

# Applied Biosystems™ Presence Absence Analysis Module USER GUIDE

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# Getting Started

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

## About the software

The Presence/Absence (PA) module of the Applied Biosystems™ Analysis Software performs analysis of presence/absence experiments run on Applied Biosystems™ real-time qPCR instruments. The presence/absence method is used to determine the presence of a target sequence in samples.

PCR reactions for presence/absence experiments contain primers and probes that amplify the target and a reagent to detect amplification of the target. The reactions can be set up in three different ways:

- **IPC setup** – Use an internal positive control (IPC) to monitor the PCR progress and ensure that a negative result is not caused by failed PCR in the sample. PCR reactions contain two primer/probe sets: One to detect the unknown target (unknown target primer set and TaqMan probe to detect the unknown target) and one to detect the IPC (IPC primer set and a VIC dye-labeled TaqMan probe to detect the IPC template). With this setup, there are three well types:
  - **Unknown-IPC wells** – Wells contain sample template and IPC template; the presence of the target is not known.
  - **Negative control-IPC wells** – Wells contain IPC template and water or buffer instead of sample template in the PCR reaction. Only the IPC template should amplify in negative control-IPC wells because the reaction contains no sample template. Also called IPC+.
  - **Negative control-blocked IPC wells** – Wells contain IPC blocking agent instead of sample template in the PCR reaction. No amplification should occur in negative control-blocked IPC wells because the reaction contains no sample template and amplification of the IPC is blocked. Also called no amplification control (NAC).
- **No IPC, singleplex setup** – Use Advanced Setup to omit the IPC from your presence/absence experiment. PCR reactions contain one primer/probe set. PCR reactions do not contain the IPC. With this setup, there are two well types:
  - **Unknown wells** – Wells contain sample template; the presence of the target is not known.
  - **Negative controls** – Wells contain water or buffer instead of sample template.
- **No IPC, multiplex setup** – Use Advanced Setup to omit the IPC from your presence/absence experiment and detect two targets in one reaction. PCR reactions contain two primer/probe sets. PCR reactions do not include the IPC. With this setup, there are two well types:
  - **Unknown-Unknown wells** – Wells contain sample template; the presence of the target is not known.
  - **Negative control-Negative control wells** – Wells contain water or buffer instead of sample template.

### Required experiment components

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

- **Samples** – The sample in which the presence of the target is unknown.
- **Internal Positive Controls** – A sample that is known to contain the target.
- **Replicates** – Identical reactions containing the same 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.

## About the analysis

The Applied Biosystems™ Analysis Software can calculate presence/absence calls either by analyzing the change in normalized fluorescence before and/or after the PCR (the  $R_n/\Delta R_n$  method) or by comparing threshold cycle values generated from the amplification data (the  $C_T/C_{RT}$  method). Depending on your experiment setup and analysis settings, the software generates presence/absence calls using the data collected during multiple points of the real-time experiment:

- **Pre-PCR read** – If using the  $R_n/\Delta R_n$  analysis method, the data collected from the pre-PCR read can be used to normalize data collected during the post-PCR read.
- **Amplification** – If using the  $C_T/C_{RT}$  analysis method, the threshold cycle ( $C_T/C_{RT}$ ) values generated from the data collected during the PCR amplification are used to make presence/absence calls.

**Note:** Regardless of the chosen analysis method, amplification data can be used to troubleshoot the PCR and failed presence/absence calls.

- **Post-PCR read** – If using the  $R_n/\Delta R_n$  analysis method, the data collected from the post-PCR read are used to make presence/absence calls.

Following the analysis, the software summarizes the results in the Presence/Absence (PA) Grid that visualizes the call data. If you are using the  $\Delta R_n$  analysis method, the results are also displayed in a Presence/Absence (PA) Results Plot. In addition, if your experiment includes amplification, you can also view the real-time data within the Amplification Plot.

Based on the presence/absence analysis, the software applies the following calls to unknowns:

- **Presence** – The target amplified above the target's threshold. The target is present in the sample.
- **Absence** – The target did not amplify above the target's threshold. The target is absent in the sample.
- **Unconfirmed** – The target exhibited amplification but was below the target threshold. The target cannot be confirmed and the sample must be retested.

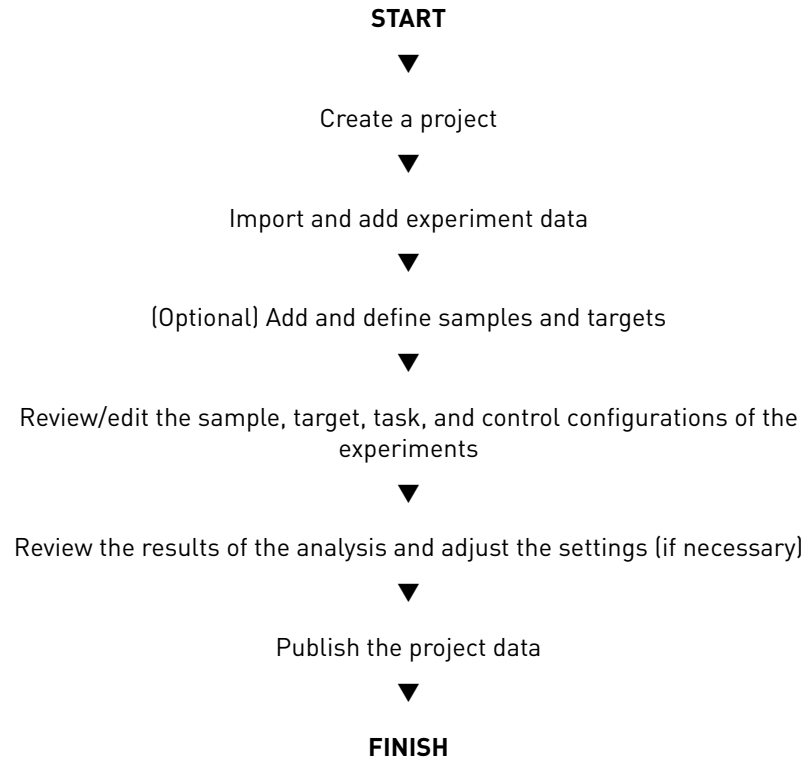
With the IPC setup, the data collected are used to make the following additional calls:

- **IPC Failed** – The IPC target did not amplify in the IPC wells.
- **IPC Succeeded** – The IPC target amplified in the IPC wells.
- **Blocked IPC Control** – The well is designated as a blocked IPC control.



## Analysis workflows

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



## Compatible Real-Time PCR System Data

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

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

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

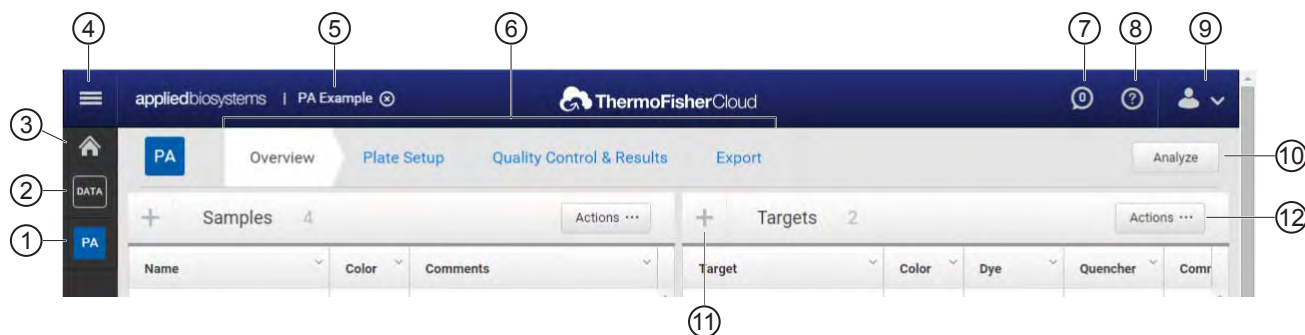
## System requirements

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

Category	Requirement
Web Browser	<ul style="list-style-type: none"><li>• Apple™ Safari™ 8 Browser</li><li>• Google™ Chrome™ Browser Version 21 or later</li><li>• Microsoft™ Internet Explorer™ Browser Version 10 or later</li><li>• Mozilla™ Firefox™ Browser Version v10.0.12 or later</li></ul>
Operating System	<ul style="list-style-type: none"><li>• Windows™ XP, Vista, 7, or 8</li><li>• Macintosh™ OS 8 or later</li></ul>
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 <a href="https://apps.lifetechnologies.com">apps.lifetechnologies.com</a> on HTTPS-443.

## About the software interface

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



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

## Best practices and tips for using the software

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

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

- Refresh your browser regularly
- Clear your browser cache





# Manage experiment data

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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: / \ < > \* ? " | : ; & % \$ @ ^ ( ) !


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

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

- Repeat step 3 until your project contains all of the desired experiment data.
- 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:
  - While viewing your project, click  (Manage Data) from the bar on the left side of the screen.
  - From the Manage Data screen, add any additional experiment data to the project.

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

- 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.




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**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.

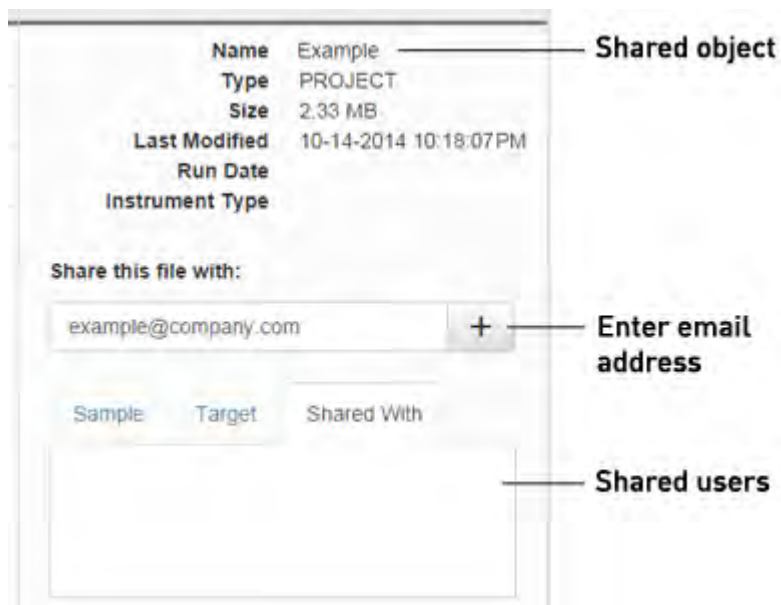
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- **Experiments** – Sharing experiment files with other users grants them full access to the data, allowing them to import the data to their own projects or download the experiment data file.
- **Folders** – Sharing a folder with another user grants 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	
Applied Biosystems™ 7500 and 7500 Fast Real-Time PCR System	v2.0.5 or later	.eds
Applied Biosystems™ StepOne™ and StepOnePlus™ Real-Time PCR System	v2.0.1, v2.1, or later	
Applied Biosystems™ ViiA™ 7 Real-Time PCR System	v1.1 or later	
Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System	v1.1.1 or later	
Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System	v1.0 or later	
Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System		
Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System	v1.0 or later	
Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System		

# 3

## Set up the project

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- Import sample information from design files ..... 19

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

### Manage samples and targets

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

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

## Import 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.

## Import sample information from design files

This Applied Biosystems™ Analysis Software Analysis Module does not support sample import by design file. To populate your project with samples, enter them directly into the Samples list in the Overview screen. For more information on entering sample information, see “Manage samples and targets” on page 18.



# Edit experiment properties

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

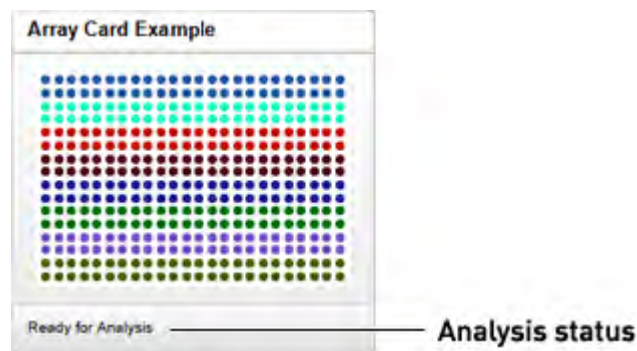
## Review and edit the plate setups

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

To review the plate setup information for your project:

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


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



## Apply samples and targets

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

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

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

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



Sample	NAC
Target	TGFB ×
Task	NTC ▾
Internal Positive Control	IPC ▾ <input checked="" type="checkbox"/> Blocked
Comments	


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

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

## Specify and assign tasks

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

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

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

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

Available tasks include:

- Unknown** – The task for wells that contain a sample with unknown target content.
- 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.




Sample	NAC
Target	TGFB ×
Task	NTC
Internal Positive Control	IPC <input checked="" type="checkbox"/> Blocked
Comments	

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

## Specify and assign controls

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

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

- From the Plate Setup screen, select the experiment that you want to modify.
- From the Edit Plate screen, click **Views** , then select **IPC** to color the plate setup according to control assignment.
- Select the wells of the plate layout to which you want to apply the control.

- When the wells are selected, select the target from the Internal Positive Control dropdown list to designate the well as a control for the related assay. If the well contains a blocked reaction, select **Blocked**.

The screenshot shows a form with the following fields:

- Sample:** A text input field containing "NAC".
- Target:** A dropdown menu with "TGFB" selected and a close button (x).
- Task:** A dropdown menu with "NTC" selected and a downward arrow.
- Internal Positive Control:** A dropdown menu with "IPC" selected and a downward arrow, followed by a checked checkbox and the text "Blocked".
- Comments:** An empty text input field.

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

## Presence absence controls

The Applied Biosystems™ Analysis Software supports multiple control configurations for presence/absence analysis. The following are common approaches for the arrangement of control and unknown assays for experiments:

**IPC setup** – An internal positive control (IPC) is used to monitor the PCR progress and ensure that a negative result is not caused by failed PCR in the sample. Each PCR reaction contains two primer/probe sets: one to detect the unknown target and one to detect the IPC template.

Well types include:

- **Unknown-IPC wells** – Contain sample template and IPC template; the presence of the target is not known.
- **Negative control-IPC wells** – Contain IPC template and water or buffer instead of sample template in the PCR reaction. Only the IPC template should amplify in negative control-IPC wells because the reaction contains no sample template. Also called IPC+.
- **Negative control-blocked IPC wells** – Contain IPC blocking agent instead of sample template in the PCR reaction. No amplification should occur in negative control-blocked IPC wells because the reaction contains no sample template and amplification of the IPC is blocked. Also called no amplification control (NAC).

**No IPC, singleplex setup** – Each PCR reaction contains one primer/probe set. PCR reactions do not use an IPC.

Well types include:

- **Unknown wells** – Contain sample template; the presence of the target is not known.
- **Negative controls** – Contain water or buffer instead of sample template.



**No IPC, multiplex setup** – Each PCR reactions contain two primer/probe sets, but the reactions do not use an IPC. Well types include:

- **Unknown-Unknown wells** – Contain sample template; the presence of the target is not known.
- **Negative control-Negative control wells** – Contain water or buffer instead of sample template.

## Assign a passive reference dye for an experiment

If the passive reference assignments of one or more of your experiments are set improperly, you can use the Applied Biosystems™ Analysis Software to correct the problem prior to analysis.

1. When viewing a project, click **Plate Setup** at the top of the screen to view the Plate Setup screen.
2. From the Plate Setup screen, select the experiment that you want to modify.
3. From the Edit Plate screen, select the passive reference from the **Passive Ref Dye** drop-down list.
4. When finished making changes to the plate layout, click **Analyze** to reanalyze your project.

## Apply plate setup information using a template file

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

**Note:** For detailed information on the structure of template files, see “Template files” on page 27.

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 27 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.

---

## Set the PCR stage for an experiment

If the PCR stage is set incorrectly for one or more experiments in your project, you can use the Applied Biosystems™ Analysis Software to assign the PCR stage prior to analysis.

1. When viewing a project, click **Plate Setup** at the top of the screen to view the Plate Setup screen.
2. From the Plate Setup screen, select the experiment that you want to modify.
3. From the Edit Plate screen, select the correct stage of the PCR from the **PCR Stage/Step** drop-down list.
4. When finished 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	F	G
<b>Experiment data (do not edit):</b>	1	* Block Type = 96-Well						
	2	* Experiment Type = Presence Absence						
	3	* Instrument Type = QuantStudio 12K Flex Real-Time PCR System						
	4	* No. Of Wells = 96						
	5	* Set Up Well Section Info =						
<b>Column headings (do not edit):</b>	6	Well	Well Position	Sample Name	Task	Target Name	Reporter	Quencher
<b>Plate setup content (add well data in any order):</b>	7	0	A1	Sample 3	BLOCKED_IPC	IPC	VIC	TAMRA
	8	1	A2	Sample 3	NTC	TGFB	FAM	NFQ-MGB
	9	2	A3	Sample 3	BLOCKED_IPC	IPC	VIC	TAMRA
	...	...	...	...	...	...	...	...

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

For each well the file contains the following information:

- **Column A (Well)** – The numerical position of the well on the plate, where wells are numbered left to right and top to bottom. For example, on a 96-well plate, the number of well A1 is "0" and the number of well G12 is "95".
- **Column B (Well Position)** – The coordinates of the well on the plate.
- **Column C (Sample Name)** – The name of the sample within the well (up to 256-characters).
- **Column D (Task)** – The task of the sample within the well, where acceptable values include UNKNOWN, NTC, and your custom names for positive controls.
- **Column F (Reporter)** – The name of the reporter dye present in the well.
- **Column G (Quencher)** – The name of the quencher dye present in the well.
- 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.



# Review the quality data and results

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- Perform manual calls ..... 34
- Omit wells from the analysis ..... 35

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

## Configure the analysis settings

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

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

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

Group	Settings
Cq Settings	<p>Select the method (C<sub>T</sub> or C<sub>RT</sub>) that the Applied Biosystems™ Software will use to compute the Cq (C<sub>T</sub>) values for the analysis:</p> <ul style="list-style-type: none"> <li><b>CT method</b> – 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.</li> <li><b>C<sub>RT</sub> method</b> – Specify the cycle number that the software will use as the minimum parameter in defining the relative threshold for the C<sub>RT</sub> calculation.</li> </ul> <p><b>Note:</b> The Applied Biosystems™ Software allows you to import the Cq settings from a file exported from another project (click <b>Import CT Settings</b> to import the file).</p>
Advanced C <sub>T</sub> Settings	<p>Define whether individual wells will use automatic baselining. If you choose to use manual settings for one or more wells, enter the manual start and end baseline values for the appropriate targets.</p>
Flag Settings	<p>Specify the quality measures that the Applied Biosystems™ Software will compute during the analysis.</p> <ol style="list-style-type: none"> <li>In the Use column, select the check boxes for flags you want to apply during analysis.</li> <li>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.</li> </ol> <p><b>Note:</b> If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.</p> <ol style="list-style-type: none"> <li>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.</li> </ol>
Presence Absence Settings	<p>Select the method that the Applied Biosystems™ Software will use to compute the presence/absence calls for the analysis (C<sub>T</sub>/C<sub>RT</sub> or Rn/dRn):</p> <ul style="list-style-type: none"> <li><b>Use C<sub>T</sub>/C<sub>RT</sub></b> – Select to use the threshold cycle (C<sub>T</sub>) or relative threshold cycle (C<sub>RT</sub>) values to determine the presence/absence calls. If selected, choose the rule to apply the calls: <ul style="list-style-type: none"> <li>Select <b>All target level calls are presence then well level call is presence</b> to have the software assign "presence" calls only to wells that have "presence" calls for <i>all</i> assays assigned to them. For example, if a well is evaluating three targets, the software will apply a "presence" call to the well only if it achieves presence calls for target 1, target 2, <i>and</i> target 3.</li> <li>Select <b>One target level call is presence then well level call is presence</b> to have the software assign "presence" calls to wells that have presence calls for <i>any</i> assay assigned to them. For example, if a well is evaluating three targets, the software will apply a "presence" call to the well if it achieves a presence call for target 1, target 2, <i>or</i> target 3.</li> <li>Select <b>50% target level calls are presence then well level call is presence</b> to have the software assign "presence" calls to wells that have "presence" calls for ≥50% of the assays assigned to them.</li> </ul> </li> </ul>

Group	Settings
	<p>For example, if a well is evaluating three targets, the software will apply a "presence" call for the well only if the well achieves presence calls for 2/3 of the targets.</p> <p>For each target present in the project, use the <b>Call Setting</b> slider to set the <math>C_T</math> threshold that will be used to make presence/absence calls for the target.</p> <p><b>Note:</b> If desired, enter a default <math>C_T</math> value in the <b>Set default Ct</b> field to uniform setting to all targets.</p> <ul style="list-style-type: none"> <li>• <b>Use Rn/dRn</b> – Select to use the normalized fluorescence (Rn/dRn) values to determine the presence/absence calls. If selected, choose the rule to apply the calls: <ul style="list-style-type: none"> <li>– Select <b>Analyze Data from Post-PCR Read Only</b> to use data (Rn) only from the post-PCR read to determine presence/absence calls.</li> <li>– Select <b>Analyze Data from Pre-PCR Read and Post-PCR Read</b> if you included a pre-PCR read in your experiments and you want to use data (dRn) from both the pre- and post-PCR reads to determine presence/absence calls.</li> </ul> </li> </ul> <p>For each target present in the project, use the <b>Confidence</b> dropdown list to set the confidence interval that will be used to make presence/absence calls for the target, where the call is "unconfirmed" if the confidence value is less than the call setting.</p> <p><b>Note:</b> If desired, enter a default confidence value in the <b>Set default confidence</b> field to apply a uniform setting to all targets.</p>

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

## Review the quality data

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

1. If you have not already done so, click **Analyze** to analyze your project.
2. In the Applied Biosystems™ Software, click **Quality Control & Results** to view the Quality Control & Results screen.
3. Review the PA Grids for quality flags.

**Note:** The Applied Biosystems™ Software displays the total number of quality flags generated for a plate in the margin beneath the related PA Grid. You can view the identity of the quality data by mousing over the PA Grid of interest.
4. If flags or irregularities are present, or you would like to review the presence/absence for a specific experiment, click the PA Grid of interest to zoom the display.

## 5. View and modify the data in the Well table:

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

## 6. Review the data in the Well table data.

Column	Description
Well	The location of the well on the reaction plate. For example, P18 indicates that the sample is found in row P, column 18.
Omit	The omission status of the related well.
Target	The ID (a unique name or number) of the nucleic acid sequence targeted by the assay added to the well.
Sample	The ID (a unique name or number) of the sample.
$\Delta Rn/ C_t/ C_{RT}$	The $\Delta Rn$ , $C_T$ , or $C_{RT}$ calculated for the related well.
Call	The presence/absence call assigned to the well.
Manual	Indicates whether the sample was called manually.
Target Call	( $C_T/C_{RT}$ analysis only) The presence/absence call assigned to the target assigned to the well.
Well Call	( $C_T/C_{RT}$ analysis only) The presence/absence call assigned to the well according to the rules selected in the analysis settings.
Amp Status	The amplification status for the well: amplification, no amplification, reviewed, and undetermined.
Amp Score	The amplification score calculated for the well.
Cq Conf	The Cq confidence score calculated for the well.



Column	Description
Task	The task assigned to the well. A task is the function that a sample performs: <ul style="list-style-type: none"> <li>Blocked internal positive control (BLOCKED_IPC)</li> <li>Internal positive control (IPC)</li> <li>UNKNOWN</li> <li>No template control (NTC)</li> </ul>
$\Delta Rn/Ct$ Mean	The arithmetic mean generated from the $\Delta Rn$ , $C_T$ , or $C_{RT}$ values calculated for the technical replicates of the well.
$\Delta Rn/Ct$ SD	The standard deviation generated from the $\Delta Rn$ , $C_T$ , or $C_{RT}$ values calculated for the technical replicates of the well.
Dye	The reporter dye associated with the assay/target.
$C_{RT}$ Start	( $C_{RT}$ analysis only) The starting cycle used to perform the $C_{RT}$ analysis.
Flags	The number of flags generated for the well.
Quality data	The quality flags generated by the associated well.

7. Review the PA Results Plot as needed (see “PA Results” on page 45 for more information).

8. Review the amplification plots as needed (see “Amplification Plot” on page 41 for more information on the plot and typical signal characteristics).


When reviewing the amplification data, look for:

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

9. When ready, click **Multicomponent** to review the multicomponent plot as needed (see “Multicomponent Plot” on page 42 for more information on the plot).

When reviewing the multicomponent plot, look for:


- Consistent fluorescence of the passive reference. The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- Consistent fluorescence of the reporter dye. The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- Irregular fluorescence. There should not be any spikes, dips, and/or sudden changes in the fluorescence.
- No amplification in negative control wells. There should not be any amplification in the negative control wells.

10. When ready, click  to return to the thumbnails view.

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

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

### About the quality data summary

The quality summary displays a table of the experiments included in the current analysis and the number of quality flags generated by the associated data. To display the summary, click **Quality Check & Results** at the top of the screen when viewing a project, then click .

**Note:** To examine the data that triggered a quality flags, click the link in the Name column to view the amplification data for the related plate.

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

- Change the quality settings in the analysis settings:
  - 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.

## Perform manual calls

Perform manual calls when you want to assign a presence/absence call to one or more samples:

1. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
2. In the Quality Control & Results pane, select the experiment of interest.
3. In the Review Result screen, select one or more wells within a PA Grid or Well Table.
4. Click **Actions**, then select **Manual Call**.  
In the Well Table tab, in the Method column, Manual appears next to the selected sample.
5. Repeat the steps above to assign more manual calls.
6. Click **Analyze** to reanalyze the data using the manual calls.

## 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).




# Export the results

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- Export plots for presentation and publication . . . . . 38
- Export data for use in other projects . . . . . 39

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

## Export the analyzed data from a project

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

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


**Note:** Naming the report will allow you to repeat the export if you need to do so again.
  - b. Select the file type for the exported data:
    - **.txt** - To export data to a tab-delimited text file.
    - **.csv** - To export data to a comma-separated text file.
  - c. (*CSV and TXT exports only*) Select the check boxes for the data that you want to export.
    - **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  $\Delta Rn$  values.
    - **Call Results** - Exports the results from the presence/absence results, including all call data.
    - **Analysis Settings** - Exports the analysis settings configurations used to generate the analyzed data, including the threshold settings for individual QC flags.
    - **QC Summary** - Exports a summary of the quality metrics (flags) generated by the data analysis.

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



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

## Export data for use in other projects

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

- Export a template file

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

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



# Screens and plots

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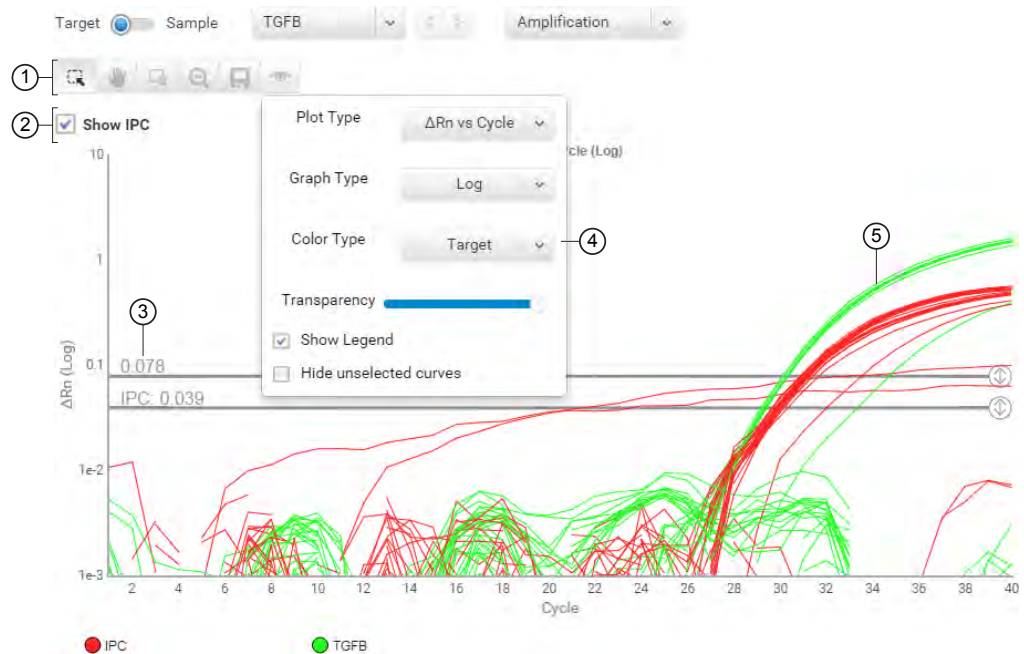
The Applied Biosystems™ Analysis Software provides the following screens and plots that can be used to edit and visualize experiment setups and results that have been added to your project.



## Amplification Plot

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

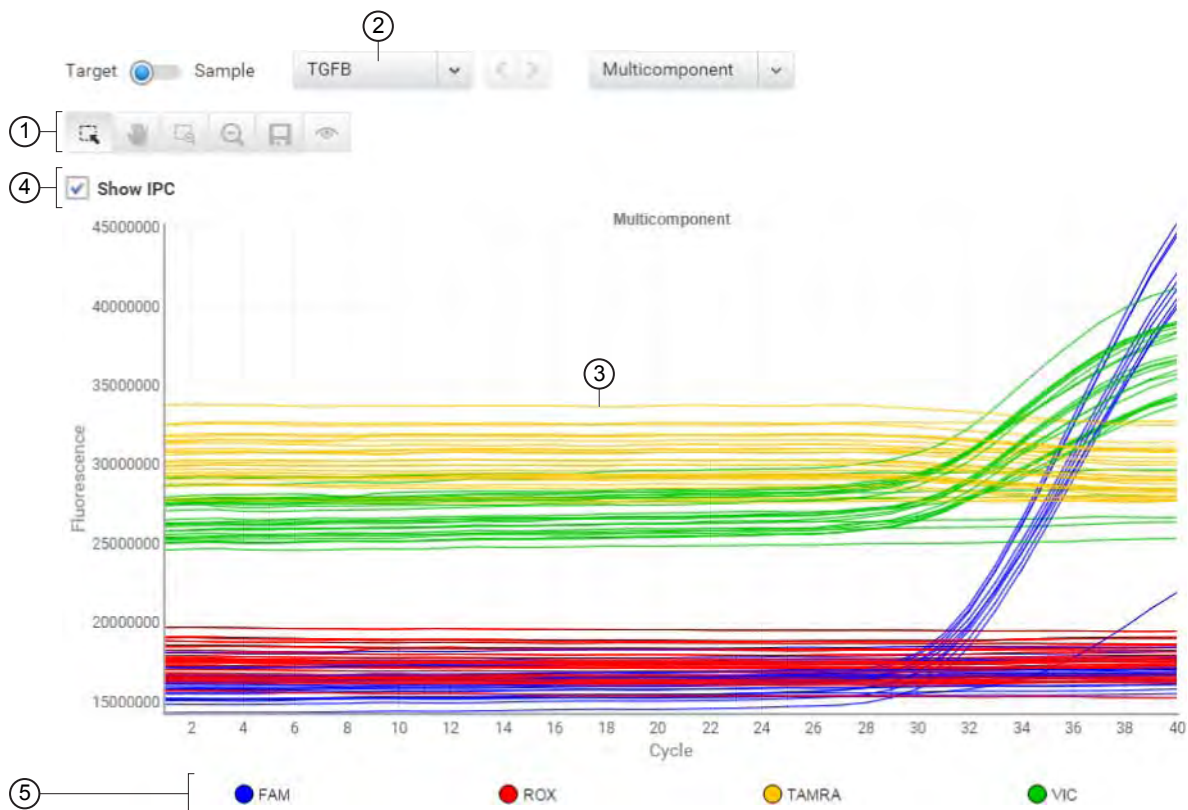
- **$\Delta R_n$  vs Cycle** –  $\Delta R_n$  is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification ( $\Delta R_n = R_n - \text{baseline}$ ). This plot displays  $\Delta R_n$  as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **$R_n$  vs Cycle** –  $R_n$  is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference. This plot displays  $R_n$  as a function of cycle number. You can use this plot to identify and examine irregular amplification.
- **$C_T$  vs Well** –  $C_T$  ( $C_q$ ) is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays  $C_T$  as a function of well position. You can use this plot to locate outlying amplification (outliers).



- ① **Toolbar** – Contains the following tools for controlling the plot:
  - Select individual data points from the plot.
  - Allows you to click and manually move the position of the plot.
  - Zoom the plot to the selected area.
  - Zooms out the plot to show all data points.
  - Saves the plot as an image (.png or .jpg).
  - Allows you to adjust the display options for the plot.
- ② **Show IPC** checkbox – Click to toggle the presence of the internal positive control (IPC) data within the plot.
- ③ **Threshold** – The threshold (calculated or manual) that is currently applied to the project data.
- ④ **View Options** – The view options for the Amplification Plot. Use the drop-down lists to display the type of plot displayed by the software ( **$\Delta R_n$  vs Cycle**,  **$R_n$  vs Cycle**, or  **$C_T$  vs Well**), the scale of the y-axis (**log** or **linear**), and the color scheme for the plot.
- ⑤ **Amplification curves** – Normalized fluorescence for individual wells throughout the course of the thermal cycling protocol.

## Multicomponent Plot

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



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

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

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

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

④ **Show IPC** – Click to toggle the presence of the internal positive control (IPC) data within the plot.

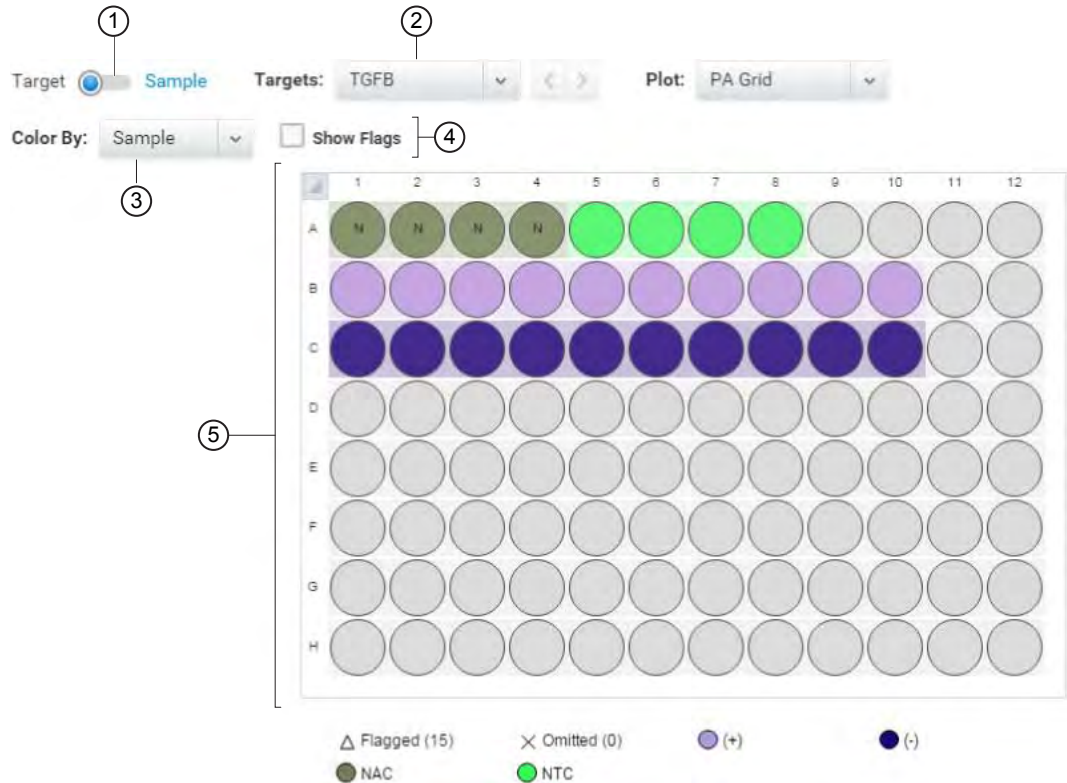
⑤ **Legend** – Fluorescent dyes present in the analyzed data.

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.

## PA Grid

The Presence/Absence (PA) Grid displays the assay-specific setup and analysis properties for the plate document in a well format corresponding to the type of reaction plate used for the run. The PA Grid can be customized in a variety of ways to visualize different aspects of the results and quality data. The following figure displays the default view of the PA Grid.



- ① **Target/Sample switch** – Select an option to toggle the data view of the PA Grid between target and sample assignment.
- ② **Target/Sample drop-down list** – After selecting an option using the Target/Sample switch, select a target or sample from the list to view the associated data within the PA Grid.
- ③ **View drop-down list** – Adjusts the color scheme of the PA Grid according to the selection:
  - Well Call** – Select to color the PA Grid wells according to the calls assigned on a per well basis.
  - Target Call** – Select to color the PA Grid wells according to the aggregate call assigned to the wells based on the analysis settings.
  - $\Delta R_n/C_T/C_{RT}$  Range** – Select to color the PA Grid wells according to their calculated  $\Delta R_n$ ,  $C_T$ , or  $C_{RT}$ . Wells for which the software could not determine a  $C_T$  (negative controls (NTC) and undetermined samples) are displayed in white.
- Sample** – Select to color the PA Grid wells according to *sample* assignments.
- Target** – Select to color the PA Grid wells according to *target* assignments.
- Task** – Select to color the PA Grid wells according to *task* assignments.
- ④ **Show Flags checkbox** – Select to toggle the presence of the internal positive control (IPC) data within the plot.
- ⑤ **Plate grid** – A top-down representation of the plate used to run the reactions, where each circle corresponds to an individual well.

When viewing the PA Grid the Results Table displays the following:

Column	Description
Well	The location of the well on the reaction plate. For example, P18 indicates that the sample is found in row P, column 18.
Omit	The omission status of the related well.
Target	The ID (a unique name or number) of the nucleic acid sequence targeted by the assay added to the well.
Sample	The ID (a unique name or number) of the sample.
$\Delta Rn/ C_t/ C_{RT}$	The $\Delta Rn$ , $C_T$ , or $C_{RT}$ calculated for the related well.
Call	The presence/absence call assigned to the well.
Manual	Indicates whether the sample was called manually.
Target Call	( $C_T/C_{RT}$ analysis only) The presence/absence call assigned to the target assigned to the well.
Well Call	( $C_T/C_{RT}$ analysis only) The presence/absence call assigned to the well according to the rules selected in the analysis settings.
Amp Status	The amplification status for the well: amplification, no amplification, reviewed, and undetermined.
Amp Score	The amplification score calculated for the well.
Cq Conf	The Cq confidence score calculated for the well.
Task	The task assigned to the well. A task is the function that a sample performs: <ul style="list-style-type: none"> <li>Blocked internal positive control (BLOCKED_IPC)</li> <li>Internal positive control (IPC)</li> <li>UNKNOWN</li> <li>No template control (NTC)</li> </ul>
$\Delta Rn/C_t$ Mean	The arithmetic mean generated from the $\Delta Rn$ , $C_T$ , or $C_{RT}$ values calculated for the technical replicates of the well.
$\Delta Rn/C_t$ SD	The standard deviation generated from the $\Delta Rn$ , $C_T$ , or $C_{RT}$ values calculated for the technical replicates of the well.
Dye	The reporter dye associated with the assay/target.
$C_{RT}$ Start	( $C_{RT}$ analysis only) The starting cycle used to perform the $C_{RT}$ analysis.
Flags	The number of flags generated for the well.
Quality data	The quality flags generated by the associated well.

## PA Results

If you are using the  $\Delta Rn$  analysis method, the software displays the results of the analysis in the Presence/Absence (PA) Results plot, which contrasts normalized reporter fluorescence ( $\Delta Rn$ ) and well position (where the wells on the reaction plate are numbered left-to-right and top-to-bottom beginning with well 1 at position A1).















- ① **Target/Sample** switch – Toggles the plot between the target-centric to sample-centric views.
- ② **Target/Sample** drop-down list – Selects the target or sample data displayed by the plot.
- ③ **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.
- Note:** Selecting or deselecting the **Show IPC** option in the display options shows or hides the data points for the internal positive controls (IPC).
- ④ **Presence/absence data** – The normalized fluorescence for individual wells, colored according to the calls applied to them.
- ⑤ **Legend** – The calls available in the analysis.
- ⑥ **IPC Threshold** – The threshold value calculated from the Negative Control- Blocked IPC reactions.
- ⑦ **Target Threshold** – The threshold value calculated from the Negative Control- IPC reactions. If the target's intensity is:
  - Above the target threshold, the call is present (regardless of the intensity of the IPC).
  - Below the target threshold, and the IPC's intensity is above the IPC threshold, the call is absent.
  - Below the target threshold, and the IPC's intensity is below the IPC threshold, the call is unconfirmed.

## Well Table

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

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

Column	Description
Well	The location of the well on the reaction plate. For example, P18 indicates that the sample is found in row P, column 18.
Omit	The omission status of the related well.
Target	The ID (a unique name or number) of the nucleic acid sequence targeted by the assay added to the well.
Sample	The ID (a unique name or number) of the sample.
$\Delta R_n$ / $C_t$ / $C_{RT}$	The $\Delta R_n$ , $C_t$ , or $C_{RT}$ calculated for the related well.
Call	The presence/absence call assigned to the well.
Manual	Indicates whether the sample was called manually.
Target Call	( $C_t/C_{RT}$ analysis only) The presence/absence call assigned to the target assigned to the well.
Well Call	( $C_t/C_{RT}$ analysis only) The presence/absence call assigned to the well according to the rules selected in the analysis settings.
Amp Status	The amplification status for the well: amplification, no amplification, reviewed, and undetermined.
Amp Score	The amplification score calculated for the well.
Cq Conf	The Cq confidence score calculated for the well.

Column	Description
Task	The task assigned to the well. A task is the function that a sample performs: <ul style="list-style-type: none"><li>• Blocked internal positive control (BLOCKED_IPC)</li><li>• Internal positive control (IPC)</li><li>• UNKNOWN</li><li>• No template control (NTC)</li></ul>
$\Delta Rn/Ct$ Mean	The arithmetic mean generated from the $\Delta Rn$ , $C_T$ , or $C_{RT}$ values calculated for the technical replicates of the well.
$\Delta Rn/Ct$ SD	The standard deviation generated from the $\Delta Rn$ , $C_T$ , or $C_{RT}$ values calculated for the technical replicates of the well.
Dye	The reporter dye associated with the assay/target.
$C_{RT}$ Start	( $C_{RT}$ analysis only) The starting cycle used to perform the $C_{RT}$ analysis.
Flags	The number of flags generated for the well.
Quality data	The quality flags generated by the associated well.



# Quality flags

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## AMPNC (Amplification in negative control) quality flag

The AMPNC ( $\mathcal{N}^{\text{C}}$ ) 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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)], and confirm the fluorescence signal increased for the flagged negative control well. If the fluorescence signal did not increase, omit the well from analysis.

Possible Cause	Recommended Action
Contamination in one or more PCR reaction components	<ul style="list-style-type: none"> <li>• 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.</li> <li>• Decontaminate the work area and pipettors.</li> </ul>
Unstable reaction mix	<ul style="list-style-type: none"> <li>• Use a hot-start enzyme.</li> <li>• If you are not using a hot-start enzyme, run the reactions as soon as possible after you prepare them.</li> </ul>
Poor primer and/or probe design	Redesign the primers and/or probe.

## AMPSCORE (Low signal in linear phase) quality flag

The AMPSCORE ( $\mathcal{N}^{\text{L}}$ ) 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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)], and check the shape of the curve. If the curve is atypical, consider omitting the flagged well(s) from analysis.

## BADROX (Bad passive reference signal) quality flag

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


If a well is flagged, confirm the results:

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

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

## BLFAIL (Baseline algorithm failed) quality flag

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

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

If a well is flagged, confirm the results:

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

## CQCONF (Calculated confidence in the C<sub>q</sub> value is low) quality flag

The CQCONF (cc) quality flag indicates that the calculated confidence for the C<sub>q</sub>/C<sub>T</sub> 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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)], and check the shape of the curve. If the curve is atypical, consider omitting the flagged well(s) from analysis.

## CTFAIL (C<sub>q</sub> algorithm failed) quality flag


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

The CTFail (f) quality flag indicates that the automatic C<sub>q</sub> algorithm failed for the given well, and the software cannot calculate the threshold cycle (C<sub>q</sub>).

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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)] and check for:
  - Amplification too early
  - Amplification too late
  - Low amplification
  - No amplification
3. If the amplification looks acceptable, set the threshold and baseline manually.
4. Click **Analyze** to reanalyze the data.
5. Evaluate the results. If the adjustments do not produce a valid C<sub>q</sub>, consider omitting the well from analysis.

## DRNMIN (Detection of minimum $\Delta R_n$ due to abnormal baseline) quality flag

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


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

If a well is flagged, confirm the results:

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

## EXPFAIL (Exponential algorithm failed) quality flag


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

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

If a well is flagged, confirm the results:

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

## HIGHSD (High standard deviation in replicate group) quality flag

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

If a replicate group is flagged, confirm the results:

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

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

## IPCFAIL (Abnormal amplification of the internal positive control) quality flag

The IPCFAIL (~~IPC~~) quality flag indicates that the well is designated as a control (NTC, Negative Control, or Positive Control) and it has failed:

- **Positive Control/IPC** – The well has generated a call that is inconsistent with the call that would be assigned by the software to an unknown with the same reporter dye intensities. For example, a positive control for a homozygous genotype (AA) that instead exhibits amplification associated with either the heterozygous (AB) or alternate homozygous (BB) genotypes.
- **Negative Control/NTC** – The software has detected amplification within the negative control.

If a well is flagged, confirm the results:

1. Select the flagged well in the plate layout or well table.
2. View the amplification plot [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)], and review the amplification curve associated with the control assay for abnormalities. Determine whether the control failed to amplify or if the control amplified but failed to cross the detection threshold.
3. Omit the well or ignore the flag if it appears to be inappropriate.

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

## NOAMP (No amplification) quality flag

The NOAMP (AMP) 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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)], and make sure that the fluorescence signal did not increase in the well.
4. View the multicomponent plot, and look for fluorescence signal higher than the background.

Possible Cause	Recommended Action
Missing template.	Repeat the experiment, and make sure to include all reaction components.
Target is not expressed in the sample.	<ul style="list-style-type: none"> <li>• If this occurs in just one sample, it may be correct.</li> <li>• 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.</li> </ul>

## NOISE (Noise higher than others in plate) quality flag

The NOISE (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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)] and check for a noisy amplification curve in the flagged wells.
3. In the multicomponent plot:
  - a. From the Color by drop-down list, select Dye to color the data according to the dye.
  - b. Check for a drop in ROX™ signal relative to the reporter dye and compare flagged wells with unflagged wells.
  - c. If there is a drop in the ROX™ signal compared to the reporter dye, consider omitting the flagged well(s) from analysis.

## NOSAMPLE (No sample assigned to well) quality flag

The NOSAMPLE (su) 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 (E) 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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)] or the well table, and note the threshold cycle.
3. View the multicomponent plot, and review the data for a plateau over one or more cycles. A plateau indicates saturation of the instrument's detectors. If the signal plateaus before the threshold cycle, omit the well(s).

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

## OUTLIERRG (Outlier in replicate group) quality flag

The OUTLIERRG (RGD) 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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)], and compare the data from the flagged well to the data from the unflagged replicates. If the  $C_q$  or the amplification curve for the flagged well vary significantly, carefully consider omitting the flagged well from analysis.

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

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

The PRFDROP (PD) quality flag indicates that the fluorescent 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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)], and check the shape of the curve. If the curve is atypical, consider omitting the flagged well(s) from analysis.

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

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

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

If a well is flagged, confirm the results:

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

## REPFAIL (Wells have same sample and targets, but they have different final call) quality flag

The REPFAIL (RPL) quality flag indicates that the designated well is a technical replicate (wells that contain identical sample and PCR reaction components), but the manual or automatic call assigned to the well is different than its siblings. Variations of calls among replicate wells can indicate the presence of one or more outliers within the population.

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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)], and compare the data from the flagged well to the data from the unflagged replicates. If the C<sub>q</sub> or the amplification curve for the flagged well vary significantly, carefully consider omitting the flagged well from analysis.

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

## SPIKE (Noise spikes) quality flag

The SPIKE (S<sup>PK</sup>) 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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)], and evaluate whether the noise spike adversely affects the baseline or  $C_q$ .
3. If the baseline is adversely affected, set the baseline and threshold values manually.
4. Click **Analyze** to reanalyze the data.
5. Evaluate the results. If the adjustments do not produce a valid  $C_q$ , consider omitting the well from analysis.

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

## THOLDFAIL (Thresholding algorithm failed) quality flag

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

The THOLDFAIL (T<sup>HF</sup>) 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 [ $\Delta R_n$  vs. Cycle (Linear) or  $\Delta R_n$  vs. Cycle (Log)], and check for:
  - Amplification too early
  - Amplification too late
  - Low amplification
  - No amplification for all wells with this target
3. If the amplification looks acceptable, set the baseline and threshold manually.



## Chapter 8 Quality flags

*THOLDFAIL (Thresholding algorithm failed) quality flag*

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



# Documentation and support

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

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# Glossary

assay information files	<p>Assay information files are delivered on Information CDs that accompany TaqMan™ assay orders. Each assay information file contains reference information about the associated order and technical details of all assays in the shipment.</p> <p>You can import an assay information file into the Applied Biosystems™ Analysis Software to add supplementary assay information to a project. Assay information files are available in three formats (.html, .txt, and .xml), but the Applied Biosystems™ Analysis Software supports only .txt and .xml files.</p> <hr/> <p><b>IMPORTANT!</b> The assay information file must include an assay ID (in the Assay ID column) for each assay listed in the file. The software matches the assay IDs in the assay information file with the existing assay IDs in the project.</p> <hr/> <p><b>IMPORTANT!</b> When you import an assay information file, information from the file populates the corresponding columns in the Assays list in the Overview screen. All data in the Overview screen are replaced for all assays that are identified in the assay information file. If the assay information file does not contain information for an assay, the existing data in the Overview screen is unaffected.</p> <hr/>
amplification plot	<p>Display of data collected during the cycling stage of PCR amplification. The amplification plot can be viewed as:</p> <ul style="list-style-type: none"><li>• Baseline-corrected normalized reporter (<math>\Delta R_n</math>) vs. cycle</li><li>• Normalized reporter (<math>R_n</math>) vs. cycle</li></ul>
assays	<p>A PCR reaction mix that contains primers to amplify a target and a reagent to detect the amplified target.</p>
automatic baseline	<p>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.</p>
automatic threshold	<p>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 (<math>C_q</math>).</p>
baseline	<p>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.</p>



baseline-corrected normalized reporter ( $\Delta R_n$ )	<p>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 <math>\Delta R_n</math> vs Cycle amplification plot, <math>\Delta R_n</math> is calculated at each cycle as:</p> $\Delta R_n (\text{cycle}) = R_n (\text{cycle}) - R_n (\text{baseline}), \text{ where } R_n = \text{normalized reporter}$
biological replicates	<p>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).</p> <p>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 <math>C_q</math> 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.</p>
$C_{RT}$	See relative threshold cycle ( $C_{RT}$ ).
cycle threshold	See threshold cycle ( $C_T$ ).
cycling stage	See threshold cycle ( $C_T$ ).
$C_T$	See threshold cycle ( $C_T$ ).
delta $R_n$ ( $\Delta R_n$ )	See baseline-corrected normalized reporter ( $\Delta R_n$ ).
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.
multicomponent plot	A plot of the complete spectral contribution of each dye for the selected well(s) over the duration of the PCR run.
negative control (NC)	See no template control (NTC).
no template control (NTC)	In the software, the task for targets in wells that contain water or buffer instead of sample. No amplification should occur in negative control wells. Also called negative control (NC).
nonfluorescent quencher-minor groove binder (NFQ-MGB)	Molecules that are attached to the 3' end of TaqMan™ MGB probes. When the probe is intact, the nonfluorescent quencher (NFQ) prevents the reporter dye from emitting fluorescence signal. Because the NFQ does not fluoresce, it produces lower background signals, resulting in improved precision in quantification. The minor groove binder moiety (MGB) increases the melting temperature ( $T_m$ ) without increasing probe length. It also allows the design of shorter probes.

normalized reporter (Rn)	Fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference (usually ROX™ dye).
omit well	An action that you perform before reanalysis to omit one or more wells from analysis. Because no algorithms are applied to omitted wells, omitted wells contain no results. You can add wells back in to the analysis; no information is permanently discarded.
outlier	A data point that deviates significantly from the values of an associated group (for example, the other technical replicates for a sample).
passive reference	A dye that produces fluorescence signal independent of PCR amplification, and that is added to each reaction at a constant concentration. Because the passive reference signal should be consistent across all wells, it is used to normalize the reporter dye signal to account for non-PCR related fluorescence fluctuations caused by minor well-to-well differences in volume. Normalization to the passive reference signal generally results in data with noticeably high precision among technical replicates.
plate grid (plate layout)	<p>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.</p> <p>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.</p>
projects	<p>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.</p> <p><b>Note:</b> Projects do not contain the data from experiments uploaded to the repository; they link the data for analysis without affecting the original data files.</p>
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.
Relative Threshold algorithm	Well-based analysis ( $C_{RT}$ ) based on the PCR reaction efficiency and fitted to the amplification curve.
relative threshold cycle (CRT)	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 <i>normalized reporter (Rn)</i> .
ROX dye	A dye used as the passive reference.
run method	The reaction volume and the thermal profile (thermal cycling parameters) for the instrument run.
sample	The biological tissue or specimen that you are testing for a target gene.
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.
technical replicates	Reactions that contain identical components and volumes, and that evaluate the same sample; important for evaluating precision.
thermal profile	The part of the run method that specifies the temperature, time, ramp, number of cycles, and data collection points for all steps and stages of the instrument run.
threshold	In amplification plots, the threshold is the level of fluorescence above the baseline and within the exponential amplification region. For the Baseline Threshold algorithm, the threshold can be determined automatically (see <i>automatic threshold</i> ), or it can be set manually (see <i>manual threshold</i> ).
threshold cycle (C <sub>T</sub> )	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.

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