applied biosystems

Applied Biosystems™ Relative Quantitation Analysis Module

USER GUIDE

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

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1 Getting Started

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.

Getting started

The Applied Biosystems™ Analysis Software supports both the Comparative C_T ($\Delta\Delta C_T$) and Relative Standard Curve methods of relative quantitation of gene expression.

Comparative C_T ($\Delta\Delta C_T$) method

The comparative C_T ($\Delta\Delta C_T$) method is used to determine the relative target quantity in samples. With the comparative C_T method, the Applied Biosystems™ Analysis Software measures amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the endogenous control. The software determines the relative quantity of target in each sample by comparing normalized target quantity in each sample to normalized target quantity in the reference sample.

Comparative C_T experiments are commonly used to:

- Compare expression levels of a gene in different tissues.
- Compare expression levels of a gene in a treated sample vs. an untreated sample.
- Compare expression levels of wild-type alleles vs. mutated alleles.

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

- **Sample** – The sample in which the quantity of the target is unknown.
- **Reference sample** – The sample used as the basis for relative quantitation results. For example, in a project of drug effects on gene expression, an untreated control would be an appropriate reference sample. Also called calibrator.
- **Endogenous control** – A target or gene that should be expressed at similar levels in all samples you are testing. The endogenous control is used to normalize fluorescence signals for the target you are quantifying. Housekeeping genes can be used as endogenous controls.
- **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.

Relative standard curve method

The Relative Standard Curve method is used to determine relative target quantity in samples using a standard curve as the basis for comparison. With the relative standard curve method, the instrument measures the amplification of the target and endogenous control within unknown samples, a reference sample, and in a standard dilution series. During the analysis, the measurements are normalized using the endogenous control, then data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates the quantities of the target and endogenous control in the unknown and reference samples. For each sample, the target quantity is normalized by the endogenous control quantity (quantity of target/quantity of endogenous control). The normalized quotient from each sample is divided by the quotient from the reference sample to obtain the relative quantification (fold change). The software determines the relative quantity of target in each sample by comparing target quantity in each sample to target quantity in the reference sample.

Relative Standard Curve experiments are commonly used to:

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

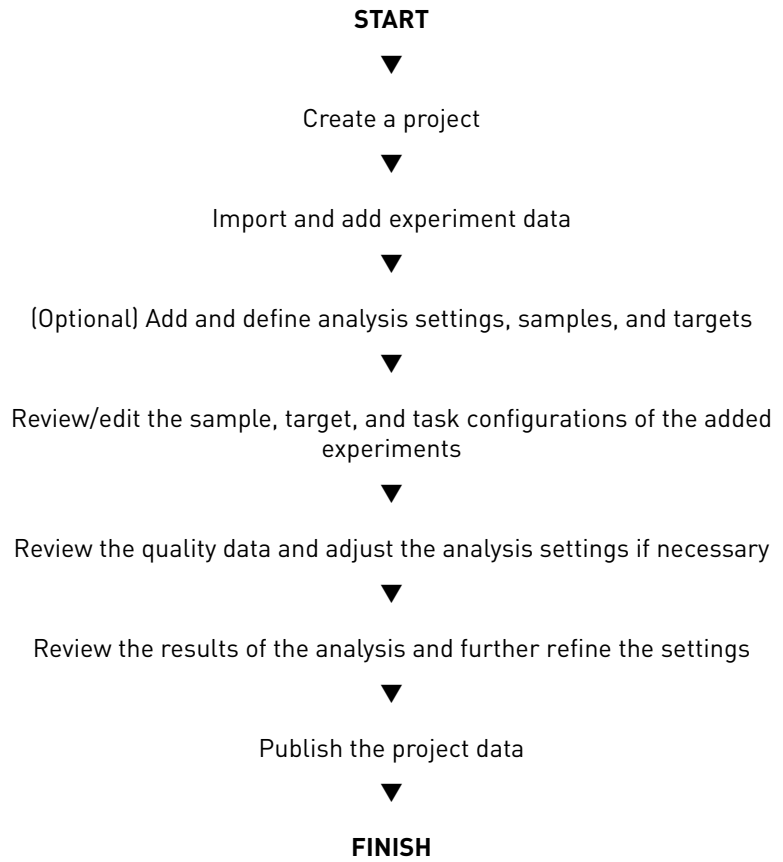
The following components are required to perform a relative standard curve analysis and must be present on all experiments added to the project:

- **Sample** – The tissue group that you are testing for a target gene.
- **Reference sample** (also called a calibrator) – The sample used as the basis for relative quantification results. For example, in a study of drug effects on gene expression, an untreated control is an appropriate reference sample.
- **Standard** – A sample that contains known quantities of the target; used in quantification experiments to generate standard curves.
- **Standard dilution series** – A set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards.

- **Endogenous control** – A gene that is used to normalize template input differences, and sample-to-sample or run-to-run variation.
- **Replicates** – The total number of identical reactions containing identical components and identical volumes.
- **Negative Controls** – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in the negative control wells.

Analysis workflow

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



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	• 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	• 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.

Compatible Real-Time PCR System Data

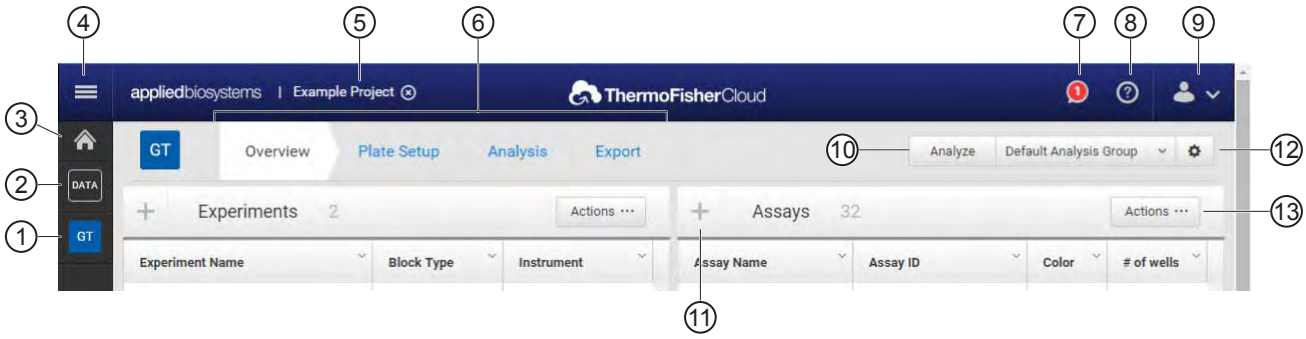
The Applied Biosystems™ Analysis Software can import and analyze data generated by any of the supported instruments listed in the following table. The software versions listed in the table represent only those tested for use with the Applied Biosystems™ Software. Data generated by versions other than 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	
	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		

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 (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 **Settings** – Click to edit the analysis settings for the project.

⑬ **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

2 Manage your projects and experiment data

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	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	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 DATA (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	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	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.

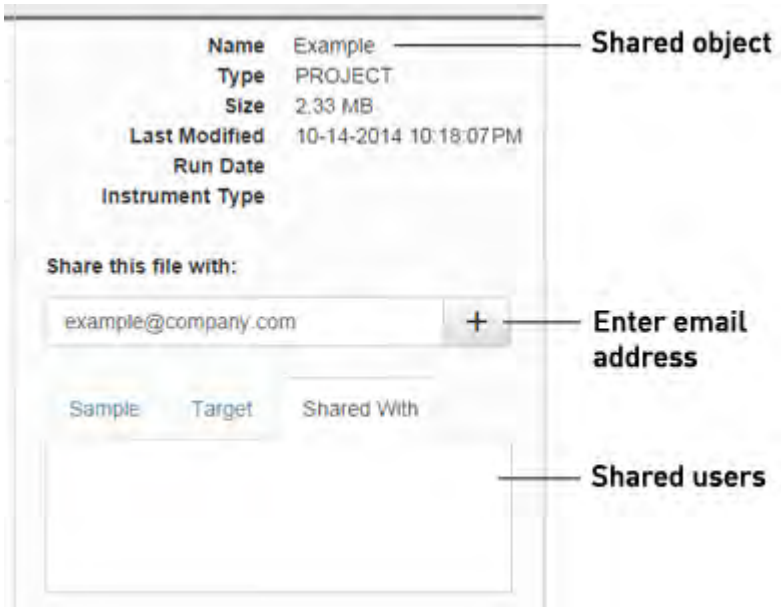
  IMPORTANT! A project is locked (preventing access) when it is open (in use) by any user with shared access to the project. For example, User A shares a project with two colleagues (User B and User C), User B opens the project and begins data analysis (the project is locked and unavailable to Users A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

3 Set up the project

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

Create or edit an analysis group

When a project is created, the Applied Biosystems™ Analysis Software generates the default analysis group from the analysis settings of the experiments added to the project. If desired, you can create additional analysis groups to explore different analysis setting configurations (for example, manual versus automatic thresholding, stringent versus relaxed quality thresholds, etc).

1. From the Analysis Groups table in the Overview screen, do one of the following:
   - Select **Actions ▸ Add** to create a new analysis group.
   - Select an existing group, then select **Actions ▸ Edit Analysis Settings**. Go to step 4.
2. From the Analysis Settings dialog box, enter the following information, then click **Next**.
   - **Group Name** – Enter a name for the analysis group (up to 50 characters).
   - (*Optional*) **Description** – Enter a description for the analysis group (up to 256 characters).
   - **Samples** or **Experiments** – Select the option to determine the basis by which the Applied Biosystems™ Software will apply the analysis group.

     For example, by selecting "Sample", the software allows you to apply the analysis group to a subset of the samples within the project. Conversely, by selecting "Experiment", the software allows you to apply the analysis group to only some of the experiments or reaction plates added to the project.
3. From the Analysis Group: Content dialog box, select the samples or experiments to which the analysis group will apply, then click **Next**.

4. From the Analysis Group: Analysis Setting dialog box, modify the analysis settings as desired.

Group	Settings
Endogenous Controls	Select the method that the Applied Biosystems™ Software will use to normalize the data and identify the endogenous control (if used): 1. Select an option for specifying the endogenous control: • **Use specific endogenous control** – Select to specify one or more endogenous controls from the list of targets. • **Use global normalization** – Select to allow the Applied Biosystems™ Software to algorithmically normalize the C_T scores to calculate relative expression. 2. If you chose to use specific endogenous controls, select one or more targets from the list to use as the endogenous control(s) for the selected analysis group.
RQ Settings	• **Analysis Type** – Multiplex or Singleplex. In multiplex analysis, the target(s) of interest and the endogenous control are in the same well; for singleplex analysis, the target(s) of interest and the endogenous controls are in different wells. For multiplex analysis, ΔC_T (or ΔC_{RT}) is calculated at the well level. • **Reference Sample** and **Reference Biological Group** – Select the sample and/or biological group to use as the reference. The samples or other biological groups are compared to the reference to determine relative quantities of target. • **Confidence level (exclude results below this level)** – Select to calculate the RQ minimum and maximum values for the selected confidence level. Select the confidence level to use. (RQ min/max values are derived using standard error.) • **Limit by standard deviations** – Select to calculate the RQ minimum and maximum values for the selected number of standard deviations. Select the number of standard deviations to use. (RQ min/max values are derived using standard deviation.) • **Benjamin-Hochberg false discovery rate for p-values** – Select to use the Benjamin-Hochberg statistical method to adjust the p-values for the analysis. • **Maximum allowed CT** – Enter the maximum allowed C_T (or C_{RT}) value. Any value greater than this is rounded down to this value. • **Include maximum CT values in calculation** – Select whether to include the rounded values in any calculations.
Efficiency	For targets with amplification efficiency other than 100%, click in the Efficiency (%) column, then enter a percentage value between 1% and 150% to correct the amplification efficiency.
Cq Settings	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 group: • **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. **Note:** If you are using a global, standardized set of C_T settings for baselining and thresholding, you can apply the global settings using a CT Settings file. To generate the file, click **Export CT Settings** after you set the Cq settings as desired. The exported file can then be used to set the settings for subsequent projects by importing it.

Group	Settings
Flag Settings	Specify the quality measures that the Applied Biosystems™ Software will compute during the analysis. 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. **Note:** If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting. 3. In the Reject column, select the check boxes if you want the software to reject wells with the flag. Rejected wells are not considered for data analysis.
Inter-plate Calibrator Settings	Specify whether the Applied Biosystems™ Software will perform the analysis using an inter-plate calibrator. An IPC is a positive qPCR control, template, and assay that can be added to qPCR experiments in a project to provide a method for normalization. When the experiments are added for a collective analysis, a comparison of inter-plate calibrator performance can be used to account for minor variations in instrument performance. 1. Identify the target and sample combination to use as the inter-plate calibrator. **IMPORTANT!** The inter-plate calibrator must be present on the reaction plates of all experiments added to your project. Include at least three technical replicates on each reaction plate to ensure optimal performance of the calibrator. 2. Click **Add Inter-plate Calibrator Settings**, then double-click the table cells in the Target and Sample columns to select the inter-plate calibrator target and sample. 3. Repeat the previous step to add additional inter-plate calibrators to the analysis settings. 4. If desired, select **Allow calculation of delta Cq across all plates in the analysis group** to activate cross-plate calculation of ΔCq values. **Note:** To remove an inter-plate calibrator setting, select the appropriate row from the table, click **Delete Inter-plate Calibrator Settings**, then click **OK**.

Group	Settings
SC Settings	If you are using a relative standard curve to perform relative quantitation, specify standard curve settings that the Applied Biosystems™ Software will use to perform the analysis . The Relative Standard Curve is generated from a standard dilution series that is either present on the reaction plate with the unknowns or run separately on another plate. In both cases, data from the standards are normalized to the amplification of an endogenous control and then used to generate the standard curve for quantitation. To construct the curve, you must use the SC Settings to specify the location of the standards and select the curve from those detected by the software. 1. Select the location of the standard curve: • **On Plate Standard Curve** – Select if the standard curve reactions are located on the same reaction plate as the unknowns. If you want to use an on-plate standard curve to analyze other experiments, click **Export** and save the file. • **External Standard Curves** – Select if the standard curve reactions are located on a different reaction plate that was run separately. IMPORTANT! Before you can import an external standard curve, you must export it from the related experiment. 2. (*External curve only*) Click **Import**, then select the experiment from which you want to import the standard curve data. 3. Select the standard curve that you want to use from the Standard Curves table. Note: To remove an external standard curve from an analysis, select the curve from the Standard Curves table, click **Delete**, then click **OK**.

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

6. Click **Analyze** to reanalyze your 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.

Manage biological groups

If your project uses biological replicates, assign biological replicate groups to the samples to associate the data. The use of biological groups is optional and applicable when an experiment uses reactions with identical components and volumes to 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 project, the results are calculated by combining the results of the separate biological samples and treating this collection as a single population (that is, as one sample).

You can use the Applied Biosystems™ Analysis Software to do the following:

- **Create** a new biological group:
  a. From the Bio Groups table in the Overview screen, select **Actions** ▸ **Add**.

  b. In the Add New Biological Group dialog box, enter a name for the new group (up to 256 characters), then click **OK**.

  c. Edit the properties of the biological group directly in the BioGroups table, then click **OK**.
     – (Optional) Select a color from the drop-down menu. (The color is shown in the box plot.)
     – Enter up to a 72-character description of the biological group.

- **Update** an existing biological group by editing the group directly in the table.

  Note: Alternately, select a biological group from the table, then select **Actions** ▸ **Update**.

- **Assign** samples to biological groups:
  a. From the Samples table in the Overview screen, select one or more samples from the list, then select **Actions** ▸ **Assign**.

  b. In the Update Sample dialog box, select the desired biological group, then click **OK**.

- **Delete** a biological group:
  a. From the Bio Groups table in the Overview screen, select the biological group of interest, then select **Actions** ▸ **Delete**.

  b. In the confirmation dialog box, click **OK** to delete the group.

Configure the analysis settings

The Applied Biosystems™ Analysis Software applies analysis settings through the use of analysis groups. You can either edit the analysis settings for the default analysis group or create additional groups to capture changes to the settings for later comparison.

See "Create or edit an analysis group" on page 17 for more information.

Import sample information from design files

For convenience, the Applied Biosystems™ Software can import sample information directly from design files exported from projects or created using a text editor or spreadsheet application. Design files are formatted as either tab-delimited (.txt) or as comma-separated (.csv) text. The following figure illustrates the structure of the exported file.

	1	2	3	4	5	6	7	8
A	sample name	group						
B	Adipose1	Adipose						
C	Adipose2	Adipose						
D	Bladder1	Bladder						
E	Bladder2	Bladder						

Use the following guidelines when editing the file:

- **Row A** – The first row of the file must contain the **sample name** and **group** column headers.
- **Column 1 (sample name)** – For each row, enter a name for a single sample (up to 100-characters).
- **Column 2 (group)** – For each row, enter the name of the biological group to which you want to assign the sample.

  Note: If you are not using biological groups, leave the second column blank. The Applied Biosystems™ Software does not import blank entries.
- If the samples included in the design 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.

You can perform the following related actions from the Overview screen:

- **Create** a design file:

  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.

  a. From the Samples table in the Overview screen, click **Actions ▸ Export Design File**.

b. In the Export Sample Settings dialog box, select the format for the exported file (.txt or .csv), then click **OK**.

c. Using a text editor or spreadsheet application, open the sample design file and edit it as needed.

- **Import** a design file:
  a. From the Samples table in the Overview screen, click **Actions ▸ Import Design File**.

  b. Locate the sample design file with the sample information, then click **Open**.

If the import is successful, the sample(s) are populated to the samples in the table. If a sample of the same name is already present in the project, it is overwritten with the information from the sample design file.

Note: Sample name matching is not case-sensitive. For instance, if the sample in the project is "fly", then both "fly" and "Fly" in the sample design file will match.

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.

Define an endogenous control for the analysis

In the Applied Biosystems™ Analysis Software, the endogenous control is assigned within, and is specific to, each analysis group.

1. From the Analysis Groups table in the Overview screen, select an existing group, then select **Actions ▸ Edit Analysis Settings**.

2. Click **Endogenous Controls** to view the settings.

3. Select an option for specifying the endogenous control:
   - **Use specific endogenous control** – Select to specify a specific endogenous control from the list of targets.
   - **Use global normalization** – Select to allow the Applied Biosystems™ Software to algorithmically normalize the C_T scores to calculate relative expression.

4. If you chose to use a specific endogenous control, select one or more targets from the list to use as the control(s) for the selected analysis group.

5. Click **Finish**.

4 Edit experiment properties

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.

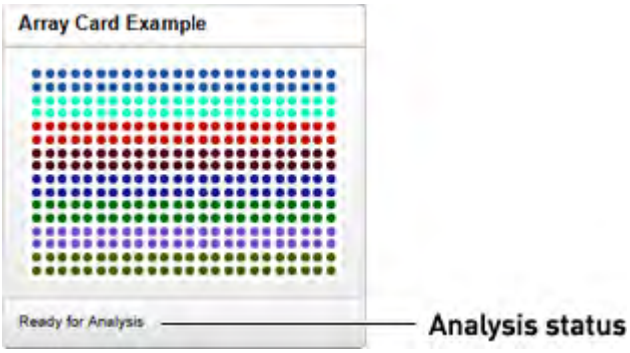
Review and edit the plate setups

After configuring your project with all necessary samples, targets, and biological groups, 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.

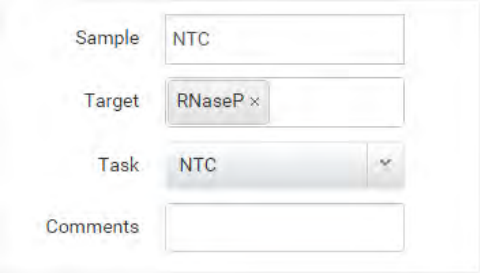


Apply samples and targets

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.



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

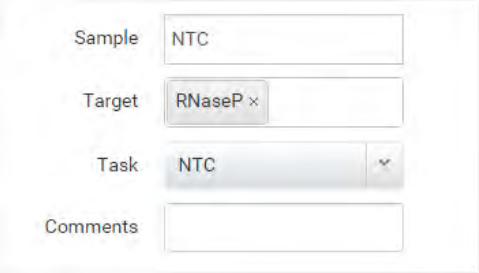
If the task assignments of one or more of your experiments contain errors or are missing, you can use the Applied Biosystems™ Analysis Software to correct the problem prior to analysis.

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.
4. 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.
   - **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.



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

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
Experiment data (do not edit):	**1**	* Block Type = 96-Well Block (0.2mL)				
	2	* Experiment Type = Relative Quantitation				
	3	* Instrument Type = 7900HT Real-Time PCR System				
	4	* No. Of Wells = 96				
Column headings (do not edit):	**5**	Set Up Well Section Inf				
	6	Well	Well Position	Sample Name	Task	Target Name
Plate setup content (add well data in any order):	**7**	80	G9	Testes3	UNKNOWN	Hs00169663_m1
	8	95	H12	Testes3	UNKNOWN	Hs00608224_m1
	9	14	B3	Testes1	UNKNOWN	Hs00609297_m1
	...	...	...	...	...	...

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
- 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.

    Note: For OpenArray™ plates, wells are identified through the combination of the sector coordinates on the plate, and the coordinates of the well within the sector. For example, the position "b2d10" refers to the through-hole at position D10 within sector B2 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** or **NTC**.
  - **Column E (Target Name)** – The name of the assay added to the well, or the identity of the target sequence (up to 256-characters).
- 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.

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 28.

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 28 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.

5 Review the raw data

After adding experiments to your project, use the Data Review screen to make a first quality pass of your analyzed project data. The plots and features of the screen can help you review your project for irregular amplification and other common PCR problems.

Review the quality data

After the Applied Biosystems™ Analysis Software processes your project, you can use the Data Review 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 **Data Review** to view the Data Review screen.
3. From the drop-down list at the top of the screen, choose the way that you would like to organize and review the quality data:
   - **Targets** – Groups and displays the quality data by target name.
   - **Samples** – Groups and displays the quality data by sample name.
   - **Plates** – Groups and displays the quality data by experiment/plate.

   Note: The Plates view is common to all real-time instrument software manufactured by Thermo Fisher Scientific.
4. 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.
5. If flags or irregularities are present, or you would just like to review the amplification data for a specific target, sample, or experiment, click the amplification plot of interest to zoom the display.

6. Configure the display options for the amplification plot. Click , then select from the available options:

Group	Select...	Description
Plot Type	dRn vs Cycle	Displays ΔRn as a function of cycle number, where ΔRn is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
	Rn vs Cycle	Displays Rn as a function of cycle number, where Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. You can use this plot to identify and examine irregular amplification.
	CT vs Well	Displays C_T (C_q) as a function of well position, where C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. You can use this plot to locate outlying amplification (outliers).
Graph Type	Linear	Displays the data on a linear scale.
	Log	Displays the data on a logarithmic scale.
Color Type	Well	Colors the data for each well according to its position on the reaction plate.
	Sample	Colors the data for each well according to the sample that it contains.
	Flag Status	Colors the data for each well according to whether it generates quality flags.
	Amp Status	Colors the data for each well according to the amplification status that it is assigned.

7. If the amplification plot that you are viewing includes data from more than 384 wells, use the histogram beneath the Amplification Plot to view the data of interest:
   - Click and drag the anchor icon ( ) to the desired location in the histogram to display the curves from the 384 wells with values nearest to the position of the icon.
   - Select the heading of a column in the Well Table that contains numerical content (such as, Amp Score or C_T/C_{RT} without Flags) to change the x-axis content of the histogram.

   Note: Selecting the heading of a column in the Well Table that contains less than 384 data points hides the histogram. The feature is present only when the plot contains more than 384 amplification curves.

8. If the data set that you are viewing consists of a large number of data points, use the Outlier Wheel to organize, filter, and review the data for irregular amplification:
   a. In the right-side pane of the Review Plate screen, select **View By ▸ Outlier Wheel**.

   b. From the Sort By dropdown list, select the attribute by which you want the Applied Biosystems Software to filter the displayed data set.

c. While viewing the data set within the Outlier Wheel, select a segment of the wheel to view the associated data within the Amplification Plot and Well Table.

   Note: Click the center of the wheel to deselect the data.

d. If desired, double-click a segment of the Outlier Wheel to review the related subset of the displayed data.

   As you select and filter the displayed data, the Applied Biosystems Software lists the filters that you apply at the bottom of the Outlier Wheel Plot. To remove a filter, either click **Undo** to remove the last filter applied or click the **X** beside a desired filter to remove it.

e. At any time while reviewing your data, click **Show Table** to view the tabular data for the datapoints present in the Outlier Wheel Plot.

See "Outlier Wheel Plot" on page 62 for more information on the Outlier Wheel plot.

9. Review the amplification plots as needed.

   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.

10. When ready, click **Multicomponent** to review the multicomponent plot as needed.

    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.

11. View and modify the data in the Well table:

Tool	Use this tool to...
Mouse/cursor	To select: • 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 Results table, the corresponding data points are selected in the amplification plot.

Tool	Use this tool to...
Group by drop-down menu	Select how to group the samples in the Well table. For example, if you select **Target**, the samples are grouped according to the nucleic acid sequence they target.
Actions drop-down list	**Bookmark/Clear Bookmark** for wells in the project. The bookmarks persist in the Data Review and Results screens, so you can easily find bookmarked wells.
	Omit/UnOmit well from the analysis. After you omit or un-omit a well, click **Analyze** to reanalyze the project. For omitted wells, the software: • Does not display data or tasks in the Well table. • 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.
Flag Details	Select **Show Flag Details** to display the results of each quality flag in an individual column. When unselected, the table displays the results of the quality analysis in a single column.
or	Expand or collapse the Well table.

12. Review the data in the Well table data.

Column	Use this column to...
(Bookmark)	View whether or not the well has been bookmarked.
Omit	View the omission status of the related well.
C_T /C_{RT}	View the C_T /C_{RT} calculated for the related well.
Amp Status	View the amplification status as determined by the software, where possible states are amplification, no amplification, reviewed, and undetermined.
Amp Score	View the amplification score calculated for the well.
Cq Conf	View the confidence value calculated by the software for the C_Q (C_T) for the given well.
Sample	View the ID (a unique name or number) of the sample.
Target	View the ID (a unique name or number) of the nucleic acid sequence targeted by the assay added to the 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.
Plate	View the barcode of the reaction plate used to run the reaction. If no barcode is present, the software displays the name of the experiment file to which the data belongs.
Baseline Start	View the start and endpoints of the range of PCR cycles used as the baseline in the calculation the C_T for the related well.
Baseline End	

Column	Use this column to...
Task	View the task assigned to the well. A task is the function that a sample performs: • Unknown • No template control (NTC) (control identifier)
Flags	View the number of flags generated for the well.

13. When ready, click < to return to the QC thumbnails.

14. Review the amplification data for specific targets, samples, or experiments as needed.

15. Click ☰ in the toolbar 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.

    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.

Using the Amplification Plot Histogram

When an Amplification Plot for a specific sample, target, or plate includes data for more than 384 data points, the Applied Biosystems™ Software displays a subset of the data (shown in color) for active viewing against a background of the full dataset (shown in grey). The range of active data in the plot is controlled through a histogram of a numerical attribute associated with the well data, which is located beneath the plot (see below).

- Click and drag the anchor icon ( ) to the desired location in the histogram to display the curves from the 384 data points with values nearest to the position of the icon.

- Select the heading of a column in the Well Table that contains numerical content (such as, Amp Score or C_T/C_{RT} without Flags) to change the x-axis content of the histogram.

  Note: Selecting the heading of a column in the Well Table that contains less than 384 data points hides the histogram. The feature is present only when the plot contains more than 384 amplification curves.

About the quality data summary

The quality summary displays a table of the quality flags supported by the software. For each sample, target, or plate, the table lists the flag frequency and location for any experiment that is added to a project. 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.

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).

IMPORTANT! You cannot omit all wells that belong to a reference sample, that belong to a biological group, or that serve as the endogenous control for the project.

Note: To restore an omitted well, select the well from a plot or table, then select **Actions ▸ UnOmit**.

6 Review the analyzed data

After reviewing the data, use the Analysis screen to review the results of the analyzed project. The screen provides a variety of plots to help you characterize the analyzed data and to better visualize the relationships between the calculated expression of the evaluated targets.

Review the analyzed data and plots

After you have reviewed the quality data for your project, view the results of the analysis in the Analysis screen. As with the quality check, 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 **Analysis** to view the Analysis screen.
3. Review the Box Plot as needed (see "Box Plot" on page 42).
   a. Click **View Options**, then select **CT vs Sample** from the Plot Type drop-down list.
   b. Click the magnification factor to determine the percentage of the plot displayed onscreen at once.

c. Scroll down to review the data in the Well table data.

Column	Use this column to...
Sample	View the ID (a unique name or number) of the sample.
Biological Group	View the biological group (a unique name or number) to which the sample belongs.
Mean	View the arithmetic average of the technical replicate C_T values.
Min	View the minimum technical replicate C_T value for the test sample calculated using the confidence level set in the analysis settings.
Max	View the maximum technical replicate C_T value for the test sample calculated using the confidence level set in the analysis settings.
Median	View the median C_T value for the technical replicates of the sample.
Q1	View the 1st Quartile (Q_1) for the sample replicate group, which is calculated as the numeric midpoint between the lowest and median C_T values of the technical replicates. **Note:** The 1st Quartile defines lower boundary of the inter-quartile region (IQR), which is defined as the difference between the 3rd and 1st quartile.
Q3	View the 3rd Quartile (Q_3) for the sample replicate group, which is calculated as the numeric midpoint between the median and maximum C_T values of the technical replicates.

Note: To filter the table data, click (Filter) within any column heading, configure the rules as desired, then click **Filter**.

Note: If desired, you can view the quality data for any selected sample or well by selecting the element in the plot or table and clicking **View QC**.

4. When ready, click to review the Correlation Plot as needed (see "Correlation Plot" on page 42).
   a. If you are using biological groups and more than 100 samples are present in the analysis group currently in use, select a group from the drop-down list to view the corresponding data.
   b. Click **View Options**, then select the data arrangement that you want to view (**Matrix View** or **Tabular View**).
   c. Select the type of plot that you want to display (**CT** or **ΔCT**)
5. When ready, click to review the Heatmap Plot as needed (see "Heatmap Plot" on page 45).
   a. Click **View Options**, then select the distance measure (Euclidean Distance or Pearson's Correlation) from the Distance Measure drop-down menu.
   b. Select the clustering method (Average Linkage, Complete Linkage, or Single Linkage) from the Clustering Method drop-down menu.

c. Change the plot type from the Map Type menu. Select a data display option (Global (ΔC_T), Global (ΔC_T Plus), Sample-centric, or Target-centric) from the Map Type drop-down menu.

d. Select a color scheme (Red Blue, Red Green, or Green Orange) from the Color Scheme drop-down menu.

6. When ready, click to review the Relative Quantitation Plot as needed (see "RQ Plot" on page 43).
   a. Click **View Options**, then select a data display option (RQ vs Sample, RQ vs Biological Group or RQ vs Target) from the Plot Type drop-down menu.
   b. Change the graph type by selecting a plot scale (Linear, Log 10, Log 2, or Ln) from the Graph Type drop-down menu.
   c. Change the plot options:
      - To view error bars showing RQ Max and RQ Min, select **Show Error Bars**.
      - To view labels on the graph, select the appropriate label option.
   d. Click the magnification factor to determine the percentage of the plot displayed onscreen at once.
   e. Scroll down to review the data in the Well table data.

Column	Use this column to...
Target	View the ID (a unique name or number) of the nucleic acid sequence targeted by the assay.
Sample	View the ID (a unique name or number) of the sample.
Biological Group	View the biological group (a unique name or number) to which the sample belongs.
Max C_T/C_{RT}	View the maximum C_q defined by the "Maximum allowed CT" limit in the RQ Settings analysis settings.
C_T/C_{RT} Mean	View the arithmetic average of the technical replicate C_q values.
Adjusted C_T/C_{RT} Mean	View the average of the technical replicate C_q values that have been adjusted based on the "Maximum allowed CT" limit defined in the RQ Settings analysis settings. **Note:** Wells with C_q scores greater than the "Maximum allowed CT" value are adjusted to the specified C_q limit.
C_T/C_{RT} SE	View the sample standard deviation of the sample replicate group level C_q values.
$\Delta C_T/\Delta C_{RT}$ Mean	View the arithmetic average of the technical replicate C_q values for the sample replicate group. **Note:** The $\Delta C_T/\Delta C_{RT}$ mean is calculated at the reaction plate level and represents the mean difference between the target C_q values and the endogenous control C_q values for all the technical replicates for that sample that are present on the plate.
$\Delta C_T/\Delta C_{RT}$ SE	View the sample standard deviation of the sample replicate group level C_q values. **Note:** The $\Delta C_T/\Delta C_{RT}$ SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level C_q value variation between the target and the endogenous control.

Column	Use this column to...
F-Factor	View the calculated F-Factor for the replicate group associated with the reference sample. The F-Factor is used to calculate the RQ confidence intervals in the $\Delta\Delta C_T$ calculation (displayed as error bars in the RQ plot). The value is calculated differently depending on the RQ Settings in the analysis settings for your project. • If you specified a **Confidence level** setting, then the F-Factor is the *t-value* of the Student's t-distribution calculated from the: – *Degrees of freedom* that characterize the distribution of the replicate population. If the project contains no biological groups, degrees of freedom is calculated per Technical Test Sample as: #Technical Replicates $Wells_{Target}$ + #Technical Replicates $Wells_{Endo}$ - 2. Otherwise, the value is calculated per Biological Test Sample as: #Technical Replicates Groups $_{Test\ Sample}$ - 1. – *Probability* associated with the two-tailed Student's t-distribution (determined by the RQ Settings in the analysis settings for your project). • If you specified a **Limit by standard deviations** setting, then the F-Factor is equal to the setting (1, 2, or 3).
$\Delta\Delta C_T/\Delta\Delta C_{RT}$	View the calculated $\Delta\Delta C_T$ value for the replicate group associated with the reference sample.
$\Delta\Delta C_T/\Delta\Delta C_{RT}$ ± F-Sigma	View the calculated $\Delta\Delta C_T$ value added to or subtracted from the F-Sigma value calculated for the replicate group associated with the reference sample.
RQ	View the calculated relative level of gene expression for the replicate group that is associated with the test sample.
RQ Min	View the minimum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The minimum includes the variability associated with the endogenous control and targets in only the test samples.
RQ Max	View the maximum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The maximum includes the variability associated with the endogenous control and targets in only the test samples.

Note: To filter the table data, click (Filter) within any column heading, configure the rules as desired, then click **Filter**.

7. When ready, click to review the Volcano Plot as needed (see "Volcano Plot" on page 46).

Box Plot

The box plot displays the distribution of C_q values for each sample or for each target, making it easy to view the variation in values among biological groups.

Below the box plots is the Results Details table, showing the following information:

Column	Use this column to...
Sample	View the ID (a unique name or number) of the sample.
Biological Group	View the biological group (a unique name or number) to which the sample belongs.
Mean	View the arithmetic average of the technical replicate C_q values.
Min	View the minimum technical replicate C_q value for the test sample calculated using the confidence level set in the analysis settings.
Max	View the maximum technical replicate C_q value for the test sample calculated using the confidence level set in the analysis settings.
Median	View the median C_q value for the technical replicates of the sample.
Q1	View the 1st Quartile (Q_1) for the sample replicate group, which is calculated as the numeric midpoint between the lowest and median C_q values of the technical replicates. **Note:** The 1st Quartile defines lower boundary of the inter-quartile region (IQR), which is defined as the difference between the 3rd and 1st quartile.
Q3	View the 3rd Quartile (Q_3) for the sample replicate group, which is calculated as the numeric midpoint between the median and maximum C_q values of the technical replicates.

Changing the Box Plot display

Correlation Plot

The Correlation plots display the correlation between the target genes in one or more samples or biological groups. There are two correlation plots: the scatter plot and the signal correlation plot.

- The scatter plot shows the correlation of C_q for all targets for a pair of samples or biological groups.
- The signal correlation plot shows the correlation coefficient (r) for all pairs of samples or biological groups in the project. The plot is color-coded based on |r|, (the absolute value of r), indicating the strength of the correlation: green indicates highly correlated (either negative or positive) and red indicates low correlation (either negative or positive). Each cell represents a different scatter plot.

RQ Plot

The RQ (Relative Quantitation) Plot displays the results of the relative quantitation calculations in the gene expression profile. Three plots are available:

- **RQ vs Target** – Groups the relative quantitation (RQ) values by target. Each sample is plotted for each target. You can view the plot as the following graph types: linear, log10, Ln, log2.
- **RQ vs Sample** (present when the plot displays results by Samples) – Groups the relative quantitation (RQ) values by sample. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.
- **RQ vs BioGroup** – (present when the plot displays results by Biogroups) – Groups the relative quantitation (RQ) values by biological replicate group. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.

IMPORTANT! If one or more assay efficiencies are set less than 100%, then the Applied Biosystems™ Software performs the gene expression calculation using equivalent C_q values, where the software adjusts the C_qs of each target proportionally to achieve equivalent efficiency. The resulting equivalent C_qs calculated for the affected targets reflect the values expected if the assays performed at 100% efficiency.

Below the gene expression plot is the Results Details table, showing the following information:

Column	Use this column to...
Target	View the ID (a unique name or number) of the nucleic acid sequence targeted by the assay.
Sample	View the ID (a unique name or number) of the sample.
Biological Group	View the biological group (a unique name or number) to which the sample belongs.
Max C_T/C_{RT}	View the maximum C_q defined by the "Maximum allowed CT" limit in the RQ Settings analysis settings.
C_T/C_{RT} Mean	View the arithmetic average of the technical replicate C_q values.
Adjusted C_T/C_{RT} Mean	View the average of the technical replicate C_q values that have been adjusted based on the "Maximum allowed CT" limit defined in the RQ Settings analysis settings. **Note:** Wells with C_q scores greater than the "Maximum allowed CT" value are adjusted to the specified C_q limit.
C_T/C_{RT} SE	View the sample standard deviation of the sample replicate group level C_q values.
$\Delta C_T/\Delta C_{RT}$ Mean	View the arithmetic average of the technical replicate C_q values for the sample replicate group. **Note:** The $\Delta C_T/\Delta C_{RT}$ mean is calculated at the reaction plate level and represents the mean difference between the target C_q values and the endogenous control C_q values for all the technical replicates for that sample that are present on the plate.

Column	Use this column to...
$\Delta C_T/\Delta C_{RT}$ SE	View the sample standard deviation of the sample replicate group level C_q values. **Note:** The $\Delta C_T/\Delta C_{RT}$ SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level C_q value variation between the target and the endogenous control.
F-Factor	View the calculated F-Factor for the replicate group associated with the reference sample.
$\Delta\Delta C_T/\Delta\Delta C_{RT}$	View the calculated $\Delta\Delta C_T$ value for the replicate group associated with the reference sample.
$\Delta\Delta C_T/\Delta\Delta C_{RT}$ ± F-Sigma	View the calculated $\Delta\Delta C_T$ value added to or subtracted from the F-Sigma value calculated for the replicate group associated with the reference sample.
RQ	View the calculated relative level of gene expression for the replicate group that is associated with the test sample.
RQ Min	View the minimum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The minimum includes the variability associated with the endogenous control and targets in only the test samples.
RQ Max	View the maximum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The maximum includes the variability associated with the endogenous control and targets in only the test samples.

Heatmap Plot

The heat map is a representation of the level of expression of many targets (genes) across a number of samples. The targets and samples are arranged according to the similarity of their gene expression.

Below the Heatmap Plot is the Results Details table, showing the following information:

Column	Use this column to...
Sample	View the ID (a unique name or number) of the sample.
Biological Group	View the biological group (a unique name or number) to which the sample belongs.
Target	View the ID (a unique name or number) of the nucleic acid sequence targeted by the assay.
C_T/C_{RT} Mean	View the arithmetic average of the technical replicate C_q values.
Adjusted C_T/ C_{RT} Mean	View the average of the technical replicate C_q values that have been adjusted based on the "Maximum allowed CT" limit defined in the RQ Settings analysis settings. **Note:** Wells with C_q scores greater than the "Maximum allowed CT" value are adjusted to the specified C_q limit.
$\Delta C_T/\Delta C_{RT}$ Mean	View the arithmetic average of the technical replicate ΔC_q values for the sample replicate group. **Note:** The $\Delta C_T/\Delta C_{RT}$ mean is calculated at the reaction plate level and represents the mean difference between the target $\Delta C_T/\Delta C_{RT}$ values and the endogenous control $\Delta C_T/\Delta C_{RT}$ values for all the technical replicates for that sample that are present on the plate.
$\Delta C_T/\Delta C_{RT}$ SE	View the sample standard deviation of the sample replicate group level C_q values. **Note:** The $\Delta C_T/\Delta C_{RT}$ SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level C_q value variation between the target and the endogenous control.
$\Delta C_T/\Delta C_{RT}$ + Control Median	View the arithmetic average of the technical replicate $\Delta C_T/\Delta C_{RT}$ values for the sample replicate group added to the control median.

Volcano Plot

The volcano plot displays the p-value versus the fold change for each target in a biological group, relative to the reference group. Green and red dots represent targets with a fold change outside (greater or lesser than) the fold change boundary. Compare the size of the fold change (x-axis) to the statistical significance level (y-axis) on the volcano plot.

Note: You must have biological groups assigned (so that p-values can be calculated) before you can view data on the volcano plot.

IMPORTANT! If one or more assay efficiencies are set to <100%, then the Applied Biosystems™ Software performs the gene expression calculation using *equivalent C_q* values, where the software adjusts the C_qs of each target proportionally to achieve equivalent efficiency. The resulting equivalent C_qs calculated for the affected targets reflect the values expected if the assays performed at 100% efficiency.

Below the plot is the Results Details table, showing the following information:

Column	Use this column to...
Biological Group	View the biological group (a unique name or number) to which the sample belongs.
Target	View the ID (a unique name or number) of the nucleic acid sequence targeted by the assay.
C_T/C_{RT} Mean	View the arithmetic average of the technical replicate C_q values.
Adjusted C_T/C_{RT} Mean	View the average of the technical replicate C_q values that have been adjusted based on the "Maximum allowed CT" limit defined in the RQ Settings analysis settings. **Note:** Wells with C_q scores greater than the "Maximum allowed CT" value are adjusted to the specified C_q limit.
ΔC_T/ΔC_{RT} Mean	View the arithmetic average of the technical replicate C_q values for the sample replicate group. **Note:** The ΔC_T/ΔC_{RT} mean is calculated at the reaction plate level and represents the mean difference between the target C_q values and the endogenous control C_q values for all the technical replicates for that sample that are present on the plate.
ΔC_T/ΔC_{RT} SE	View the sample standard deviation of the sample replicate group level C_q values. **Note:** The ΔC_T/ΔC_{RT} SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level C_q value variation between the target and the endogenous control.
$\Delta\Delta C_T$/$\Delta\Delta C_{RT}$	View the calculated $\Delta\Delta C_T$ value for the replicate group associated with the reference sample.
RQ	View the calculated relative level of gene expression for the replicate group that is associated with the test sample.
RQ Min	View the minimum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The minimum includes the variability associated with the endogenous control and targets in only the test samples.

Column	Use this column to...
RQ Max	View the maximum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The maximum includes the variability associated with the endogenous control and targets in only the test samples.
P-Value	View the probability (P-value) that the observed RQ (fold change) in gene expression for the replicate group associated with the test sample is not differentially expressed due to treatment or condition.
Result	View the assignment for the replicate group that is associated with the test sample, where possible states are: down-regulated, up-regulated, insignificant, or flat.

View and modify the Volcano Plot

The Applied Biosystems™ Software allows you to view the gene expression data in volcano plot that displays the p-value statistics data for each biological group.

1. View details of the Volcano Plot:
   a. In the Analysis screen, click
   b. Move the pointer over a point to view information about it.
2. If necessary, change the group displayed in the plot:
   From the **Group** drop-down menu, select a different group to compare to the reference group.
3. If necessary, change the boundaries displayed on the plot. To change the:
   - *Fold-change Boundary* (x-axis) – Enter a number greater than or equal to 1 in the **Fold Change Boundary** field, then click **Apply**.
     Targets having a fold change greater than the upper boundary are colored red; targets with a fold change less than the lower boundary are colored green.
   - *P-value Boundary* (y-axis) – Enter a number from 0 to 1 in the **P-Value Boundary** field, then click **Apply**.

Melt Curve Plot

The melt curve plot (also called a dissociation curve plot) displays data collected during a melt curve stage. Peaks in the melt curve can indicate the melting temperature (T_m) of a target or can identify nonspecific PCR amplification. The software displays the melt curve plot only for those experiments with a PCR method that includes a melt curve stage (a gradual temperature ramp configured for data collection).

Two views are available:

- **Normalized reporter (Rn) vs. temperature** — The normalized reporter view visualizes the rise in fluorescence throughout the temperature ramp. The normalized reporter (Rn), displayed on the y-axis, is calculated as the fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference.
- **Derivative reporter (–Rn') vs. temperature** — The derivative reporter view allows you to visualize the maximum rate of change in fluorescence during the temperature ramp. The derivative reporter, displayed on the y-axis, is calculated as the negative first derivative of the normalized fluorescence (Rn) generated by the reporter during PCR amplification.

Omitting wells and samples

You may omit wells from analysis if you do not want to consider data generated by the well.

1. Select one or more wells to omit from analysis.
2. Omit the selected wells:
   - **Well Table tab** - Select the wells, then select **Actions ▸ Omit**.
   - **Plate Layout tab** - Select the wells, then click **Omit**.
3. Click **Analyze** to reanalyze the data without the omitted wells.

IMPORTANT! You cannot omit all wells that belong to a reference sample, that belong to a biological group, or that serve as the endogenous control for a project.

7 Export the results

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.
      - **Biological Group Results** - Exports gene expression analysis results organized and analyzed by biological group.
      - **Sample Results** - Exports gene expression analysis results organized and analyzed by individual samples.
      - **Well Results** - Exports gene expression analysis results for the individuals wells of every reaction plate used in the analysis.
      - **Amplification Data** - Exports amplification results for each well in the project, such as cycle numbers, and Rn or ΔRn values.

- **Volcano Plot Data** - Exports gene expression data used to generate the Volcano Plot, including Fold Change, P-Value, Fold Change Boundary, P-Value Boundary, and the Result.
- **Targets/Samples/Plates QC (with flags)** - Exports a summary of the quality metrics (flags) generated by the data analysis. The option allows you to selectively export the quality data organized by target, sample, or plate.

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 50).

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

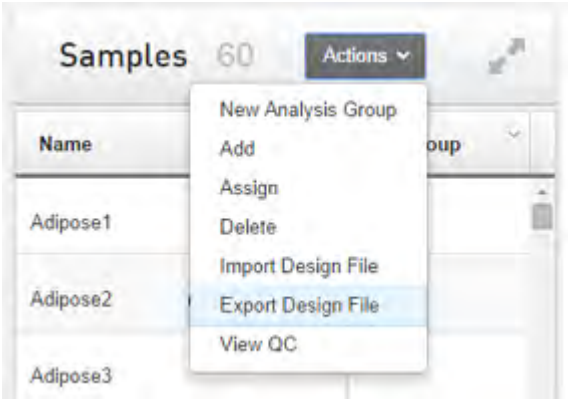
The Applied Biosystems™ Analysis Software allows you to export the following data from a project for use in other analyses.

- Export a sample design file

  A sample design file is a tab-or comma-delimited file (*.txt or *.csv) that contains a list of sample names and the corresponding names of the biological groups to which the samples belong. You can use exported sample design files to quickly import the sample information into other experiments.

  a. Open the project that contains experiment with the sample information of interest, then click **Overview**.

b. From the Samples table in the Overview screen, click **Actions** ▸ **Export Design File**.



c. In the Export Sample Settings dialog box, select the format for the exported file (.txt or .csv), then click **OK**.

d. Select the location and name for the exported file, then click **Save**.

- 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.

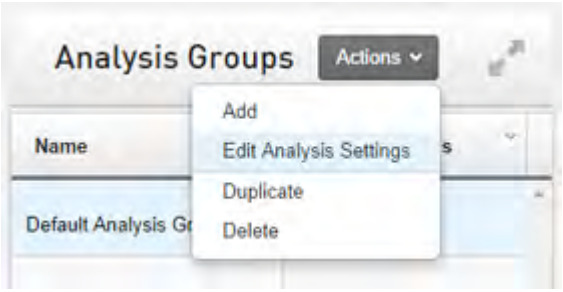
  c. From the Edit Plate screen, click **Actions** ▸ **Download Template**, then save the file to the desired location.

- Export the amplification efficiency settings

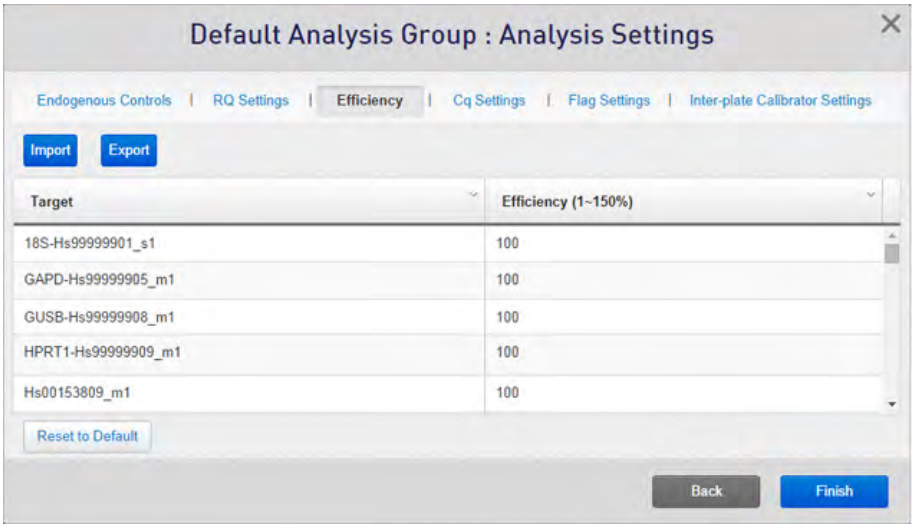
  Amplification efficiency files associate targets with the specific amplification efficiencies of the corresponding assays. Once exported, you can use the file to import the settings to other projects (especially useful for projects involving large numbers of targets).

  a. Open the project that includes the analysis settings with the desired efficiency data, then select **Overview**.

b. From the Overview screen, select the analysis group that contains the efficiency data, then click **Edit Analysis Settings**.



c. From the Analysis Settings dialog box, click **Efficiency** to view the assay efficiency settings, then click **Export**.



d. In the Export Sample Settings dialog box, select the format for the exported file (.txt or .csv), then click **OK**.

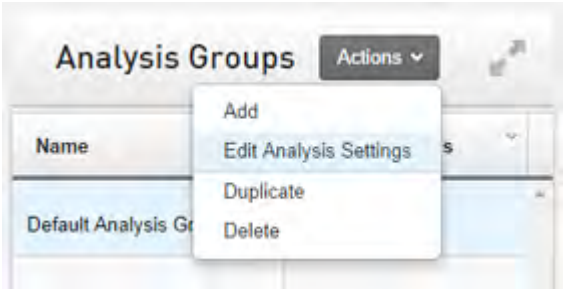
e. Select the location and name for the exported file, then click **Save**.

- Export the C_T settings

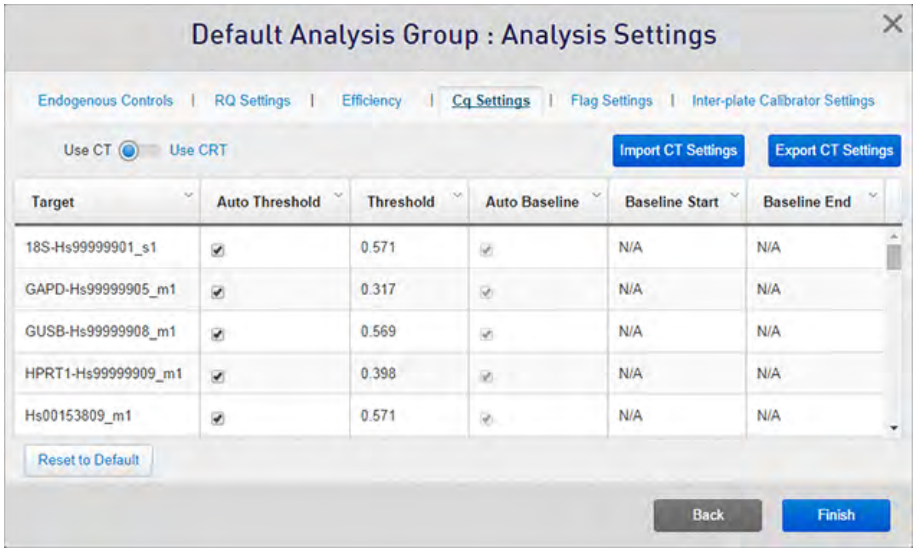
  The C_T settings file associates targets with specific methods for calculating baseline and threshold values (manual or automatic). Once exported, you can use the file to import the settings to other projects (especially useful for projects involving large numbers of targets).

  a. Open the project that includes the analysis settings with the desired C_T settings, then select **Overview**.

b. From the Overview screen, select the analysis group that contains the C_T settings, then select **Action ▸ Edit Analysis Settings**.



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



d. In the Export CT Settings dialog box, select the format for the exported file (.txt or .csv), then click **OK**.

e. Select the location and name for the exported file, then click **Save**.

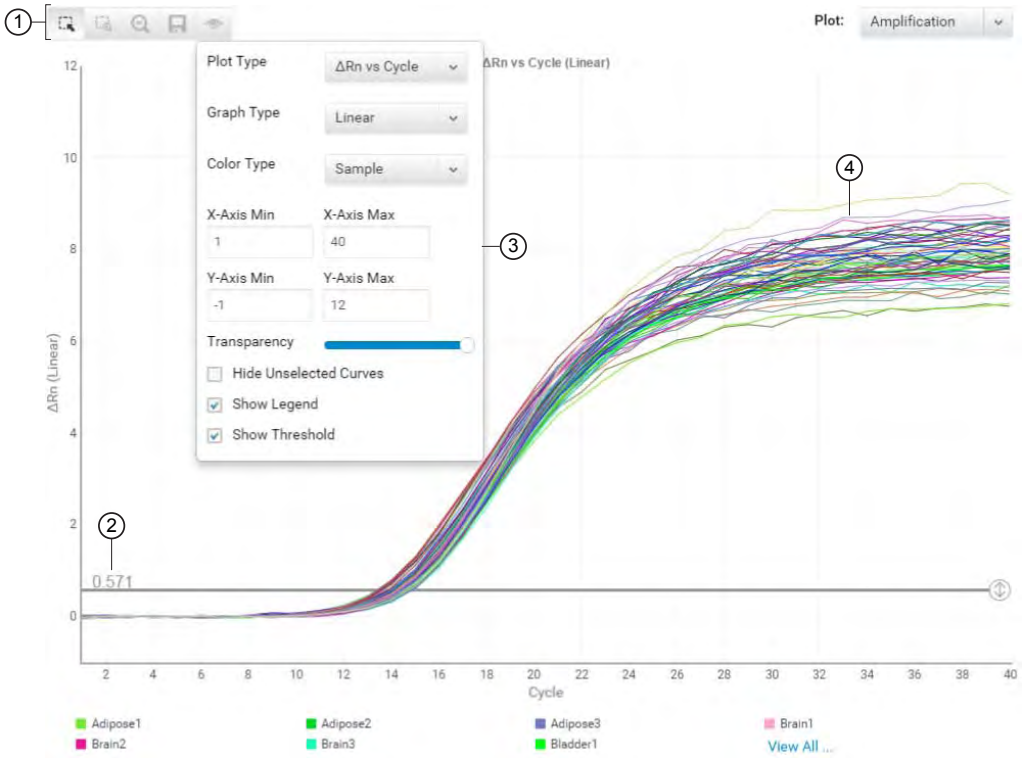
8 Screens and plots

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 (ΔRn = Rn – 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.
- **CT 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.
- – 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 **Threshold** – The threshold (calculated or manual) that is currently applied to the project data.

③ **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 **CT vs Well**), the scale of the y-axis (**log** or **linear**), and the color scheme for the plot.

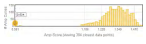
④ **Amplification curves** – Normalized fluorescence for individual wells throughout the course of the thermal cycling protocol.

Note: When the Amplification Plot displays a large data set, the Applied Biosystems™ Software alters the appearance of curves to better identify them:

- **Inactive data** – Displayed using solid, grey curves
- **Omitted data** – Wells omitted automatically/manually are displayed using Long-dashed, grey curves (— — —)
- **Unselected data** – Wells not part of the current selection are displayed using short-dashed, grey curves (- - - - - -)

Viewing large data sets

When an Amplification Plot for a specific sample, target, or plate includes data for more than 384 data points, the Applied Biosystems™ Software displays a subset of the data (shown in color) for active viewing against a background of the full data set (shown in grey). The range of active data in the Amplification Plot is controlled through a histogram, located beneath the plot, which displays the well data for the experiment tabulated by an associated numerical attribute (such as Amp Score or C_T/C_{RT} without Flags).



The position of the anchor icon ( ) in the histogram controls the range of active data displayed by the Amplification Plot, where the software displays the curves from the 384 data points with values nearest to the position of the icon. To change the range of active data, click and drag the icon to view the data nearest the desired location.

Note: Alternatively, click / below the Well Table to advance the active data range displayed by the Amplification Plot.

The content of the histogram is determined by the table heading selected in the Well Table. By default, the histogram tabulates data by Amp Score as shown above. To change the data displayed on the x-axis of the histogram, select the heading of a column in the Well Table that contains numerical content (such as, Amp Score or C_T/C_{RT} without Flags).

Note: Selecting the heading of a column in the Well Table that contains less than 384 data points hides the histogram. The feature is present only when the plot contains more than 384 amplification curves.

Box Plot

The box plot displays the distribution of C_q values for each sample or for each target, making it easy to view the variation in values among biological groups.

Below the box plots is the Results Details table, showing the following information:

Column	Use this column to...
Sample	View the ID (a unique name or number) of the sample.
Biological Group	View the biological group (a unique name or number) to which the sample belongs.
Mean	View the arithmetic average of the technical replicate C_q values.
Min	View the minimum technical replicate C_q value for the test sample calculated using the confidence level set in the analysis settings.

Column	Use this column to...
Max	View the maximum technical replicate C_q value for the test sample calculated using the confidence level set in the analysis settings.
Median	View the median C_q value for the technical replicates of the sample.
Q1	View the 1st Quartile (Q_1) for the sample replicate group, which is calculated as the numeric midpoint between the lowest and median C_q values of the technical replicates. **Note:** The 1st Quartile defines lower boundary of the inter-quartile region (IQR), which is defined as the difference between the 3rd and 1st quartile.
Q3	View the 3rd Quartile (Q_3) for the sample replicate group, which is calculated as the numeric midpoint between the median and maximum C_q values of the technical replicates.

Correlation Plot

The Correlation plots display the correlation between the target genes in one or more samples or biological groups. There are two correlation plots: the scatter plot and the signal correlation plot.

- The scatter plot shows the correlation of C_q for all targets for a pair of samples or biological groups.
- The signal correlation plot shows the correlation coefficient (r) for all pairs of samples or biological groups in the project. The plot is color-coded based on $|r|$, (the absolute value of r), indicating the strength of the correlation: green indicates highly correlated (either negative or positive) and red indicates low correlation (either negative or positive). Each cell represents a different scatter plot.

Heatmap Plot

The heat map is a representation of the level of expression of many targets (genes) across a number of samples. The targets and samples are arranged according to the similarity of their gene expression.

Below the Heatmap Plot is the Results Details table, showing the following information:

Column	Use this column to...
Sample	View the ID (a unique name or number) of the sample.
Biological Group	View the biological group (a unique name or number) to which the sample belongs.
Target	View the ID (a unique name or number) of the nucleic acid sequence targeted by the assay.
C_T/C_{RT} Mean	View the arithmetic average of the technical replicate C_q values.

Column	Use this column to...
Adjusted C_T/ C_{RT} Mean	View the average of the technical replicate C_q values that have been adjusted based on the "Maximum allowed CT" limit defined in the RQ Settings analysis settings. **Note:** Wells with C_q scores greater than the "Maximum allowed CT" value are adjusted to the specified C_q limit.
$\Delta C_T/\Delta C_{RT}$ Mean	View the arithmetic average of the technical replicate ΔC_q values for the sample replicate group. **Note:** The $\Delta C_T/\Delta C_{RT}$ mean is calculated at the reaction plate level and represents the mean difference between the target $\Delta C_T/\Delta C_{RT}$ values and the endogenous control $\Delta C_T/\Delta C_{RT}$ values for all the technical replicates for that sample that are present on the plate.
$\Delta C_T/\Delta C_{RT}$ SE	View the sample standard deviation of the sample replicate group level C_q values. **Note:** The $\Delta C_T/\Delta C_{RT}$ SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level C_q value variation between the target and the endogenous control.
$\Delta C_T/\Delta C_{RT}$ + Control Median	View the arithmetic average of the technical replicate $\Delta C_T/\Delta C_{RT}$ values for the sample replicate group added to the control median.

Melt Curve Plot

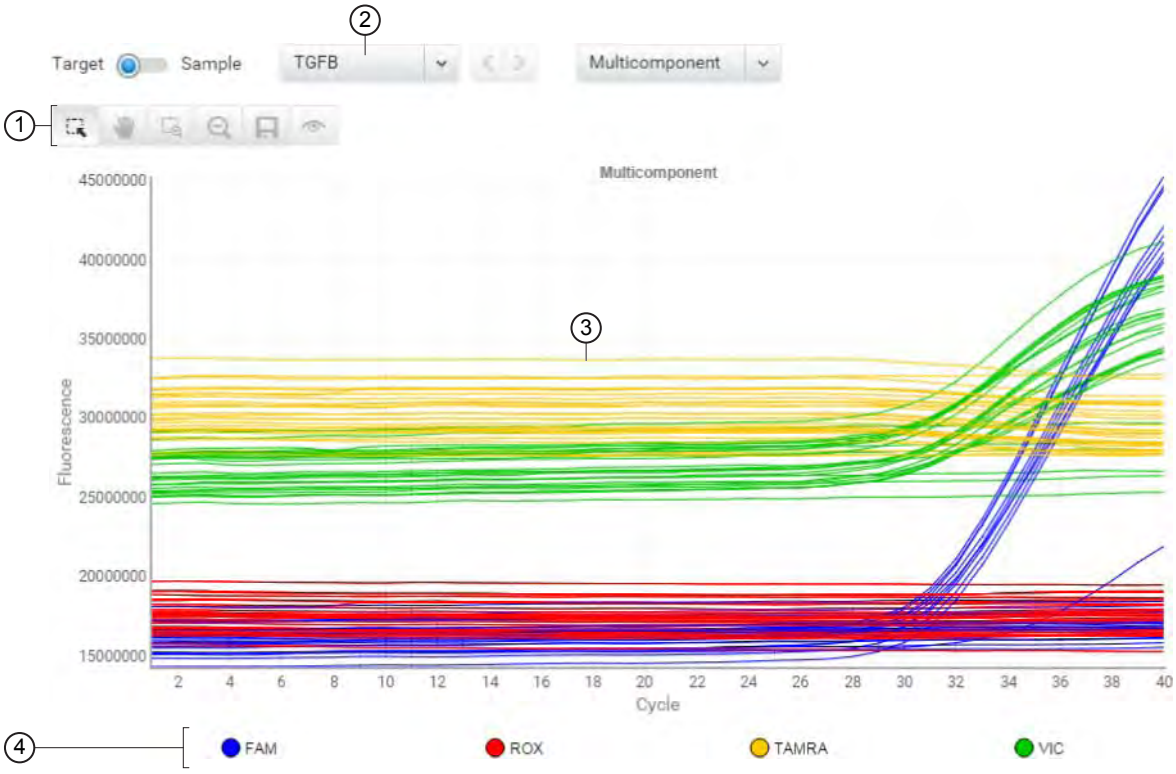
The melt curve plot (also called a dissociation curve plot) displays data collected during a melt curve stage. Peaks in the melt curve can indicate the melting temperature (T_m) of a target or can identify nonspecific PCR amplification. The software displays the melt curve plot only for those experiments with a PCR method that includes a melt curve stage (a gradual temperature ramp configured for data collection).

Two views are available:

- **Normalized reporter (Rn) vs. temperature** — The normalized reporter view visualizes the rise in fluorescence throughout the temperature ramp. The normalized reporter (Rn), displayed on the y-axis, is calculated as the fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference.
- **Derivative reporter (–Rn') vs. temperature** — The derivative reporter view allows you to visualize the maximum rate of change in fluorescence during the temperature ramp. The derivative reporter, displayed on the y-axis, is calculated as the negative first derivative of the normalized fluorescence (Rn) generated by the reporter during PCR amplification.

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.

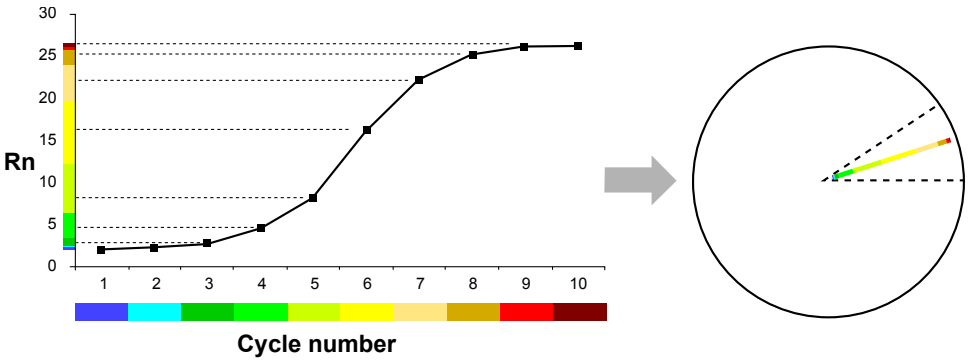
Outlier Wheel Plot

The Outlier Wheel is a data review tool that can greatly simplify the quality control review of very large data sets, especially data generated by OpenArray experiments. When a sample, target, or plate data set consists of thousands of data points, you can use the Outlier Wheel to organize and review the data set for irregular amplification.

The Outlier Wheel simplifies the review process by visualizing the amplification data of a given data set in a circular format, where each amplification curve in the set is represented as a colored line extending from a central axis. As shown in the following figure, each line is broken into colored segments that map to regions of the amplification curve, where:

- **Segment length** indicates the increase in normalized fluorescence (R_n) at a given PCR cycle.
- **Segment color** indicates the PCR cycle number at which signal was collected.

In the example below, the amplification curve exhibits little growth in R_n during cycle 1 (shown in cyan). Therefore, the projection of that section of the curve on the R_n scale translates into a small segment (cyan) in the corresponding line on the Outlier Wheel. In contrast, the amplification curve exhibits a large increase in R_n during cycle 5 (shown in light green) that translates into a much larger line segment (light green) in the corresponding line.



The combination of line segment color and length for each data point in the Outlier Wheel allows you to understand the shape of the related amplification curve and where most amplification occurs during the PCR. For example, reactions that amplify during the early cycles of the PCR appear primarily blue or green (early cycle colors), whereas reactions that amplify later appear primarily orange or red (later cycle colors), because those respective colors represent the cycles during which the maximum increase in Rn occurs.

RQ Plot

The RQ (Relative Quantitation) Plot displays the results of the relative quantitation calculations in the gene expression profile. Three plots are available:

- **RQ vs Target** – Groups the relative quantitation (RQ) values by target. Each sample is plotted for each target. You can view the plot as the following graph types: linear, log10, Ln, log2.
- **RQ vs Sample** (present when the plot displays results by Samples) – Groups the relative quantitation (RQ) values by sample. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.
- **RQ vs BioGroup** – (present when the plot displays results by Biogroups) – Groups the relative quantitation (RQ) values by biological replicate group. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.

IMPORTANT! If one or more assay efficiencies are set less than 100%, then the Applied Biosystems™ Software performs the gene expression calculation using equivalent C_q values, where the software adjusts the C_qs of each target proportionally to achieve equivalent efficiency. The resulting equivalent C_qs calculated for the affected targets reflect the values expected if the assays performed at 100% efficiency.

Below the gene expression plot is the Results Details table, showing the following information:

Column	Use this column to...
Target	View the ID (a unique name or number) of the nucleic acid sequence targeted by the assay.
Sample	View the ID (a unique name or number) of the sample.
Biological Group	View the biological group (a unique name or number) to which the sample belongs.
Max C_T/C_{RT}	View the maximum C_q defined by the "Maximum allowed CT" limit in the RQ Settings analysis settings.
C_T/C_{RT} Mean	View the arithmetic average of the technical replicate C_q values.
Adjusted C_T/C_{RT} Mean	View the average of the technical replicate C_q values that have been adjusted based on the "Maximum allowed CT" limit defined in the RQ Settings analysis settings. **Note:** Wells with C_q scores greater than the "Maximum allowed CT" value are adjusted to the specified C_q limit.
C_T/C_{RT} SE	View the sample standard deviation of the sample replicate group level C_q values.
$\Delta C_T/\Delta C_{RT}$ Mean	View the arithmetic average of the technical replicate C_q values for the sample replicate group. **Note:** The $\Delta C_T/\Delta C_{RT}$ mean is calculated at the reaction plate level and represents the mean difference between the target C_q values and the endogenous control C_q values for all the technical replicates for that sample that are present on the plate.

Column	Use this column to...
$\Delta C_T/\Delta C_{RT}$ SE	View the sample standard deviation of the sample replicate group level C_q values. **Note:** The $\Delta C_T/\Delta C_{RT}$ SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level C_q value variation between the target and the endogenous control.
F-Factor	View the calculated F-Factor for the replicate group associated with the reference sample.
$\Delta\Delta C_T/\Delta\Delta C_{RT}$	View the calculated $\Delta\Delta C_T$ value for the replicate group associated with the reference sample.
$\Delta\Delta C_T/\Delta\Delta C_{RT}$ ± F-Sigma	View the calculated $\Delta\Delta C_T$ value added to or subtracted from the F-Sigma value calculated for the replicate group associated with the reference sample.
RQ	View the calculated relative level of gene expression for the replicate group that is associated with the test sample.
RQ Min	View the minimum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The minimum includes the variability associated with the endogenous control and targets in only the test samples.
RQ Max	View the maximum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The maximum includes the variability associated with the endogenous control and targets in only the test samples.

Volcano Plot

The volcano plot displays the p-value versus the fold change for each target in a biological group, relative to the reference group. Green and red dots represent targets with a fold change outside (greater or lesser than) the fold change boundary. Compare the size of the fold change (x-axis) to the statistical significance level (y-axis) on the volcano plot.

Note: You must have biological groups assigned (so that p-values can be calculated) before you can view data on the volcano plot.

IMPORTANT! If one or more assay efficiencies are set to <100%, then the Applied Biosystems™ Software performs the gene expression calculation using *equivalent C_q* values, where the software adjusts the C_qs of each target proportionally to achieve equivalent efficiency. The resulting equivalent C_qs calculated for the affected targets reflect the values expected if the assays performed at 100% efficiency.

Below the plot is the Results Details table, showing the following information:

Column	Use this column to...
Biological Group	View the biological group (a unique name or number) to which the sample belongs.
Target	View the ID (a unique name or number) of the nucleic acid sequence targeted by the assay.
C_T/C_{RT} Mean	View the arithmetic average of the technical replicate C_q values.
Adjusted C_T/C_{RT} Mean	View the average of the technical replicate C_q values that have been adjusted based on the "Maximum allowed CT" limit defined in the RQ Settings analysis settings. **Note:** Wells with C_q scores greater than the "Maximum allowed CT" value are adjusted to the specified C_q limit.
ΔC_T/ΔC_{RT} Mean	View the arithmetic average of the technical replicate C_q values for the sample replicate group. **Note:** The ΔC_T/ΔC_{RT} mean is calculated at the reaction plate level and represents the mean difference between the target C_q values and the endogenous control C_q values for all the technical replicates for that sample that are present on the plate.
ΔC_T/ΔC_{RT} SE	View the sample standard deviation of the sample replicate group level C_q values. **Note:** The ΔC_T/ΔC_{RT} SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level C_q value variation between the target and the endogenous control.
$\Delta\Delta C_T$/$\Delta\Delta C_{RT}$	View the calculated $\Delta\Delta C_T$ value for the replicate group associated with the reference sample.
RQ	View the calculated relative level of gene expression for the replicate group that is associated with the test sample.
RQ Min	View the minimum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The minimum includes the variability associated with the endogenous control and targets in only the test samples.

Column	Use this column to...
RQ Max	View the maximum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The maximum includes the variability associated with the endogenous control and targets in only the test samples.
P-Value	View the probability (P-value) that the observed RQ (fold change) in gene expression for the replicate group associated with the test sample is not differentially expressed due to treatment or condition.
Result	View the assignment for the replicate group that is associated with the test sample, where possible states are: down-regulated, up-regulated, insignificant, or flat.

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.

Table 1 Box Plot Well Table

Column	Definition
Sample	The ID (a unique name or number) of the sample.
Biological Group	The biological group (a unique name or number) to which the sample belongs.
Mean	The arithmetic average of the technical replicate C_T values.
Min	The minimum technical replicate C_T value for the test sample calculated using the confidence level set in the analysis settings.
Max	The maximum technical replicate C_T value for the test sample calculated using the confidence level set in the analysis settings.

Column	Definition
Median	The median C_T value for the technical replicates of the sample.
Q1	The 1st Quartile (Q_1) for the sample replicate group, which is calculated as the numeric midpoint between the lowest and median C_T values of the technical replicates. **Note:** The 1st Quartile defines lower boundary of the inter-quartile region (IQR), which is defined as the difference between the 3rd and 1st quartile.
Q3	The 3rd Quartile (Q_3) for the sample replicate group, which is calculated as the numeric midpoint between the median and maximum C_T values of the technical replicates.

Table 2 Relative Quantitation Plot Well Table

Column	Definition
Target	The ID (a unique name or number) of the nucleic acid sequence targeted by the assay.
Sample	The ID (a unique name or number) of the sample.
Biological Group	The biological group (a unique name or number) to which the sample belongs.
Max C_T/C_{RT}	The maximum C_q defined by the "Maximum allowed CT" limit in the RQ Settings analysis settings.
C_T/C_{RT} Mean	The arithmetic average of the technical replicate C_q values.
Adjusted C_T/C_{RT} Mean	The average of the technical replicate C_q values that have been adjusted based on the "Maximum allowed CT" limit defined in the RQ Settings analysis settings. **Note:** Wells with C_q scores greater than the "Maximum allowed CT" value are adjusted to the specified C_q limit.
C_T/C_{RT} SE	The sample standard deviation of the sample replicate group level C_q values.
ΔC_T/ΔC_{RT} Mean	The arithmetic average of the technical replicate C_q values for the sample replicate group. **Note:** The ΔC_T/ΔC_{RT} mean is calculated at the reaction plate level and represents the mean difference between the target C_q values and the endogenous control C_q values for all the technical replicates for that sample that are present on the plate.
ΔC_T/ΔC_{RT} SE	The sample standard deviation of the sample replicate group level C_q values. **Note:** The ΔC_T/ΔC_{RT} SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level C_q value variation between the target and the endogenous control.

Column	Definition
F-Factor	The calculated F-Factor for the replicate group associated with the reference sample. The F-Factor is used to calculate the RQ confidence intervals in the $\Delta\Delta C_T$ calculation (displayed as error bars in the RQ plot). The value is calculated differently depending on the RQ Settings in the analysis settings for your project. • If you specified a **Confidence level** setting, then the F-Factor is the *t-value* of the Student's t-distribution calculated from the: – *Degrees of freedom* that characterize the distribution of the replicate population. If the project contains no biological groups, degrees of freedom is calculated per Technical Test Sample as: #Technical Replicates $Wells_{Target}$ + #Technical Replicates $Wells_{Endo}$ - 2. Otherwise, the value is calculated per Biological Test Sample as: #Technical Replicates Groups $_{Test\ Sample}$ - 1. – *Probability* associated with the two-tailed Student's t-distribution (determined by the RQ Settings in the analysis settings for your project). • If you specified a **Limit by standard deviations** setting, then the F-Factor is equal to the setting (1, 2, or 3).
$\Delta\Delta C_T/\Delta\Delta C_{RT}$	The calculated $\Delta\Delta C_T$ value for the replicate group associated with the reference sample.
$\Delta\Delta C_T/\Delta\Delta C_{RT}$ ± F-Sigma	The calculated $\Delta\Delta C_T$ value added to or subtracted from the F-Sigma value calculated for the replicate group associated with the reference sample.
RQ	The calculated relative level of gene expression for the replicate group that is associated with the test sample.
RQ Min	The minimum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The minimum includes the variability associated with the endogenous control and targets in only the test samples.
RQ Max	The maximum relative level of gene expression in the test samples calculated using the confidence level set in the analysis settings. **Note:** The maximum includes the variability associated with the endogenous control and targets in only the test samples.

9 Quality flags

AMPNC (Amplification in negative control) quality flag

The AMPNC (NC) 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 [ΔRn vs. Cycle (Linear) or ΔRn 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	• 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	• 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 (ROX) 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 Cq 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 Cq 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 (Cq 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 (↓) 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 ΔRn due to abnormal baseline) quality flag

The DRNMIN ( ) quality flag indicates that the normalized fluorescence (ΔRn) 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 ΔRn 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 µL 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 (ROX) 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.

MAXCT (C_q above maximum) quality flag

The MAXCT (C_T) quality flag indicates that the mean C_q for the replicate group is above the maximum allowed value.

The maximum allowed value is set in the Flag Settings tab of the Analysis Settings dialog box. If the mean C_q of the replicate group is above the maximum, the software adjusts it to the maximum allowed value (shown in the Adjusted C_q column in the well table).

MPOUTLIER (ΔC_q outlier in multiplex experiment) quality flag

The MPOUTLIER quality flag indicates that the ΔC_T for the targets in the well is less than the current flag setting.

If a replicate group is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification plot [ΔRn vs. Cycle (Linear) or ΔRn vs. Cycle (Log)] for both targets in the well, and make sure that they both amplified.
3. Only for experiments analyzed with the Baseline Threshold algorithm, set the baseline and threshold values manually.
4. Click **Analyze** to reanalyze the project.
5. Evaluate the results. If the adjustments do not produce a valid C_q, consider omitting the well from analysis.

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

The MTP (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 [ΔRn vs. Cycle (Linear) or ΔRn 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.	• 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 (RGO) 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 [ΔRn vs. Cycle (Linear) or ΔRn 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: • 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 Cq/Ct) 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 (SPK) 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 [ΔRn vs. Cycle (Linear) or ΔRn 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 (THD) 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 [ΔRn vs. Cycle (Linear) or ΔRn 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.

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

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Glossary

assay information files

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.

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.

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.

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.

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amplification efficiency (EFF%)

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:

- 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

Display of data collected during the cycling stage of PCR amplification. The amplification plot can be viewed as:

- Baseline-corrected normalized reporter (ΔRn) vs. cycle
- Normalized reporter (Rn) vs. cycle

analysis group

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.

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_q).
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 (ΔRn)	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 ΔRn vs Cycle amplification plot, ΔRn is calculated at each cycle as: ΔRn (cycle) = Rn (cycle) - Rn (baseline), where Rn = 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.
box plot	Display of the distribution of C_q values in each sample or for each target. For each box in the plot: • The solid box shows the range of the middle 50% of the C_q values for the target or the sample. • The horizontal black bar shows the median C_q value. • The black circle shows the mean C_q value. • The ends of the vertical lines (or "whiskers") show the maximum and minimum C_q values, unless outliers are present.

- Mild outliers are displayed as open circles and represent samples or targets with C_q values up to 1.5X the inter-quartile region (IQR). The IQR is the difference between the 3rd quartile and the 1st quartile. There is one circle for each C_q in this range.
- Extreme outliers are displayed as open triangles and represent samples or targets with C_q values up to 3.0X the IQR. There is one triangle for each C_q in this range.

comparative C_T ($\Delta\Delta C_T$) method

Method for determining relative target quantity ("RQ" or fold change) in samples. The software measures amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the endogenous control or global normalization. The software determines the RQ (fold change) of target in each sample by comparing normalized target quantity in each sample to normalized target quantity in the reference sample.

correlation coefficient

A measure of the strength of the linear relationship between two variables. The software calculates the correlation coefficient (r) for either C_q or ΔC_q for all targets in a pair of samples or a pair of biological groups, for all samples and biological groups in the study. *r* can range from -1 to 1.

- If r is close to 0, there is no relationship between the C_q values for the two samples or groups.
- If r is positive, as the C_q value for one sample (or groups) increases, so does the other.
- If r is negative, as the C_q value for one sample (or group) increases, the other decreases (often called an "inverse" correlation).

C_{RT}

See relative threshold cycle (C_{RT}).

C_{RT} algorithm

See Relative Threshold algorithm.

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.

delta Rn (ΔRn)

See baseline-corrected normalized reporter (ΔRn).

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.

endogenous control

A gene (or genes) used to correct for different amounts of starting material of RNA. Many genes can be candidates for endogenous controls, but the consistency of expression in different samples and different treatments should be validated experimentally.

Global normalization is an alternate normalization method.

For more information about selecting an endogenous control, see the application note *Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies* (Pub. no. CO16806) available from the Thermo Fisher Scientific web site.

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.

gene expression plot

Display of RQ versus sample or RQ versus target. RQ for the targets in the reference sample or group is always 0. The error bars indicate RQ minimum and maximum.

global control mean

The mean C_q of the endogenous controls for the project. If you are using global normalization, the global control mean is the median of all values used for global normalization.

global normalization

A method to correct for different amounts of starting material of RNA. Global normalization first finds the assays common to every sample and then uses the median C_q of those assays as the normalization factor, on a per sample basis. It has been shown that this type of normalization is only valid if a large number (384 or greater) of genes are profiled. As an alternative to global normalization, one or more targets can be selected as the endogenous control.

Global normalization is described in Mestdagh P.,Van Vlierberghe P., De Weer A., *et al.* 2009. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biology* 10, R64.

heat map

A representation of the level of expression of many targets (genes) across a number of comparable samples. The targets and samples are clustered according to the similarity of their gene expression, using unsupervised hierarchical clustering. The color indicates a change from the mean ΔC_T (ΔC_{RT}) value. Red or yellow is an increase, green or blue is a decrease.

The "zero" point for the color scale (representing no change in expression) is set differently for each plot type:

- **Global (ΔC_T)** - The mean ΔC_T for all targets in the project.
- **Global (ΔC_T Plus)** - For studies using an endogenous control, the median of all (ΔC_T + the global control mean) values for all targets in the project. For studies using global normalization, the median of all (ΔC_T + the global control median) values for all targets in the project.
- **Target-centric** - The median of all ΔC_T values for all samples for that target (data points for a given target can only be compared relative to other data points for that target).
- **Sample-centric** - For each sample, the middle expression is set as the mean ΔC_T for all targets in the sample.

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 quantity — The average C_q of the target gene less the average C_q of the endogenous control(s) or normalization factor.

normalized quantity mean — The relative standard curve equivalent of the ΔC_T (or ΔC_{RT}) mean value found in comparative C_T experiments (computed as the geometric mean).

normalized quantity SE — The relative standard curve equivalent of the ΔC_T (or ΔC_{RT}) SE value found in comparative C_T experiments (computed as the geometric standard error of the mean).

normalized reporter (Rn)	Fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference (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 to not contain the data from experiments uploaded to the repository; they link the data for analysis without affecting the original data files.
p-value	The probability that the observed RQ (fold change) differs from the null hypothesis by chance. For a gene expression project, the null hypothesis is: the gene is not differentially expressed due to the treatment or condition. In other words, the p-value is the probability that RQ ≠1 is not due solely to chance. Traditionally, researchers reject a hypothesis if the p-value is less than 0.05. • A low p-value indicates there is evidence against the null hypothesis, and thus more evidence that the gene is differentially expressed. • A high p-value indicates little or no evidence against the null hypothesis, thus less evidence that the gene is differentially expressed.
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.

reference sample	In comparative C_T ($\Delta\Delta C_T$) experiments or gene expression studies, the sample used as the basis of comparison for relative quantification results. Also called the calibrator. For a gene expression project using biological groups, a biological group is used as the reference rather than a sample.
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.
Relative Threshold algorithm	Well-based analysis (C_{RT}) based on the PCR reaction efficiency and fitted to the amplification curve.
relative threshold cycle (CR_T)	The PCR cycle number for the threshold calculated from the modeled amplification efficiency profile.
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 *normalized reporter (Rn)*.
ROX dye	A dye used as the passive reference.
RQ minimum and maximum	The relative target quantity (RQ) minimum and maximum and values define the error associated with the reported RQ value for a target. These values are computed using confidence or standard deviation: • Confidence: – RQ_{min}= 2 -(RQ-SE) – RQ_{max}= 2 -(RQ+SE) where SE is the standard error for the RQ. • Standard deviation: – RQ_{min}= 2 -(RQ-SD) – RQ_{max}= 2 -(RQ+SD) where SD is the standard deviation for the RQ.
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.

scatter plot

Display showing the correlation between C_q or ΔC_T (C_q or ΔC_{RT}) for targets in a pair of samples or biological groups. The scatter plot represents Pearson's product-moment correlation coefficient (r) for each target for a pair of samples or biological groups.

- Targets on or along the line of reference indicate a correlation between the samples or biological groups.
- Targets that are scattered in the plot, away from the line of reference, indicate weak or no correlation.

The line of reference is fixed in the software.

score

A measure of the stability of the expression of a candidate or endogenous control compared to all the other selected candidate or endogenous controls. The score is the average pairwise variation of the selected candidate or endogenous control compared to all the other candidate or endogenous control genes. The lower the score, the more stable the expression of that target relative to all other targets in the comparison.

The score is calculated as follows:

- For control *i*, calculate ΔC_T (or $\Delta C_{RT})_{ij}$ for all samples using another control *j* as the normalizer, and calculate the standard deviation (SD_{ij}) of the ΔC_T (or $\Delta C_{RT})_{ij}$ values
- Repeat the calculation for all other candidate controls, j = 1…N-1 and use the average of all SD_{ij}'s as the stability score for control *i*.

Note: A minimum of two controls are needed to calculate a score. Because the score is relative to other controls, when you only have two controls, the score is the same for both controls.

For more information, see Vandesompele J., De Preter K., Pattyn F., *et al.* 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* 3, research0034.

signal correlation plot

Display of the correlation coefficient (r) for every pair of samples or biological groups in the project. Each cell represents a different scatter plot, colored to indicate the strength of the correlation.

- Red cells represent a low absolute value for r (|r|), indicating low correlation (either negative or positive) between samples or groups.
- Green cells represent a high absolute value for r (|r|), indicating high correlation (either negative or positive) between samples or groups.

target

The nucleic acid sequence to amplify and detect.

target color

In the software, a color assigned to a target to identify the target in the Endogenous Controls plot and analysis plots.

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 *automatic threshold*), or it can be set manually (see *manual threshold*).
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.
volcano plot	A display of p-value (biological significance) versus fold change (statistical significance) for targets in a biological group compared to the reference group. The plot has boundaries indicating a specified fold change and p-value. Targets with fold change outside the specified fold change are colored, making it easy to identify significant changes in gene expression.