applied biosystems

Applied Biosystems™ High Resolution Melt Curve Analysis Module

USER GUIDE

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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 High Resolution Melt (HRM) module of the Applied Biosystems™ Analysis Software performs high-resolution melt analysis of experiments run on Thermo Fisher Scientific real-time qPCR instruments. The HRM analysis is a post-PCR analysis method used for identifying genetic variation in nucleic acid sequences. Simple and fast, this method is based on PCR melt (dissociation) curve techniques and is enabled by the recent availability of improved double-stranded DNA (dsDNA)–binding dyes along with next-generation real-time PCR instrumentation and analysis software. HRM analysis can discriminate DNA sequences based on their composition, length, GC content, or strand complementarity.

The Applied Biosystems™ Analysis Software can perform:

- **Mutation scanning experiments** – Screen DNA samples for new single-base changes, insertions/deletions, or other unknown mutations. The mutation scanning experiment product can be used for subsequent sequencing reactions.
- **Methylation studies** – Determine the percentage of methylated DNA in unknown samples.
- **Genotyping experiments** – Determine the genotype of a DNA sample. For all types of experiments, the software compares the melt curves of unknown samples against the melt curves of positive controls to identify groups of variants.

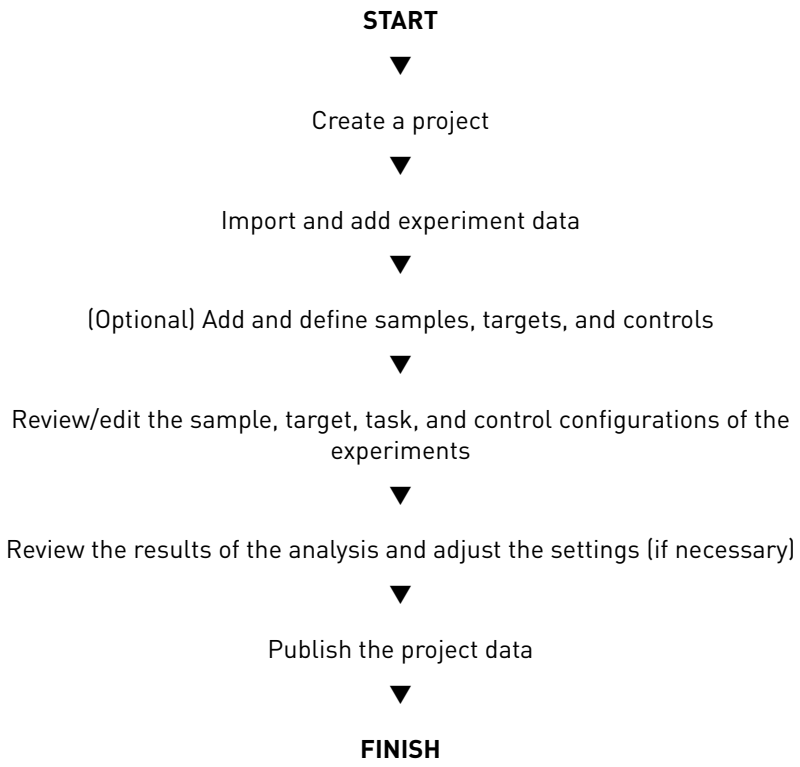
Required experiment components

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

- **Samples** – A sample in which the genotype, variant content, or percentage methylation is unknown.
- **Replicates** – The total number of identical reactions containing identical samples, components, and volumes.
- **Positive Controls** – A sample that contains a known genotype, target sequence, or standard. The type of samples used as positive controls depends on the type of experiment:
  - **Mutation scanning experiments** – One or more samples with the wild type sequence are used as the control. For unknowns, the call is either "wild type" (if it matches the control) or "variant X".
  - **Methylation studies** – Methylated DNA standards that contain from 0% to 100% methylated DNA are used as the positive controls. The software identifies the percentage methylation of the variants based on their comparison to the standards.
  - **Genotyping experiments** – Three samples are used as controls: one homozygous for Allele 1, one homozygous for Allele 2, and one heterozygous for both alleles (Allele 1 and Allele 2). The software identifies the genotypes of the unknown variants.
- **Negative Controls** – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

Analysis workflows

The following figure shows the general workflow for analyzing high resolution melt 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™ 7500 and 7500 Fast Real-Time PCR System	v2.0.5 or later	.eds
Applied Biosystems™ StepOne™ and StepOnePlus™ Real-Time PCR System	v2.0.1, v2.1, or later	
Applied Biosystems™ ViiA™ 7 Real-Time PCR System	v1.1 or later	
Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System	v1.1.1 or later	
Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System	v1.0 or later	
Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System		
Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System	v1.0 or later	
Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System		

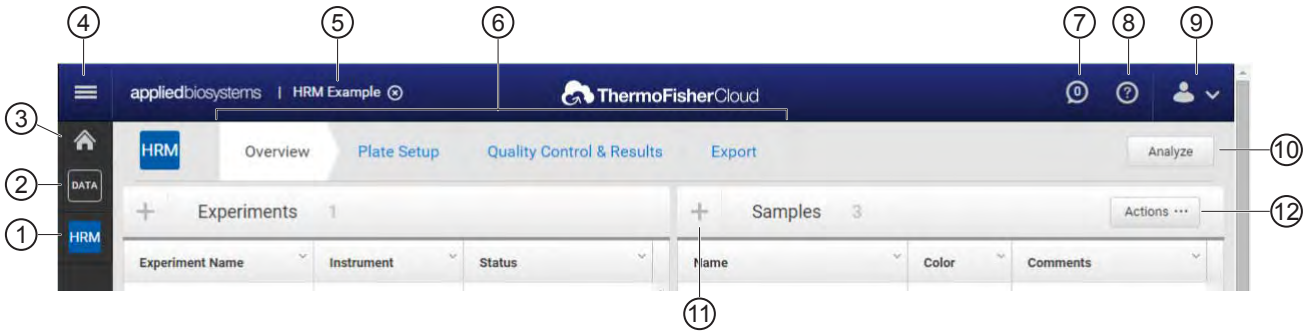
System requirements

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

Category	Requirement
Web Browser	• Apple™ Safari™ 8 Browser • Google™ Chrome™ Browser Version 21 or later • Microsoft™ Internet Explorer™ Browser Version 10 or later • Mozilla™ Firefox™ Browser Version v10.0.12 or later
Operating System	• Windows™ XP, Vista, 7, or 8 • Macintosh™ OS 8 or later
Network Connectivity	An internet connection capable of 300kbps/300kbps (upload/download) or better. If your network employs a firewall that restricts outbound traffic, it must be configured to allow outbound access to apps.lifetechnologies.com on HTTPS-443.

About the software interface

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



① **Analysis Modules** – Click to analyze the current project using the selected module.

② DATA (Data Manager) – Click to view the Data Manager, which can be used to view, add, or remove data from the current project.

③ (Project Manager) – Click to view the Project Manager, which can be used to modify the current project or open another.

④ (Account Management Menu) – Click to manage your application licenses or storage.

⑤ **Project name** – The name of the current project.

Note: Click ⊗ to close the project.

⑥ **Project tabs** – Click to view the settings, data, or plot(s) for the current project.

⑦ 1 (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

2 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: / \ < > * ? " | : ; & % $ @ ^ ( ) !

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

To import experiment data stored on...	Action
Your computer	1. Click **Import from local**. 2. From the Open dialog box, select one or more experiment files (.sds or .eds), then click **Open**. **Note:** Ctrl- or Shift-click to select multiple files. Wait for the Applied Biosystems™ Software to upload the selected data. 3. Click **Close** prompted that the import is complete.
Thermo Fisher Cloud	1. Click **Import from Thermo Fisher Cloud**. 2. Select one or more experiment files (.sds or .eds) from the table, then click **Add**. 3. When you are done adding files to the queue, click **OK**. 4. Click **Close** prompted that the import is complete.

4. Repeat step 3 until your project contains all of the desired experiment data.

5. Click the appropriate analysis module on the left side of the screen to begin the analysis.

Manage projects and experiment data

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

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

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

To import experiment data stored on...	Action
Your computer	1. Click **Import from local**. 2. From the Open dialog box, select one or more experiment files (.sds or .eds), then click **Open**. **Note:** Ctrl- or Shift-click to select multiple files.
Thermo Fisher Cloud	1. Click **Import from Thermo Fisher Cloud**. 2. Select one or more experiment files (.sds or .eds) from the table, then click **Add**. 3. When you are done adding files to the queue, click **OK**.

c. Wait for the Applied Biosystems™ Software to import the selected data. When you are prompted that the upload is complete, click **Close**.

- **Delete** projects, experiments, or folders:
  a. Select the experiments from the Files in this project table that you want to remove.
  b. From the Manage Data screen, select **Actions** ▸ **Delete**.
  c. When prompted, click **OK** to remove the experiment(s) from your project.

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

Share experiments, folders, and projects

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

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

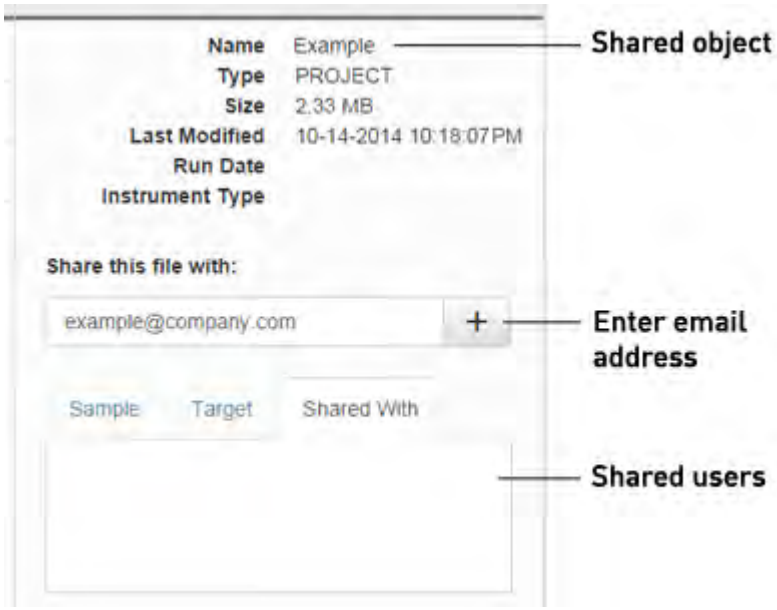
  IMPORTANT! A project is locked (preventing access) when it is open (in use) by any user with shared access to the project. For example, User A shares a project with two colleagues (User B and User C), User B opens the project and begins data analysis (the project is locked and unavailable to Users A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

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

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

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

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



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

IMPORTANT! To share multiple files:

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

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

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

About experiment data/files

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

Experiment files contain the following information:

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

File compatibility

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

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

Real-Time PCR System	Supported software version(s)	File extension
Applied Biosystems™ 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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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.

Manage HRM controls

The Applied Biosystems™ Analysis Software populates the Overview screen with the controls present in the experiments added to the project.

If necessary, you can add, edit, or remove controls as needed before the analysis:

- **Create** a new control:
  a. From the Controls table in the Overview screen, click **Actions** ▸ **Add**.
  b. In the New Control dialog box, enter a name for the new control (up to 256 characters), then edit the properties of the new control.
  c. Click **OK**.
- **Update** an existing control by editing the entry directly in the table.

  Note: Alternately, select a control from the table, then select **Actions** ▸ **Update**.
- **Delete** a control:
  a. From the Controls table in the Overview screen, select the control of interest, then click **Actions** ▸ **Delete**.
  b. In the confirmation dialog box, click **OK** to delete the control.

HRM controls

For all types of experiments, the Applied Biosystems™ Analysis Software compares the melt curves of unknown samples against the melt curves of positive controls to identify groups of variants.

The type of sample used as the positive controls depends on the type of experiment:

- **Mutation scanning experiments** – One or more samples with the wild type sequence are used as the control. For unknowns, the call is either "wild type" (if it matches the control) or "variant X".
- **Methylation studies** – Methylated DNA standards that contain from 0% to 100% methylated DNA are used as the positive controls. The software identifies the percentage methylation of the variants based on their comparison to the standards.
- **Genotyping experiments** – Three samples are used as controls: one homozygous for Allele 1, one homozygous for Allele 2, and one heterozygous for both alleles (Allele 1 and Allele 2). The software identifies the genotypes of the unknown variants.

High-resolution melt (HRM) dyes

The melt profile of a PCR product is best obtained with high-resolution melt dyes (HRM dyes). HRM dyes are double-stranded dsDNA-binding dyes that have high fluorescence when bound to dsDNA and low fluorescence in the unbound state. HRM analysis uses dsDNA-binding dyes that are brighter than those previously used, and they do not inhibit PCR at high-dye concentrations. With traditional dyes (for example, SYBR™ Green I dye), only limited concentrations of the dye can be used before the dye inhibits the PCR.

Custom HRM dyes

This getting started guide describes procedures for calibrating your instrument and performing HRM experiments using the MeltDoctor™ HRM Dye. If you use a different HRM dye, prepare a custom HRM calibration plate and then calibrate your Applied Biosystems™ qPCR Instrument for that dye. When the instrument is calibrated, follow the procedures provided, but replace the MeltDoctor™ HRM Dye with your own.

Note: See the *High-Resolution Melt Curve Getting Started Guide* for your Applied Biosystems™ qPCR Instrument for instructions on performing a custom HRM dye calibration.

Note: Optimize your reactions for the HRM dye that you use, because each dye interacts uniquely with all other reaction components.

High-Resolution Melt (HRM) reagents and controls

The example experiments described in this document include the following Applied Biosystems™ MeltDoctor™ High-Resolution Melting (HRM) reagents and controls that can be used in your own experiments. For product details and ordering information, visit: **http://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-reagents/meltdoctor-hrm-reagents.html**

- **Applied Biosystems™ MeltDoctor™ HRM Master Mix** contains all PCR components (excluding template and primers) and has been formulated for optimal HRM performance across a wide range of genomic targets. It features a dNTP blend that includes dUTP, which minimizes carryover contamination by allowing amplicon degradation by uracil DNA glycosylase (UDG) in subsequent PCR reactions. The MeltDoctor™ HRM Master Mix does not require additional mixing prior to use, and was developed and optimized solely for HRM applications.
- **Applied Biosystems™ MeltDoctor™ HRM Positive Control Kit** provides nucleic acid controls that can be used to demonstrate and troubleshoot high resolution melt (HRM) analysis. The kit contains forward and reverse primers and three DNA templates representing the homozygous and heterozygous genotypes for a β-actin target gene (ACTB).
- **Applied Biosystems™ MeltDoctor™ HRM Calibration Standard** contains a DNA template and primers which, when mixed with PCR reagents and dye, can be used to prepare a plate for thermal and optical High Resolution Melt (HRM) calibration for the MeltDoctor™ HRM (High Resolution Melting) dye.
- **Applied Biosystems™ MeltDoctor™ HRM Calibration Plates** contain all the components required for dye and HRM calibration on the Applied Biosystems™ qPCR Instrument. These plates provide the thermal and optical calibration required for accurate and reproducible discrimination between melt curves.

4 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.

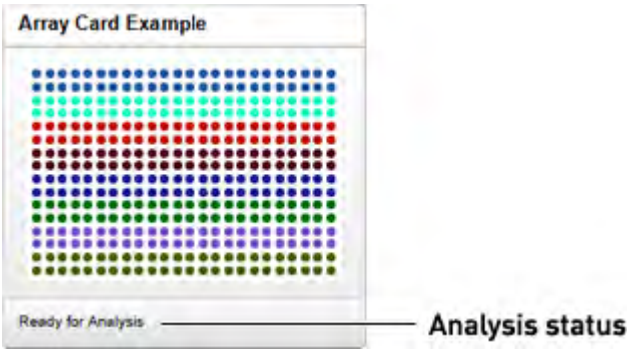
Review and edit the plate setups

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.

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

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

Specify and assign tasks

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

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

1. From the Plate Setup screen, select the experiment record that you want to modify.

2. From the Edit Plate screen, click **View**, then select **Task** to color the plate setup according to task assignment.

3. Select the wells of the plate layout to which you want to apply a task.

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

   Available tasks include:

   - **Unknown** – The task for wells that contain a sample with unknown genotype, variant content, or percentage methylation.
   - **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.
   - **Positive Controls** - Wells that contain one of the following:
     - A template known to generate a specific genotype call for one or both alleles (**Heterozygote** or **Homozygote**).
     - A wild type control (**Wild Type**).
     - Methylated DNA standards that contain from 0% to 100% methylated DNA (add a custom control label).

   Note: The Task/Control dropdown list displays the controls present in the Controls pane of the Overview screen. See “Manage HRM controls“ on page 18 for information on adding and editing controls.

5. Repeat steps 3 and 4 as needed.

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

Import an HRM calibration from an external file

You can use the Import Calibration File feature to change (overwrite) the HRM calibration file for the selected HRM experiment.

1. From the Plate Setup screen, select the experiment to which you want to apply the calibration file.
2. (Optional) From the Edit Plate screen, click **Substitute**, then select the appropriate HRM calibration file (.eds file) and click **Open**.

   IMPORTANT! The HRM calibration file must be:

   - Run on the same instrument that created the run file
   - Run in the same reaction plate type used by the run file (384-well, 96-well Fast, or 96-well standard)
   - Run with the same software version used by the run file
3. When you finish changing 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 25.

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 25 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
Experiment data (do not edit):	**1**	* Block Type = 96-Well						
	2	* Experiment Type = High Resolution Melt						
	3	* Instrument Type = StepOnePlus System						
	4	* No. Of Wells = 96						
	5	* Set Up Well Section Info =						
Column headings (do not edit):	**6**	Well	Well Position	Sample Name	Task	Target Name	Reporter	Quencher
Plate setup content (add well data in any order):	**7**	0	A1	Sample 3	Wild Type	Assay 1	SYBR	None
	8	1	A2	Sample 3	UNKNOWN	Assay 1	SYBR	None
	9	2	A3	Sample 3	UNKNOWN	Assay 1	SYBR	None
	...	...	...	...	...	...	...	...

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 E (Target Name)** – The name of the assay added to the well, or the identity of the target sequence (up to 256-characters).
  - **Column F (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.

5 Analyze the project

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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 high-resolution melt curve analysis. The plots and features of the screen can help you review your project for irregular amplification and other common qPCR 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**.

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

Group	Settings
Target Settings	Select the method (automatic or manual) that the Applied Biosystems™ Software will use to compute the pre- and post-melt ranges, grouping, and variant removal: • Define whether the pre- and post-melt range for each target will be computed automatically or manually: – **Auto Set Melt Range** – If you are using automatic settings, select the checkbox to have the software calculate the pre- and post- melt ranges for the specific target. – **Pre-Melt Start/Stop** and **Post-Melt Start/Stop** – If you are using manual settings, enter the manual pre- and post-range values for the appropriate targets. • Define whether the genotype groups for each target will be determined automatically or manually: – **Auto Determine # of Groups** – If you are using automatic settings, select the checkbox to have the software calculate the number of groups for the specific target. – **Number of Groups** – If you are using manual settings, enter the number of groups for the appropriate targets. • **Remove all Manual Variants on reanalysis** – Select to have the software omit from the analysis all wells that have been manually been labeled as a variant.
Flag Settings	Specify the quality measures that the Applied Biosystems™ Software will compute during the analysis. 1. In the Use column, select the check boxes for flags you want to apply during analysis. 2. If an attribute, condition, and value are listed for a flag, you can specify the setting for applying the flag. For example, with the default setting for the no amplification flag (NOAMP), wells are flagged if the amplification algorithm result is less than 0.1. **Note:** If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting. 3. In the Reject Well column, select the check boxes if you want the software to reject wells with the flag. Rejected wells are not considered for data analysis.

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 experiment 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. Click **Quality Control & Results** to view the Quality Control & Results screen.

3. Review the experiment data for quality flags generated during the analysis.

   Note: The Applied Biosystems™ Software displays summaries of the quality data in the margin beneath each experiment. You can view the identity of the triggered flags by mousing over an experiment of interest.

   Alternatively, to quickly find the experiments in your project that generated quality flags, click (Plate View), then click the Flags column heading to sort the experiments that generated quality flags to the top of the table. To examine the data that triggered the flag, click the corresponding link in the Name column.

   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.

4. If flags or irregularities are present, or you would like to review the melt curve data for a specific experiment, click the experiment of interest.

5. When viewing an experiment, display the Well Table:

Tool	Use this tool to...
Mouse/cursor	Select wells. To select: • An individual well, select the well in the Well table. • More than one well at a time, press the **Ctrl** key or **Shift** key when you select the wells in the Well table. When you select wells in the Well table, the corresponding data points are selected in the amplification plot.
Actions menu	**Omit/Un-Omit** well from the analysis. After you omit or un-omit a well, click **Analyze** to reanalyze the project. For omitted wells, the software: • Does not display data in the Well table (data are either Unknown or empty/blank). • Does not include the omitted wells in the analysis.
or	Expand or collapse the Well table by clicking the appropriate symbol in the vertical bar between the plot and 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.

Column	Description
Task/Control	The task assigned to the well, where the task is the function that a sample performs on the plate.
Variant	The call for the sample in well. Can be assigned by software (Auto) or manually.
Method	The method used to apply the call to the well: Auto(matic) if calculated by the Applied Biosystems™ Analysis Software or Manual if user-applied.
Silhouette Score	The *modified* silhouette score calculated for the well, which measures distinguishably of the associated melt curve relative to the other curves in the assay (Rousseeuw, 1987.). For each melt curve, the software calculates a *modified* silhouette score, which ranges from 0 to 100. A score closer to 100 indicates that a melt curve is more similar to curves assigned the same variant call than to curves called differently. Lower silhouette scores indicate that a melt curve is less similar to curves assigned the same variant call. **IMPORTANT!** The modified silhouette score differs from the standard silhouette score (Lovmar, *et. al.*, 2005.) in that the software assigns the score to each identified cluster instead of to each data point in the cluster. In addition, the modified value ranges from 0 to 100.
Tm1/Tm2/Tm3	The 1st, 2nd, and 3rd calculated melt temperature (Tm) for the well (if present). **Note:** Blank table cells indicate that the software calculated no Tm for the well at the indicated position.
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.
Ct	The C_T calculated for the related well.
Ct Mean	The arithmetic mean generated from the C_Ts calculated for the technical replicates of the well.
Ct SD	The standard deviation generated from the C_Ts calculated for the technical replicates of the well.
Flags	The number of flags generated for the well.
Quality data	The quality flags generated by the associated well.

7. When ready, click ‹ to return to the thumbnails view.

Review HRM genotyping data

The Applied Biosystems™ Analysis Software High Resolution Melt module supports the analysis of HRM genotyping experiments, where melt curve technique is used to determine the genotype of unknown DNA samples. During the analysis, the software compares the melt curves of the unknown samples against those generated from a set of DNA standards (positive controls) that represent the genotypes for the target sequence (Allele 1 homozygous, Allele 2 homozygous, and Allele 1/Allele 2 heterozygous). Based on the comparison, the software assigns calls to the unknown samples according to the fit of the unknown melt curves to those of the controls.

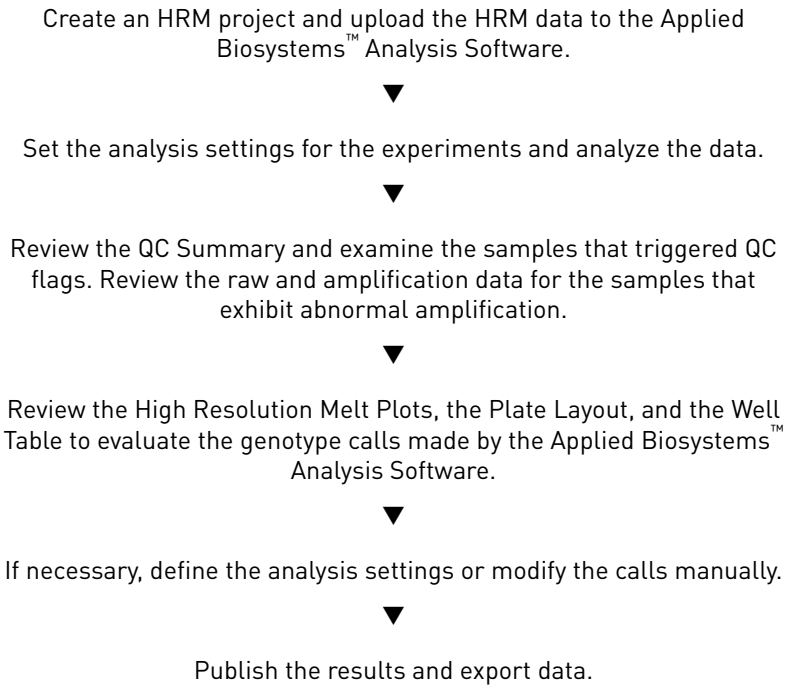
In general, review of genotyping results occurs in the following steps:

1. Define the analysis settings, then analyze the project.
2. Perform an initial review of the High Resolution Melt Plots, the Plate Layout, and the Well Table to evaluate the genotype calls made by the Applied Biosystems™ Software.
3. Perform a thorough review of the QC Summary to evaluate the samples that triggered QC flags. Review the raw data and amplification data for the samples that exhibit abnormal amplification.
4. If necessary, adjust the analysis settings or modify the calls manually.

After evaluating the results, publish the analyzed data.

Analysis workflow for HRM genotyping

The following workflow illustrates a generalized process for analyzing and reviewing HRM genotyping project data. The exact process that you use to review and quality check your data will depend on the goals and design of your experiment.



Review the controls and replicate populations for outliers

The Aligned Melt Curves plot displays the melt curves as percentage melt (0 - 100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions that you set.

1. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
2. In the Quality Control & Results pane, select **Plot ▸ Aligned Melt** to review the Aligned Melt Curve Plot.
3. If using positive controls, confirm the calls for the positive controls:
   a. From the well table, select the wells containing a positive control to highlight the corresponding melt curve in the Aligned Melt Curves Plot.

b. Confirm that the color of the line corresponds to the correct genotype.

c. Repeat steps a and b for the wells containing the other positive controls.

4. Screen the negative controls to ensure that samples failed to amplify:
   a. From the well table, select the wells containing a negative control to highlight the corresponding melt curve in the Aligned Melt Curves Plot.

   b. Confirm that the selected wells in the well table are negative controls, and not unknown samples.

   Samples that grouped with the negative controls may:
   - Contain no DNA
   - Contain PCR inhibitors
   - Be homozygous for a sequence deletion

5. Confirm the results of the samples that did not group tightly or are grouped with negative controls by retesting them.

6. If you select to run replicate reactions, carefully review your data set for curves that do not align tightly with the other samples in the group (outliers) to ensure the accuracy of the genotype calls. If outliers are present, confirm the results of the associated samples by retesting them.

7. Look for how many different variant groups (different colors) are displayed. If you see more than you were expecting, you may have sample contamination or may need to modify the analysis settings.

   Review the data:
   - **Variant groups (different colors)** – How many different variant groups are displayed? Does this number correspond to the number of variants you were expecting?
   - **Outliers** – Are there any curves within a variant group that do not cluster tightly with the other samples in that group?

Review and adjust the pre- and post-melt regions

When you analyze an HRM experiment, the software calculates the Pre- and Post-melt regions using default settings. You can review and adjust the Pre- and Post-melt regions to optimize your separation and variant calls. For most experiments, set the Pre- and Post-melt regions as close as possible to the melt transition region.

About the pre- and post-melt regions

In the Derivative Melt Curves plot and the Raw Melt Curves plot, there are two pairs of vertical lines before and after the data peak. These lines define the pre- and post-melt regions used to scale the data in the Aligned Melt Curves and Difference Plot.

- **Pre-melt region** – The pair of lines to the left of the peak indicate the pre-melt Start and Stop temperatures when every amplicon is double-stranded. Fluorescence data from the pre-melt region corresponds to 100% fluorescence in the Aligned Melt Curves Plot.
- **Active melt region** – The data peak indicates the active melt region of the plot. Data from the active melt region are used to plot the Aligned Melt Curves Plot.
- **Post-melt region** – The set of lines to the right of the peak indicate the post-melt Start and Stop temperatures when every amplicon is single-stranded. Fluorescence data from the post-melt region correspond to 0% fluorescence in the Aligned Melt Curves Plot.

Review and adjust the pre- and post-melt regions

1. Display the Derivative Plot for the experiment of interest:
   a. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Derivative Melt** to view the Derivative Melt Plot.
2. Select **Target ▸ *<assay>*** to view the melt data for the desired assay.
3. In the Derivative Melt plot, set the pre-melt region:
   a. Click and drag the Pre-melt Stop temperature line (red arrow on the left) adjacent to the start of the melt transition region.
   b. Click and drag the Pre-melt Start temperature line (green arrow on the left) approximately 0.2°C to 0.5°C from the Pre-melt Stop temperature line.

   Note: The Pre-melt region should be within a flat area where there are no large spikes or slopes present in the fluorescence levels.
4. Set the post-melt region:
   a. Click and drag the Post-melt Start temperature line (black arrow on the right) adjacent to the end of the melt transition region.
   b. Click and drag the Post-melt Stop temperature line (yellow arrow on the right) approximately 0.2°C to 0.5°C from the Post-melt Start temperature line.

Note: The Post-melt region should be within a flat area where there are no large spikes or slopes present in the fluorescent levels.

5. Click **Analyze**.

   The software reanalyzes the data using the new Pre- and Post-melt regions. The colors of the melt curves change to reflect the new results.

Confirm the genotype calls

The Applied Biosystems™ Analysis Software calls each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
2. In the Quality Control & Results pane, select **Plot ▸ Difference** to review the Difference Melt Curve Plot.
3. From the View By drop-down list, select **Well Table** to review the genotyping calls generated by the software.
4. In the well table, click the **Well** column header to sort the results according to the well position.
5. For the positive controls, review:
   - **Variant Call column** – Do all of the positive control replicates have the correct call?
   - **Cq Confidence column** – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?

   Note: If any of the controls are outliers, omit them from the HRM analysis, then reanalyze.
6. For each replicate group, review:
   - **Variant Call column** – Do all replicates have the same call?
   - **Cq Confidence column** – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?
7. Select the rows in the Results table to view the corresponding fluorescence data in the Difference Melt Plot.
8. In the Results pane, click the **Variant** column header to sort the results according to the variant call. For each variant call, review the samples that are assigned that call.

Review HRM mutation detection data

The Applied Biosystems™ Analysis Software High Resolution Melt module supports the analysis of HRM mutation detection experiments, where melt curve technique is used to screen DNA samples for new single-base changes, insertions/deletions, or other unknown mutations. During the analysis, the software compares the melt curves of unknown samples to those generated from one or more DNA samples that contain wild type sequences (positive controls). Based on the comparison, the software assigns calls to the unknown samples according to the fit of the unknown melt curves to those of the controls. For the unknowns, possible calls are either "wild type" if the sample matches a control or "variant X" if the sample does not.

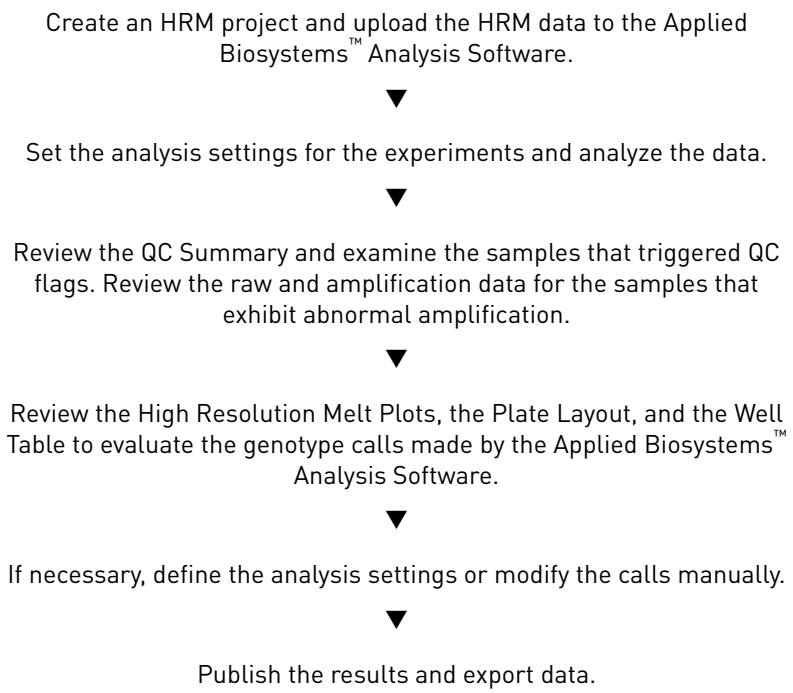
In general, review of genotyping results occurs in the following steps:

1. Define the analysis settings, then analyze the project.
2. Perform an initial review of the High Resolution Melt Plots, the Plate Layout, and the Well Table to evaluate the calls made by the Applied Biosystems™ Software.
3. Perform a thorough review of the QC Summary to evaluate the samples that triggered QC flags. Review the raw data and amplification data for the samples that exhibit abnormal amplification.
4. If necessary, adjust the analysis settings or modify the calls manually.

After evaluating the results, publish the analyzed data.

Analysis workflow for HRM mutation detection

The following workflow illustrates a generalized process for analyzing and reviewing HRM mutation detection project data. The exact process that you use to review and quality check your data will depend on the goals and design of your experiment.



Review and adjust the pre- and post-melt regions

When you analyze an HRM experiment, the software calculates the Pre- and Post-melt regions using default settings. You can review and adjust the Pre- and Post-melt regions to optimize your separation and variant calls. For most experiments, set the Pre- and Post-melt regions as close as possible to the melt transition region.

About the pre- and post-melt regions

In the Derivative Melt Curves plot and the Raw Melt Curves plot, there are two pairs of vertical lines before and after the data peak. These lines define the pre- and post-melt regions used to scale the data in the Aligned Melt Curves and Difference Plot.

- **Pre-melt region** – The pair of lines to the left of the peak indicate the pre-melt Start and Stop temperatures when every amplicon is double-stranded. Fluorescence data from the pre-melt region corresponds to 100% fluorescence in the Aligned Melt Curves Plot.
- **Active melt region** – The data peak indicates the active melt region of the plot. Data from the active melt region are used to plot the Aligned Melt Curves Plot.
- **Post-melt region** – The set of lines to the right of the peak indicate the post-melt Start and Stop temperatures when every amplicon is single-stranded. Fluorescence data from the post-melt region correspond to 0% fluorescence in the Aligned Melt Curves Plot.

Review and adjust the pre- and post-melt regions

1. Display the Derivative Plot for the experiment of interest:
   a. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Derivative Melt** to view the Derivative Melt Plot.
2. Select **Target ▸ *<assay>*** to view the melt data for the desired assay.
3. In the Derivative Melt plot, set the pre-melt region:
   a. Click and drag the Pre-melt Stop temperature line (red arrow on the left) adjacent to the start of the melt transition region.
   b. Click and drag the Pre-melt Start temperature line (green arrow on the left) approximately 0.2°C to 0.5°C from the Pre-melt Stop temperature line.

   Note: The Pre-melt region should be within a flat area where there are no large spikes or slopes present in the fluorescence levels.
4. Set the post-melt region:
   a. Click and drag the Post-melt Start temperature line (black arrow on the right) adjacent to the end of the melt transition region.
   b. Click and drag the Post-melt Stop temperature line (yellow arrow on the right) approximately 0.2°C to 0.5°C from the Post-melt Start temperature line.

Note: The Post-melt region should be within a flat area where there are no large spikes or slopes present in the fluorescent levels.

5. Click **Analyze**.

   The software reanalyzes the data using the new Pre- and Post-melt regions. The colors of the melt curves change to reflect the new results.

Review the controls and replicate populations for outliers

The Aligned Melt Curves plot displays the melt curves as percentage melt (0 - 100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions that you set.

1. Display the Aligned Melt Plot for the experiment of interest:
   a. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Aligned Melt** to view the Aligned Melt plot.
2. If using Wild type controls, confirm the calls for the positive controls:
   a. From the well table, select the wells containing a positive control to highlight the corresponding melt curve in the Aligned Melt Curves Plot.
   b. Confirm that the wild type controls cluster well and review the population for outliers.
   c. Repeat steps a and b for the wells containing the other positive controls.
3. Screen the negative controls to ensure that samples failed to amplify:
   a. From the well table, select the wells containing a negative control to highlight the corresponding melt curve in the Aligned Melt Curves Plot.
   b. Confirm that the selected wells in the well table are negative controls, and not unknown samples.

   Samples that grouped with the negative controls may:
   - Contain no DNA
   - Contain PCR inhibitors
   - Be homozygous for a sequence deletion
4. If you run replicate reactions, carefully review your data set for curves that do not align tightly with the other samples in the group (outliers). If outliers are present, confirm the results of the associated samples by retesting them.

5. Review the data:
   - **Possible mutations** – Review the curves for samples with melt curves that are different from the wild type melt curves.
   - **Unexpected peaks** – Confirm that the Derivative Melt Curve shows no unexpected Tm peaks. If the sequence you amplified contains more than one variant or a more complex mutation, you may see more than one Tm peak. Unexpected peaks can indicate contamination, primer dimers, or non-specific amplification.

Review the Difference Melt Curves Plot for outliers

The Difference Plot displays the aligned data as the difference in fluorescence between the melt curve for a reference sample and the other melt curves. You can select a control or any well as a reference. After you select the reference, the software subtracts the reference curve from the other curves.

The Difference Plot allows you to more easily see small differences between curves and identify outliers.

1. Display the Difference Plot for the experiment of interest:
   a. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Difference Melt** to view the Difference Melt Plot.
2. From the Reference drop-down menu, select a control or any well as the reference, then review:
   - **Variant clusters** – How many distinct clusters are displayed?
   - **Outliers** – How tight are the curves within each variant cluster?

   Note: Try selecting different reference samples to find the optimal display of the clusters.

Confirm the software calls

The Applied Biosystems™ Analysis Software calls each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. Display the Aligned Plot for the experiment of interest:
   a. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Aligned Melt** to view the Aligned Melt Plot.
2. In the Well Table, select **Well Table** from the View By drop-down list to review the calls generated by the software.
3. In the well table, click the **Well** column header to sort the results according to the well position.

4. For each replicate group, review:
   - **Variant Call column** – Do all replicates have the same call?
   - **Confidence column** – Are there any outliers within the replicate group? Do the values for the replicate group differ greatly from the confidence values for other replicate groups in the plate?
5. Select the rows in the Results table to view the corresponding fluorescence data in the Aligned Melt Plot.
6. In the well table, click the **Variant** column header to sort the results according to the variant call. Scan the results for samples that were not assigned the same call as the wild type control.

Review HRM methylation data

The Applied Biosystems™ Analysis Software High Resolution Melt module supports the analysis of HRM methylation experiments, where melt curve technique is used to determine the percentage of methylated DNA in unknown samples. During the analysis, the software compares the melt curves of the unknown samples against those generated from a set of methylated DNA standards (positive controls). Based on the comparison, the software assigns calls to the unknown samples according to the fit of the unknown melt curves to those of the standard ladder.

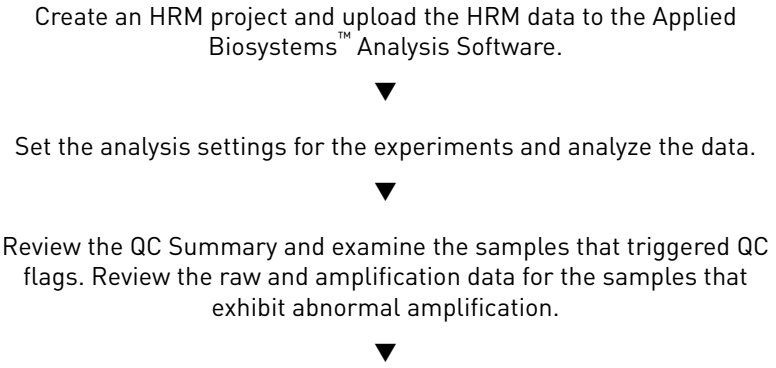
In general, review of HRM methylation results occurs in the following steps:

1. Define the analysis settings, then analyze the project.
2. Perform an initial review of the High Resolution Melt Plots, the Plate Layout, and the Well Table to evaluate the calls made by the Applied Biosystems™ Software.
3. Perform a thorough review of the QC Summary to evaluate the samples that triggered QC flags. Review the raw data and amplification data for the samples that exhibit abnormal amplification.
4. If necessary, adjust the analysis settings or modify the calls manually.

After evaluating the results, publish the analyzed data.

Analysis workflow for HRM methylation study

The following workflow illustrates a generalized process for analyzing and reviewing HRM methylation project data. The exact process that you use to review and quality check your data will depend on the goals and design of your experiment.



Review the High Resolution Melt Plots, the Plate Layout, and the Well Table to evaluate the genotype calls made by the Applied Biosystems™ Analysis Software.

▼

If necessary, define the analysis settings or modify the calls manually.

▼

Publish the results and export data.

Review the controls and replicate populations for outliers

The Aligned Melt Curves plot displays the melt curves as percentage melt (0 - 100%) over temperature. The melt curves are aligned to the same fluorescence level using the pre- and post-melt regions that you set.

1. Display the Aligned Plot for the experiment of interest:
   a. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Aligned Melt** to view the Aligned Melt Plot.
2. Screen the negative controls to ensure that samples failed to amplify:
   a. From the well table, select the wells containing a negative control to highlight the corresponding melt curve in the Aligned Melt Curves Plot.
   b. Confirm that the selected wells in the well table are negative controls, and not unknown samples.

   Samples that grouped with the negative controls may:
   - Contain no DNA
   - Contain PCR inhibitors
   - Be homozygous for a sequence deletion
3. If you run replicate reactions, carefully review your data set for curves that do not align tightly with the other samples in the group (outliers). If outliers are present, confirm the results of the associated samples by retesting them.
4. Review the data:
   - **Methylated DNA standards** – Do the melt curves for the methylated DNA standards cluster well? Are there any outliers?
   - **Define methylation range for unknowns** – Which methylated standard melt curves are above and below the melt curves for the unknowns? For example, if the melt curve for an unknown sample lies between the melt curves for the 5% and 10% methylated standards, the unknown sample contains between 5% and 10% methylated nucleotides.

Review and adjust the pre- and post-melt regions

When you analyze an HRM experiment, the software calculates the Pre- and Post-melt regions using default settings. You can review and adjust the Pre- and Post-melt regions to optimize your separation and variant calls. For most experiments, set the Pre- and Post-melt regions as close as possible to the melt transition region.

About the pre- and post-melt regions

In the Derivative Melt Curves plot and the Raw Melt Curves plot, there are two pairs of vertical lines before and after the data peak. These lines define the pre- and post-melt regions used to scale the data in the Aligned Melt Curves and Difference Plot.

- **Pre-melt region** – The pair of lines to the left of the peak indicate the pre-melt Start and Stop temperatures when every amplicon is double-stranded. Fluorescence data from the pre-melt region corresponds to 100% fluorescence in the Aligned Melt Curves Plot.
- **Active melt region** – The data peak indicates the active melt region of the plot. Data from the active melt region are used to plot the Aligned Melt Curves Plot.
- **Post-melt region** – The set of lines to the right of the peak indicate the post-melt Start and Stop temperatures when every amplicon is single-stranded. Fluorescence data from the post-melt region correspond to 0% fluorescence in the Aligned Melt Curves Plot.

Review and adjust the pre- and post-melt regions

1. Display the Derivative Plot for the experiment of interest:
   a. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Derivative Melt** to view the Derivative Melt Plot.
2. Select **Target ▸ *<assay>*** to view the melt data for the desired assay.
3. In the Derivative Melt plot, set the pre-melt region:
   a. Click and drag the Pre-melt Stop temperature line (red arrow on the left) adjacent to the start of the melt transition region.
   b. Click and drag the Pre-melt Start temperature line (green arrow on the left) approximately 0.2°C to 0.5°C from the Pre-melt Stop temperature line.

   Note: The Pre-melt region should be within a flat area where there are no large spikes or slopes present in the fluorescence levels.
4. Set the post-melt region:
   a. Click and drag the Post-melt Start temperature line (black arrow on the right) adjacent to the end of the melt transition region.
   b. Click and drag the Post-melt Stop temperature line (yellow arrow on the right) approximately 0.2°C to 0.5°C from the Post-melt Start temperature line.

Note: The Post-melt region should be within a flat area where there are no large spikes or slopes present in the fluorescent levels.

5. Click **Analyze**.

   The software reanalyzes the data using the new Pre- and Post-melt regions. The colors of the melt curves change to reflect the new results.

Review the Difference Plot for outliers

The Difference Plot displays the aligned data as the difference in fluorescence between the melt curve for a reference sample and the other melt curves. You can select any control or well as a reference to subtract the selected curve from the others.

The Difference Plot allows you to more easily see small differences between curves and identify outliers.

1. Display the Difference Plot for the experiment of interest:
   a. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Difference Melt** to view the Difference Melt Plot.
2. From the **Reference** drop-down menu, select a control or any well as the reference, then review:
   - **Variant clusters** – How many distinct clusters are displayed?
   - **Outliers** – How tight are the curves within each variant cluster?
3. Repeat step 3 to review the data using the other reference samples to find the optimal display of the clusters.

Confirm the software calls

The Applied Biosystems™ Analysis Software calls each sample according to the shape of the aligned melt curves and the Tm. Review the software calls, then omit outliers or change calls.

1. Display the Difference Plot for the experiment of interest:
   a. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
   b. In the Quality Control & Results tab, select an experiment of interest, then select all wells in the plate grid.
   c. Select **Plot ▸ Difference Melt** to view the Difference Melt Plot.
2. From the View By drop-down list, select **Well Table** to review the calls generated by the software.
3. In the well table, click the **Well** column header to sort the results according to the well position.

4. For the methylation standard controls, review:
   - **Variant Call column** – Do all of the methylation standard controls have the correct call?
   - **Confidence column** – Are there any outliers within the replicate group? Do the values for the replicate group differ from the confidence values for the other replicate groups in the plate?

   Note: If any of the controls are outliers, omit them from the HRM analysis, then reanalyze.

5. Select the rows in the Results table to view the corresponding fluorescence data in the Difference Melt Plot.

Review the Multicomponent plot

The Multicomponent Plot displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

Review the Multicomponent Plot for:

- MeltDoctor™ HRM dye or your custom reporter dye
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

1. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
2. In the Quality Control & Results tab, select all wells in the plate grid.
3. Select **Plot** ▸ **Multicomponent** to view the Multicomponent Plot.
4. Select **Target** ▸ ***<assay>***to view the data for the desired assay.
5. Select one unknown well in the plate layout to display the corresponding data in the Multicomponent Plot.

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

6. Click (View Options), then select **Color By** ▸ **Dye**.

   If the Legend is not displayed, also select **Show Legend**.
7. Confirm that the dye signals in the Multicomponent Plot increase throughout the PCR, indicating normal amplification.
8. In the Well Table or Plate Grid, select all negative control wells, then confirm that they did not amplify. If amplification has taken place, the negative controls may be contaminated.

When reviewing the Multicomponent Plot, look for:

- **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.
- **Any irregularities in the signal** – There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- **Negative control wells** – There should not be any amplification in the negative control wells.

Review the Amplification Plot

If you collected real-time data for your experiment, review the amplification data to further understand the flags triggered by the experiment data. The Amplification Plot screen displays amplification of all samples in the selected wells. Use the amplification plots to confirm the results of the experiment:

- **ΔRn vs Cycle** – This plot displays ΔRn as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle** – This plot displays Rn as a function of cycle number. You can use this plot to identify and examine irregular amplification.
- **C_T vs Well** – This plot displays C_T as a function of well position. You can use this plot to locate outlying amplification (outliers)

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

1. In the Applied Biosystems™ Analysis Software, select the **Quality Control & Results** tab.
2. In the Quality Control & Results tab, select all wells in the plate grid.
3. Select **Plot ▸ Amplification** to view the Multicomponent Plot.
4. Select **Target ▸ *<assay>*** to view the data for the desired assay.
5. In the Amplification Plot, click (View Options), then select:
   - **Plot Type ▸ ΔRn vs Cycle**
   - **Color By ▸ Sample**
   - **Graph Type ▸ Log**

   If the Legend is not displayed, also select **Show Legend**.
6. Verify that the threshold is set correctly.
7. Repeat above steps for all targets.

8. In the Amplification Plot, click (View Options), then select:
   - **Plot Type ▸ C_T vs Well**
   - **Color By ▸ Sample**
9. Confirm that the populations of technical replicates have achieved similar amplification.

When you analyze the Amplification Plot, look for:

- Outliers
- A typical amplification plot – The Applied Biosystems™ Analysis Software calculates baseline and threshold values based on the assumption that the data exhibit a typical amplification plot. A typical amplification plot has four distinct sections:
  - Plateau phase
  - Linear phase
  - Exponential (geometric phase)
  - Baseline

Perform manual calls

Perform manual calls when you want to manually assign a sample to a variant group.

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 Melt Curve Plot, Plate Layout, or Well Table.
4. Click **Actions**, then select **Manual Call**.
5. From the Manual Call dialog box, you can assign the sample to:
   - **An existing variant call** - Click **Select Existing**, select the appropriate call from the Group drop-down menu, then click **OK**.
   - **A new variant call** - Click **Create New**, enter a name for the new call in the Group field, select a color, then click **OK**.

   In the Plate Layout tab, the upper right corner of the sample well is marked with a red triangle.
   In the Well Table tab, in the Method column, Manual appears next to the selected sample.
6. Repeat the steps above to assign more manual calls.
7. 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).

6 Export the results

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- Export project data as a slide presentation 48
- Export plots for presentation and publication 49
- Export data for use in other projects 50

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.
      - **HRM Raw/HRM Difference/HRM Aligned** - Exports the results from the HRM Raw, HRM Difference, and HRM Aligned plot.
      - **Results Data** - Exports the results from the high resolution melt analysis, including calculated Tm and call data.
      - **Amplification Data** - Exports amplification results for each well in the project, such as cycle numbers, and Rn or ΔRn values.
      - **Experiment QC Summary** - Exports a summary of the quality metrics (flags) generated by the data analysis.
      - **Analysis Settings** - Exports the analysis settings configurations used to generate the analyzed data, including the threshold settings for individual QC flags.

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 48).

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.

7 Screens and plots

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- Well Table 58

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.

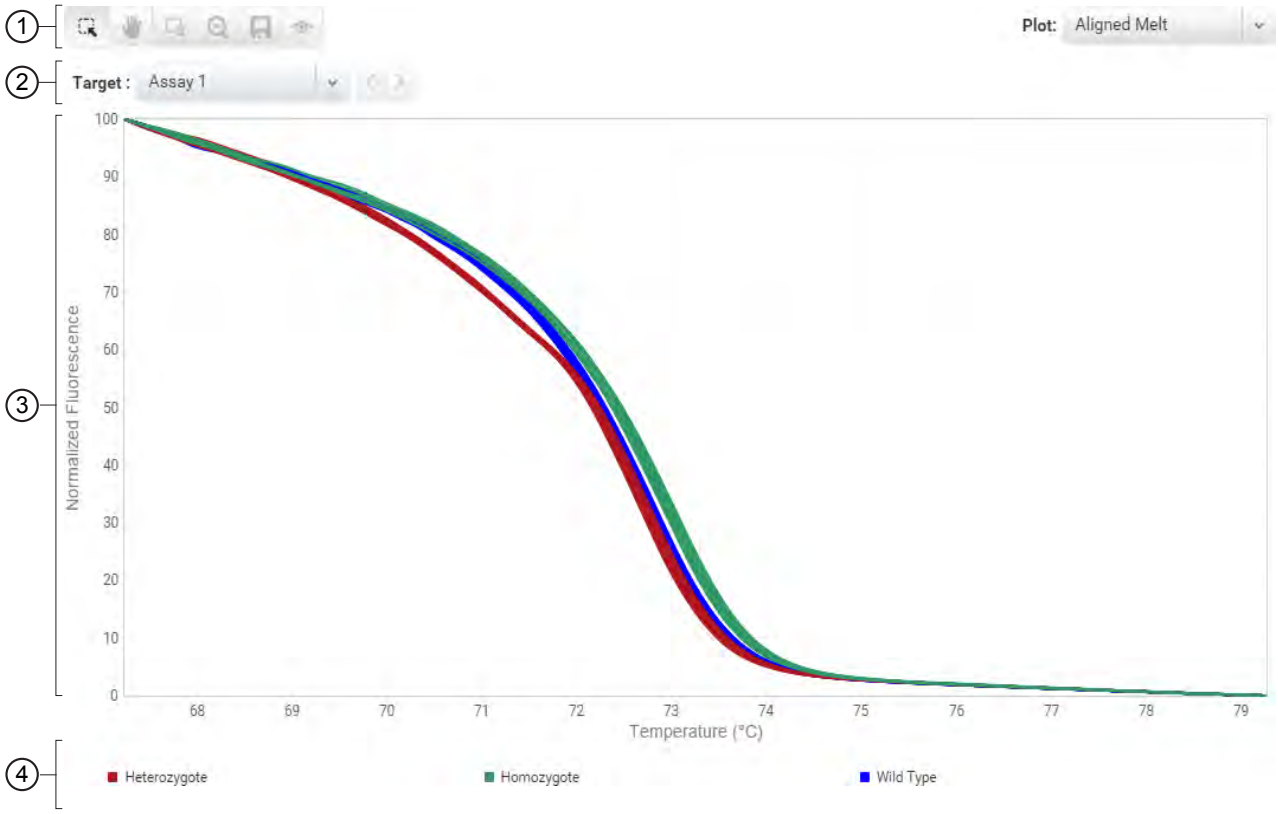
Aligned Melt Curve Plot

The Aligned Melt Curve Plot displays the melt curve data for a single experiment in a graph that contrasts percent normalized fluorescence (0-100%) versus temperature. The software uses the data captured during the pre- and post-melt regions to define the limits of the fluorescence range, where signal captured during the post-melt region defines the 0% limit and the signal captured during the pre-melt region defines the 100% limit.

Within the Aligned Melt Curve Plot, the shape of the melt profile of a PCR product is influenced by its guanine-cytosine content, length, sequence, and heterozygosity (if performing genotyping). Consequently, the characteristic slopes of melt curves can indicate differences in the nucleic acid content of the targeted amplicon.

Application	Note
Genotyping	• Heterozygous samples have a characteristically different curve shape when compared to the homozygous samples. The shape of the melt curve is an indicator of heteroduplex formation. • Homozygous samples are distinguishable from each other based on the difference in Tm values.

Application	Note
Mutation detection	The curves of samples that vary from the wild type samples may contain mutations.
Methylation study	The positions of the melt curves for the unknowns relative to those for the methylated standards can be used to estimate the percentage methylation of the samples. For example, if the melt curve for an unknown sample lies between the melt curves for the 5% and 10% methylated standards, the unknown sample contains between 5% and 10% methylated nucleotides.



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

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

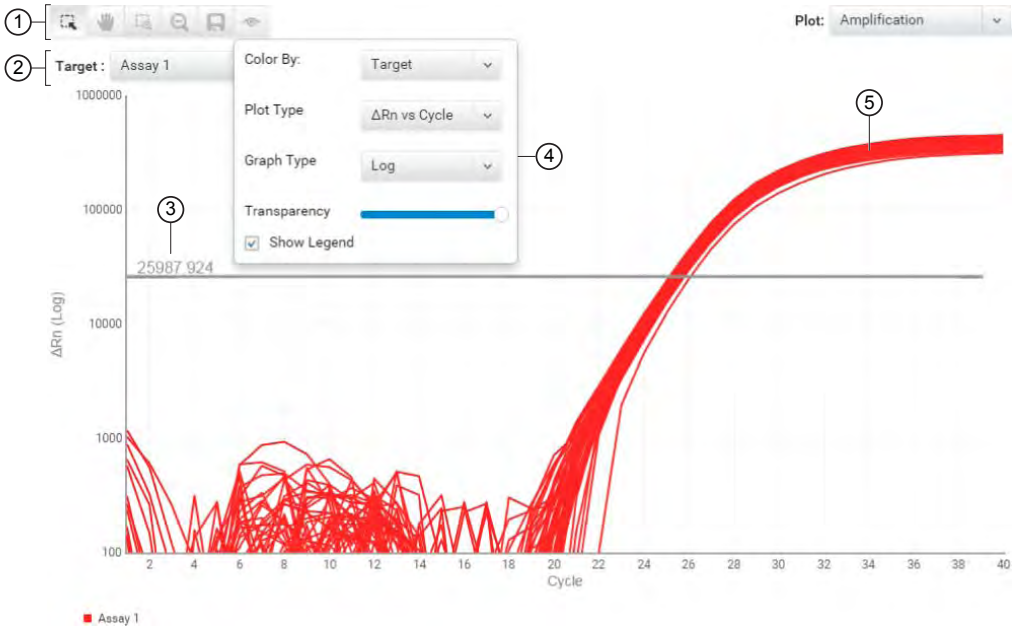
③ **Normalized fluorescence** – Normalized fluorescence signals for all wells throughout the duration of the associated thermal cycling protocol.

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

Amplification Plot

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

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



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

② **Target** drop-down list – Selects the target data displayed by the plot.

③ **Threshold** – The threshold (calculated or manual) that is currently applied to the project data.

④ **View Options** – The view options for the Amplification Plot. Use the drop-down lists to display the type of plot displayed by the software (**ΔRn vs Cycle**, **Rn vs Cycle**, or **CT 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.

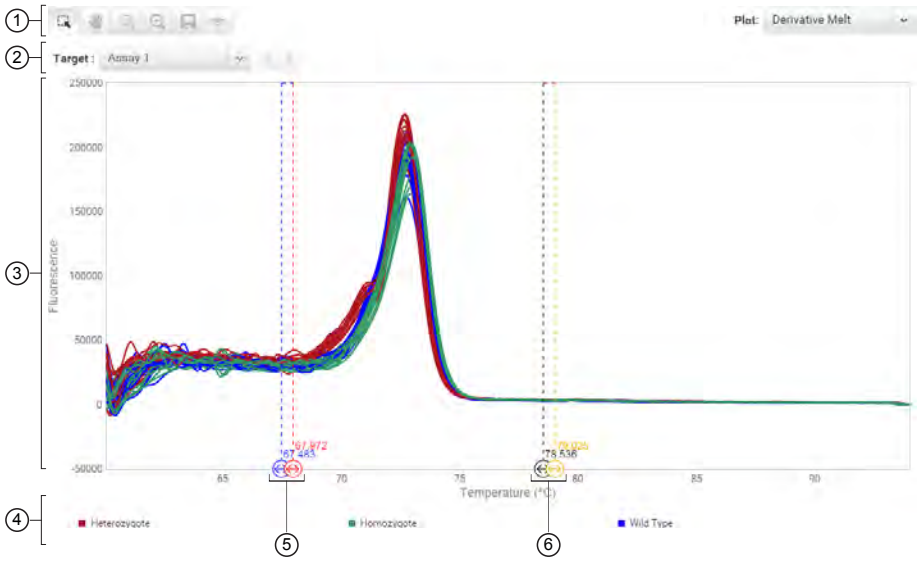
Derivative Melt Plot

The Derivative Melt Curve Plot displays the melt curve data for a single experiment in a graph that contrasts the negative derivative (-Rn') of normalized fluorescence of each well versus temperature. The Derivative Melt Plot allows you to visualize the rate of change in normalized fluorescence throughout the course of the temperature ramp. Each peak present within the plot corresponds to a maximum rate of change in fluorescence for a particular well, from which the software estimates the Tm for the related sample.

The Derivative Melt Curve Plot contains vertical bars that allow you to review and adjust the pre- and post-melt regions for optimizing separation and variant calls. See "Configure the analysis settings" on page 27 for more information on viewing and adjusting the pre- and post-melt regions.

For all experiments, note that:

- Unexpected peaks may indicate possible contamination, primer dimers, or non-specific amplification.
- The data might appear noisy because more data is collected during a high-resolution melt curve than during a standard melt curve.



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

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

③ **Derivative fluorescence** – The negative of the first derivative of the normalized fluorescence for all wells throughout the duration of the temperature ramp.

④ **Legend** – Calls/genotypes present in the in the analysis.

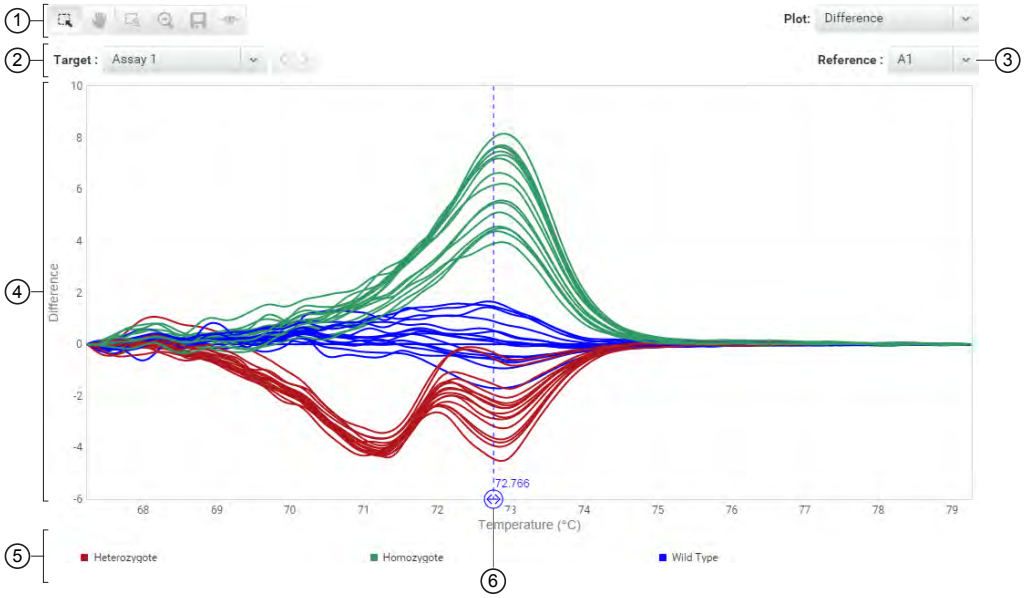
⑤ **Pre-melt region** – The pair of lines to the left of the peak indicate the Pre-melt Start and Stop temperatures when every amplicon is double-stranded. Fluorescence data from the Pre-melt region corresponds to 100% fluorescence in the Aligned Melt Curves Plot.

⑥ **Post-melt region** – The set of lines to the right of the peak indicate the Post-melt Start and Stop temperatures when every amplicon is single-stranded. Fluorescence data from the Post-melt region correspond to 0% fluorescence in the Aligned Melt Curves Plot.

Difference Melt Plot

The Difference Melt Curve Plot displays the melt curve data for a single experiment in a graph that contrasts reference-normalized fluorescence versus temperature. To generate the differenced melt curve data, the software subtracts the fluorescence of the well specified in the Reference drop-down list from each well in the experiment.

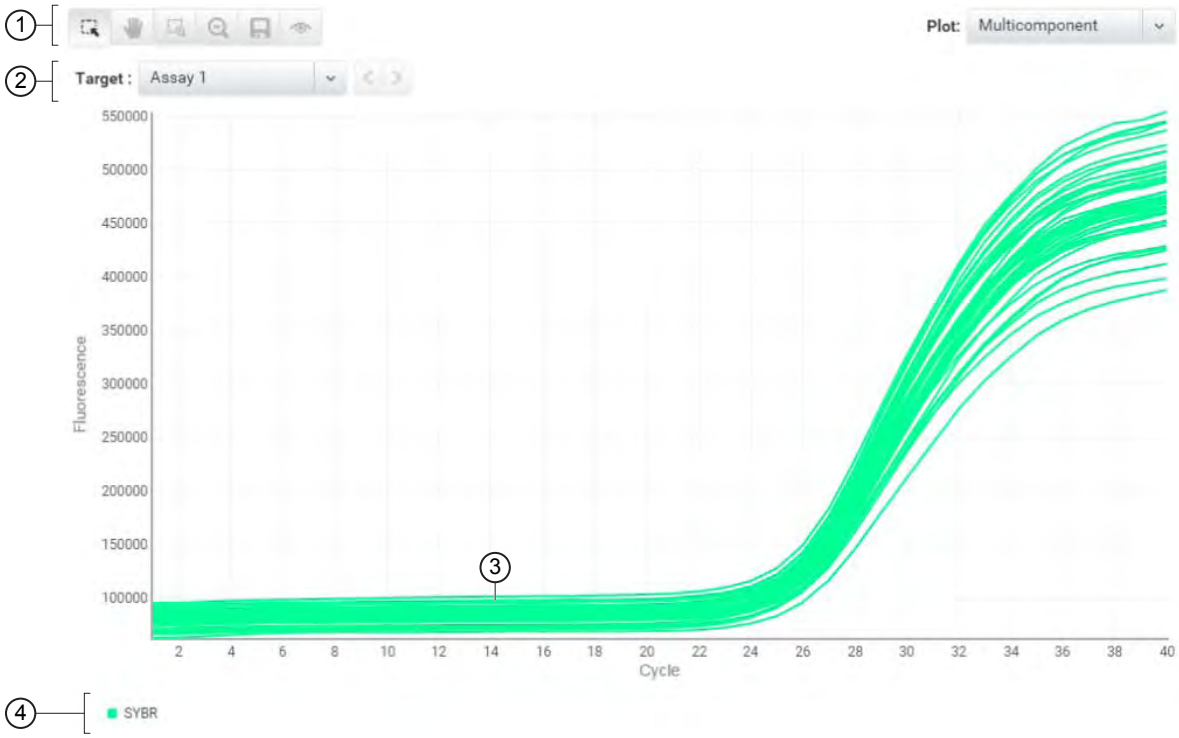
The Difference Plot allows you to more easily see small differences between curves and identify outliers among replicate populations. The plot can be used to confirm the uniform performance of control populations and technical replicates. For example, wells that contain positive controls should exhibit similar differenced melt curves.



1. **Toolbar** – Contains the following tools for controlling the plot:
   - – Select individual data points from the plot.
   - – Allows you to click and manually move the position of the plot.
   - – Zoom the plot to the selected area.
   - – Zooms out the plot to show all data points.
   - – Saves the plot as an image (.png or .jpg).
   - – Allows you to adjust the display options for the plot.
2. **Target** drop-down list – Selects the data from the target data displayed by the plot.
3. **Reference** drop-down list – Selects the well from the well to which the software will normalize the data.
4. **Normalized fluorescence** – Normalized fluorescence signals for all wells, differenced to the well selected in the Reference drop-down list, throughout the duration of the temperature ramp.
5. **Legend** – Calls/genotypes present in the in the analysis.
6. **Temperature slider** – Displays the temperature at the position of the line in the plot.

Multicomponent Plot

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



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

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

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

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

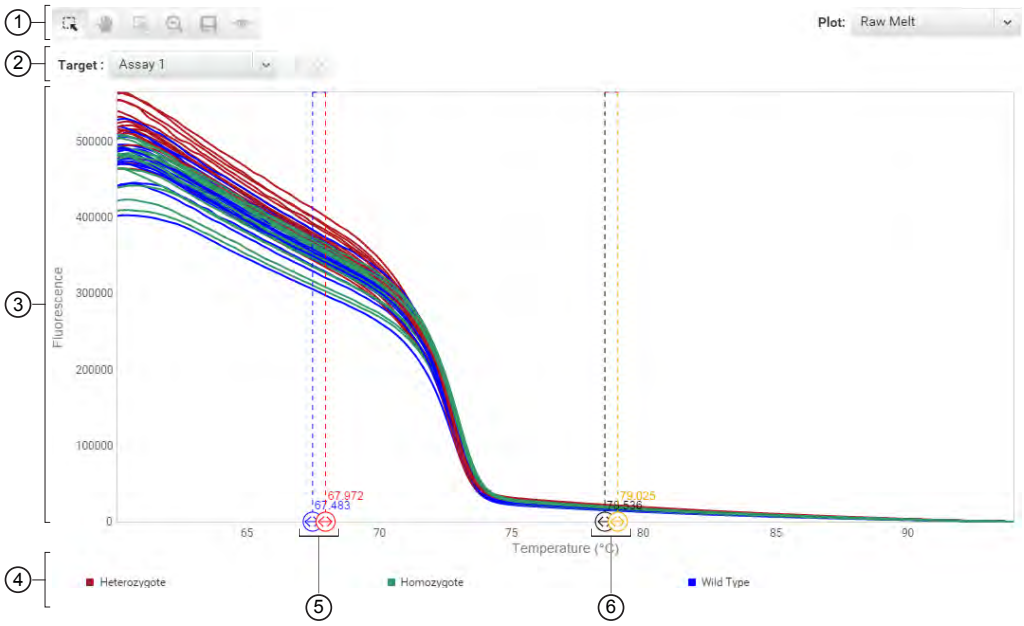
When you analyze your own experiment, confirm the following:

- The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- There should not be any spikes, dips, and/or sudden changes in the fluorescent signal.
- There should not be any amplification in negative control wells.

Raw Melt Curve Plot

The Raw Melt Curve Plot displays the melt curve data for a single experiment in a graph that contrasts normalized fluorescence (Rn) versus temperature. The Raw Melt Curve Plot allows you to visualize the decrease in normalized fluorescence for each well throughout the course of the temperature ramp. The normalized reporter (Rn), displayed on the y-axis, is calculated as the fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference.

The Raw Melt Curve Plot contains vertical bars that allow you to review and adjust the pre- and post-melt regions for optimizing separation and variant calls. See "Configure the analysis settings" on page 27 for more information on viewing and adjusting the pre- and post-melt regions.



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

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

③ **Fluorescence** – Fluorescence signals for all wells throughout the duration of the temperature ramp.

④ **Legend** – Calls/genotypes present in the in the analysis.

⑤ **Pre-melt region** – The pair of lines to the left of the peak indicate the Pre-melt Start and Stop temperatures when every amplicon is double-stranded. Fluorescence data from the Pre-melt region corresponds to 100% fluorescence in the Aligned Melt Curves Plot.

⑥ **Post-melt region** – The set of lines to the right of the peak indicate the Post-melt Start and Stop temperatures when every amplicon is single-stranded. Fluorescence data from the Post-melt region correspond to 0% fluorescence in the Aligned Melt Curves Plot.

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.
Task/Control	The task assigned to the well, where the task is the function that a sample performs on the plate.
Variant	The call for the sample in well. Can be assigned by software (Auto) or manually.
Method	The method used to apply the call to the well: Auto(matic) if calculated by the Applied Biosystems™ Analysis Software or Manual if user-applied.
Silhouette Score	The *modified* silhouette score calculated for the well, which measures distinguishably of the associated melt curve relative to the other curves in the assay (Rousseeuw, 1987.). For each melt curve, the software calculates a *modified* silhouette score, which ranges from 0 to 100. A score closer to 100 indicates that a melt curve is more similar to curves assigned the same variant call than to curves called differently. Lower silhouette scores indicate that a melt curve is less similar to curves assigned the same variant call. **IMPORTANT!** The modified silhouette score differs from the standard silhouette score (Lovmar, *et. al.*, 2005.) in that the software assigns the score to each identified cluster instead of to each data point in the cluster. In addition, the modified value ranges from 0 to 100.

Column	Description
Tm1/Tm2/Tm3	The 1st, 2nd, and 3rd calculated melt temperature (Tm) for the well (if present). **Note:** Blank table cells indicate that the software calculated no Tm for the well at the indicated position.
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.
Ct	The C_T calculated for the related well.
Ct Mean	The arithmetic mean generated from the C_Ts calculated for the technical replicates of the well.
Ct SD	The standard deviation generated from the C_Ts calculated for the technical replicates of the well.
Flags	The number of flags generated for the well.
Quality data	The quality flags generated by the associated well.

8 Quality flags

- AMPNC (Amplification in negative control) quality flag 61
- AMPSCORE (Low signal in linear phase) quality flag 61
- BADROX (Bad passive reference signal) quality flag 62
- BLFAIL (Baseline algorithm failed) quality flag 62
- CQCONF (Calculated confidence in the Cq value is low) quality flag 63
- CTFAIL (Cq algorithm failed) quality flag 63
- DRNMIN (Detection of minimum ΔRn due to abnormal baseline) quality flag 63
- EXPFAIL (Exponential algorithm failed) quality flag 64
- HIGHSD (High standard deviation in replicate group) quality flag 65
- MTP (Multiple Tm peaks for HRM) quality flag 66
- NOAMP (No amplification) quality flag 66
- NOISE (Noise higher than others in plate) quality flag 67
- NOSAMPLE (No sample assigned to well) quality flag 67
- NOSIGNAL (No signal in well) quality flag 67
- OFFSCALE (Fluorescence is offscale) quality flag 68
- OUTLIERRG (Outlier in replicate group) quality flag 68
- PRFDROP (Passive reference signal changes significantly near the Cq/Ct) quality flag 69
- PRFLOW (Average passive reference signal is below the threshold) quality flag 69
- SPIKE (Noise spikes) quality flag 70
- THOLDFAIL (Thresholding algorithm failed) quality flag 70

AMPNC (Amplification in negative control) quality flag

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

If a well is flagged, confirm the results:

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

Possible Cause	Recommended Action
Contamination in one or more PCR reaction components	• Replace all PCR reaction components with new components, then repeat the experiment. Make sure to add water or buffer instead of sample to the well. • Decontaminate the work area and pipettors.
Unstable reaction mix	• Use a hot-start enzyme. • If you are not using a hot-start enzyme, run the reactions as soon as possible after you prepare them.
Poor primer and/or probe design	Redesign the primers and/or probe.

AMPSCORE (Low signal in linear phase) quality flag

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

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

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

If a well is flagged, confirm the results:

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

BADROX (Bad passive reference signal) quality flag

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

If a well is flagged, confirm the results:

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

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

BLFAIL (Baseline algorithm failed) quality flag

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

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

If a well is flagged, confirm the results:

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

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

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

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

If a well is flagged, confirm the results:

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

CTFAIL (Cq algorithm failed) quality flag

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

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

If a well is flagged, confirm the results:

1. Select the flagged well(s) in the plate layout or well table.
2. View the amplification plot [ΔRn vs. Cycle (Linear) or ΔRn vs. Cycle (Log)] and check for:
   - Amplification too early
   - Amplification too late
   - Low amplification
   - No amplification
3. If the amplification looks acceptable, set the threshold and baseline manually.
4. Click **Analyze** to reanalyze the data.
5. Evaluate the results. If the adjustments do not produce a valid C_q, consider omitting the well from analysis.

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

A DRNMIN (DR) quality flag indicates that a given well has a:

- ΔRn less than 0.2 (or the threshold specified in the analysis settings)
  and
- Cq (C_T) less than 35 (or the threshold specified in the analysis settings)

The DRNMIN flag is designed to help identify false positive calls where a well exhibits an abnormal, but detectable, rise in fluorescence over the course of the PCR and is incorrectly assigned a positive call by the software. The flag is based on the

expectation that, given characteristic amplification, wells with lower Cq values should exhibit greater baseline-corrected normalized reporter fluorescence (ΔRn).

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 [ΔRn vs. Cycle (Linear) or ΔRn vs. Cycle (Log)], and confirm the fluorescence signal increase in the well.
3. View the multicomponent plot, and look for fluorescence signal higher than the background.

Possible Cause	Recommended Action
Missing template.	If the flag occurs in just one well of a replicate set, consider omitting the well or retesting the sample. If you retest the sample, make sure to include all reaction components.
Contamination in one or more PCR reaction components	Retest the sample. If necessary, decontaminate the work area and pipettors.

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

If a well is flagged, confirm the results:

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

HIGHSD (High standard deviation in replicate group) quality flag

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

If a replicate group is flagged, confirm the results:

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

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

MTP (Multiple Tm peaks for HRM) quality flag

The MTP (HMTP) quality flag indicates that, for the given well, the software has calculated more than one Tm value. Multiple Tm values for a given sample can result from the possible causes listed below.

If a well is flagged, review the melt curve for the data point to confirm that the dissociation/melt curve contains no unexpected Tm peaks. If you are performing a methylation experiment, the dissociation/melt curves will likely exhibit multiple peaks, where the number of peaks correlates to the number of methylation sites on the amplicon.

If unexpected peaks are present:

Possible Cause	Recommended Action
• Possible contamination • Non-specific amplification	• Replace all PCR reaction components with new components, then repeat the experiment. • Decontaminate the work area and pipettors.
Primer dimers	Redesign the primers and/or probe.

NOAMP (No amplification) quality flag

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

If a well is flagged, confirm the results:

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

Possible Cause	Recommended Action
Missing template.	Repeat the experiment, and make sure to include all reaction components.
Sample contains inhibitors or the sample concentration is too low.	• If this occurs in just one sample, it may be correct. • If this occurs in all samples of a particular type, check the concentration of the sample and if the concentration is sufficiently high try purifying the sample further.

NOISE (Noise higher than others in plate) quality flag

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

If a well is flagged, confirm the results:

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

NOSAMPLE (No sample assigned to well) quality flag

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

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

NOSIGNAL (No signal in well) quality flag

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

If a well is flagged, confirm the results:

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

OFFSCALE (Fluorescence is offscale) quality flag

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

Confirm the results:

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

Possible Cause	Recommended Action
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 ( ) quality flag indicates that the C_q for the well deviates significantly from values in the associated replicate group (only the outlier is flagged).

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

If a well is flagged, confirm the results:

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

Possible Cause	Recommended Action
Pipetting errors.	Repeat the reactions, and follow these guidelines to reduce pipetting errors: • Prepare enough master reaction mix for the entire replicate group, then transfer aliquots to all appropriate wells in the reaction plate. • Calibrate and service your pipettors regularly. • Pipette larger volumes. • Reduce the number of pipetting steps.

Possible Cause	Recommended Action
Contamination in that well.	Replace all reagents, then repeat the experiment.
Decontaminate the work area and pipettors.	Repeat the reactions, and make sure you seal the reaction plate properly.
Improper sealing or seal leaks.	

PRFDROP (Passive reference signal changes significantly near the Cq/Ct) quality flag

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

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

If a well is flagged, confirm the results:

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

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

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

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

If a well is flagged, confirm the results:

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

SPIKE (Noise spikes) quality flag

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

If a well is flagged, confirm the results:

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

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

THOLDFAIL (Thresholding algorithm failed) quality flag

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

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

If a well is flagged, confirm the results:

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

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

A Documentation and support

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Glossary

amplification efficiency (EFF%)	Calculation of the efficiency of the PCR amplification in a standard curve experiment. EFF% is calculated using the slope of the regression line in the standard curve. A slope close to -3.32 indicates optimal, 100% PCR amplification efficiency. To use amplification efficiency in a gene expression project: • On the instrument where you collected the comparative C_T ($\Delta\Delta C_T$) data that will be used in the project, run a standard curve experiment to determine the efficiency. • In the Applied Biosystems™ Analysis Software, enter the amplification efficiency in the Efficiency table in the Relative Quantification Settings tab in the Analysis Settings dialog box.
amplification plot	Display of data collected during the cycling stage of PCR amplification. The amplification plot can be viewed as: • Baseline-corrected normalized reporter (ΔRn) vs. cycle • Normalized reporter (Rn) vs. cycle
assays	A PCR reaction mix that contains primers to amplify a target and a reagent to detect the amplified target.
automatic baseline	An analysis setting for the Baseline Threshold algorithm in which the software identifies the start and end cycles for the baseline in the amplification plot.
automatic threshold	An analysis setting for the Baseline Threshold algorithm in which the software calculates the baseline start and end cycles and the threshold in the amplification plot. The software uses the baseline and threshold to calculate the threshold cycle (C_q).
baseline	In the amplification plot, the baseline is a cycle-to-cycle range that defines background fluorescence. This range can be set manually on a target-by-target basis, or automatically, where the software sets the baseline for each individual well.
Baseline Threshold algorithm	Expression estimation algorithm (C_q) which subtracts a baseline component and sets a fluorescent threshold in the exponential region for quantification.
baseline-corrected normalized reporter (ΔRn)	In experiments that contain data from real-time PCR, the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. In the ΔRn vs Cycle amplification plot, ΔRn is calculated at each cycle as: ΔRn (cycle) = Rn (cycle) - Rn (baseline), where Rn = normalized reporter

cycle threshold	See threshold cycle (C_T).
cycling stage	See threshold cycle (C_T).
C_T	See threshold cycle (C_T).
C_T algorithm	See Baseline Threshold algorithm.
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.
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).
normalized reporter (Rn)	Fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference (usually ROX™ dye).
omit well	An action that you perform before reanalysis to omit one or more wells from analysis. Because no algorithms are applied to omitted wells, omitted wells contain no results. You can add wells back in to the analysis; no information is permanently discarded.
outlier	A data point that deviates significantly from the values of an associated group (for example, the other technical replicates for a sample).
passive reference	A dye that produces fluorescence signal independent of PCR amplification, and that is added to each reaction at a constant concentration. Because the passive reference signal should be consistent across all wells, it is used to normalize the reporter dye signal to account for non-PCR related fluorescence fluctuations caused by minor well-to-well differences in volume. Normalization to the passive reference signal generally results in data with noticeably high precision among technical replicates.
plate grid (plate layout)	An illustration of the grid of wells and assigned content in the reaction plate, array card, or OpenArray™ plate. The number of rows and columns in the grid depends on the plate or card that you use. In the software, you can use the plate grid to view well assignments and results. The plate grid can be printed, included in a report, exported, and saved as a slide for a presentation.
projects	The Applied Biosystems™ Analysis Software organizes the analysis of experiment data by project, which represents the association of the raw data, all experimental setup information, and any associated settings used to perform the analysis. Once created, projects can be shared with other users and transferred to/from the repository.

	Note: Projects to not contain the data from experiments uploaded to the repository; they link the data for analysis without affecting the original data files.
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.
replicates	Identical reactions containing identical components and volumes.
reporter	A fluorescent dye used to detect amplification. With TaqMan™ reagents, the reporter dye is attached to the 5' end. With SYBR™ Green reagents, the reporter dye is SYBR™ Green dye.
Rn	See *normalized reporter (Rn)*.
ROX dye	A dye used as the passive reference.
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.
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 *automatic threshold*), or it can be set manually (see *manual threshold*).
threshold cycle (C_T)	The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.
unknown	In the software, the task for the target in wells that contain the sample being tested.

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