make any additional changes to the threshold or baseline.



Documentation and support

Customer and technical support

Visit thermofisher.com/support for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
 - Product FAQs
 - Software, patches, and updates
- Order and web support
- Product documentation, including:
 - User guides, manuals, and protocols
 - Certificates of Analysis
 - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

Life Technologies Corporation and/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.thermofisher.com/us/en/home/global/terms-and-conditions.html. If you have any questions, please contact Life Technologies at www.thermofisher.com/support.

Glossary

assay information files	<p>Assay information files are delivered on Information CDs that accompany TaqMan™ assay orders. Each assay information file contains reference information about the associated order and technical details of all assays in the shipment.</p> <p>You can import an assay information file into the Applied Biosystems™ Analysis Software to add supplementary assay information to a project. Assay information files are available in three formats (.html, .txt, and .xml), but the Applied Biosystems™ Analysis Software supports only .txt and .xml files.</p> <hr/> <p>IMPORTANT! The assay information file must include an assay ID (in the Assay ID column) for each assay listed in the file. The software matches the assay IDs in the assay information file with the existing assay IDs in the project.</p> <hr/> <p>IMPORTANT! When you import an assay information file, information from the file populates the corresponding columns in the Assays list in the Overview screen. All data in the Overview screen are replaced for all assays that are identified in the assay information file. If the assay information file does not contain information for an assay, the existing data in the Overview screen is unaffected.</p> <hr/>
amplification efficiency (EFF%)	<p>Calculation of the efficiency of the PCR amplification in a standard curve 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. To use amplification efficiency in a gene expression project:</p> <ul style="list-style-type: none">• On the instrument where you collected the comparative C_T ($\Delta\Delta C_T$) data that will be used in the project, run a standard curve experiment to determine the efficiency.• In the Applied Biosystems™ Analysis Software, enter the amplification efficiency in the Efficiency table in the Relative Quantification Settings tab in the Analysis Settings dialog box.
amplification plot	<p>Display of data collected during the cycling stage of PCR amplification. The amplification plot can be viewed as:</p> <ul style="list-style-type: none">• Baseline-corrected normalized reporter (ΔR_n) vs. cycle• Normalized reporter (R_n) vs. cycle
analysis group	<p>An analysis group is a project setting that allows you to create a profile of the analysis and quality settings for the analysis of a project. Analysis groups can be applied either globally to analyze an entire project, or exclusively to a subset of the experiments or samples added to a project. Later in the analysis, the Applied Biosystems™ Analysis Software allows you to switch between analysis groups so that you can compare the effects of changes to the analysis settings on your results.</p>

assays	A PCR reaction mix that contains primers to amplify a target and a reagent to detect the amplified target.
automatic baseline	An analysis setting for the Baseline Threshold algorithm in which the software identifies the start and end cycles for the baseline in the amplification plot.
automatic threshold	An analysis setting for the Baseline Threshold algorithm 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).
baseline	In the amplification plot, the baseline is a cycle-to-cycle range that defines background fluorescence. This range can be set manually on a target-by-target basis, or automatically, where the software sets the baseline for each individual well.
Baseline Threshold algorithm	Expression estimation algorithm (C_q) which subtracts a baseline component and sets a fluorescent threshold in the exponential region for quantification.
baseline-corrected normalized reporter (ΔR_n)	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 ΔR_n vs Cycle amplification plot, ΔR_n is calculated at each cycle as: ΔR_n (cycle) = R_n (cycle) - R_n (baseline), where R_n = normalized reporter
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 experiment uses biological replicate groups in a gene expression project, 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 C_q computations (normalizing by the endogenous control) in a singleplex experiment, the software averages technical replicates. The averages from the technical replicates are then averaged together to determine the value for that biological replicate.
cycle threshold	See threshold cycle (C_T).
cycling stage	See threshold cycle (C_T).
C_T	See threshold cycle (C_T).
C_T algorithm	See Baseline Threshold algorithm.
EFF%	See amplification efficiency (EFF%).
efficiency correction	A feature in the software that mathematically compensates for differences in amplification efficiency of the targets and endogenous controls when calculating relative quantities. The software requires you to enter amplification efficiencies to perform the correction.

To use efficiency correction in a gene expression project:

- On the instrument where you collected the comparative C_T ($\Delta\Delta C_T$) data that will be used in the project, run a standard curve experiment to determine the efficiency.
- In Applied Biosystems™ Analysis Software, enter the amplification efficiency in the Efficiency table in the Relative Quantification Settings tab in the Analysis Settings dialog box.

flag	A quality control (QC) indicator which, when applied by the software to a well during analysis, indicates a possible issue with that reaction. A summary of the flags identified in the project is displayed in the Flag Summary screen.
manual baseline	An analysis setting for the Baseline Threshold algorithm in which you enter the baseline start and end cycles for the amplification plot for a target. If you edit the baseline start and end cycles, the settings are applied to all instances of that target in the project.
manual threshold	An analysis setting for the Baseline Threshold algorithm in which you enter the threshold value and select whether to use automatic baseline or manual baseline values. The software uses the baseline and threshold values to calculate the threshold cycle (C_q).
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)	See no template control (NTC).
no template control (NTC)	In the software, the task for targets in wells that contain water or buffer instead of sample. No amplification should occur in negative control wells. Also called negative control (NC).
nonfluorescent quencher-minor groove binder (NFQ-MGB)	Molecules that are attached to the 3' end of TaqMan™ MGB 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 moiety (MGB) increases the melting temperature (T_m) without increasing probe length. It also allows the design of shorter probes.
normalized reporter (Rn)	Fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference (usually ROX™ dye).
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 data point that deviates significantly from the values of an associated group (for example, the other technical replicates for a 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 grid (plate layout)	An illustration of the grid of wells and assigned content in the reaction plate, array card, or OpenArray™ plate. The number of rows and columns in the grid depends on the plate or card that you use. In the software, you can use the plate grid to view well assignments and results. The plate grid can be printed, included in a report, exported, and saved as a slide for a presentation.
projects	The Applied Biosystems™ Analysis Software organizes the analysis of experiment data by project, which represents the association of the raw data, all experimental setup information, and any associated settings used to perform the analysis. Once created, projects can be shared with other users and transferred to/from the repository. Note: Projects do not contain the data from experiments uploaded to the repository; they link the data for analysis without affecting the original data files.
quantity	In quantification experiments, the amount of target in the samples. 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™ probes, a nonfluorescent quencher-minor groove binder (NFQ-MGB) can be used as the quencher.
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. Rejected wells contain results calculated up to the point of rejection.
replicates	Identical reactions containing identical components and 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.
Rn	See <i>normalized reporter (Rn)</i> .
ROX dye	A dye used as the passive reference.
run method	The reaction volume and the thermal profile (thermal cycling parameters) for the instrument run.
sample	The biological tissue or specimen that you are testing for a target gene.

sample design file	A tab-or comma-delimited file (*.txt or *.csv) that contains a list of sample names and the corresponding name of the biological group to which each sample belongs.
target	The nucleic acid sequence to amplify and detect.
task	In the software, the type of reaction performed in the well for the target. Available tasks include:
technical replicates	Reactions that contain identical components and volumes, and that evaluate the same sample; important for evaluating precision.
thermal profile	The part of the run method that specifies the temperature, time, ramp, number of cycles, and data collection points for all steps and stages of the instrument run.
threshold	In amplification plots, the threshold is the level of fluorescence above the baseline and within the exponential amplification region. For the Baseline Threshold algorithm, the threshold can be determined automatically (see <i>automatic threshold</i>), or it can be set manually (see <i>manual threshold</i>).
threshold cycle (C _T)	The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.
unknown	In the software, the task for the target in wells that contain the sample being tested. For quantification experiments, the unknown task is assigned to wells that contain a sample with unknown target quantities.

For support visit thermofisher.com/support or email techsupport@lifetech.com
thermofisher.com

21 March 2016

ThermoFisher
SCIENTIFIC