applied biosystems

Applied Biosystems™ Genotyping Analysis Module

USER GUIDE

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ThermoFisher SCIENTIFIC

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C.0	June 2016	Clustering normalization and minor corrections
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Contents

1 Getting Started

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

Getting started

Genotyping experiments are endpoint experiments in which:

- Data are collected at the end of the PCR process.
- Reactions are characterized by the quantity of target sequence accumulated at the end of the PCR.
- The datapoint is the normalized intensity of the reporter dye, or Rn.

Note: Some endpoint experiments also include pre-PCR datapoints. If so, the system calculates the delta Rn (ΔRn) value per the following formula:
ΔRn = Rn (post-PCR read) – Rn (pre-PCR read), where Rn = normalized reporter

Some real-time instruments provide the option of collecting real-time data for genotyping experiments. In the event that an experiment fails, the real-time data can help you determine the cause of the failure.

Optional control components

The following controls can be used to perform the genotyping analysis. If used, the components must be present on all experiments added to the project:

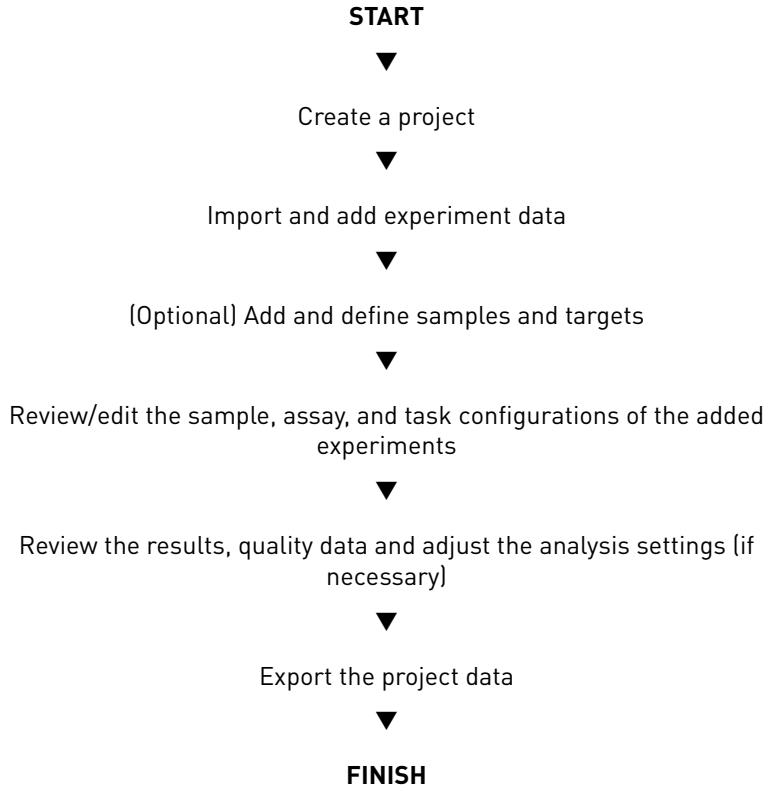
- **NTC** (No-template control) – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells. The software automatically identifies any well in the project that has a sample ID of NTC as a no-template control.

  IMPORTANT! We strongly recommend that you run two NTC wells with every assay in a project.

- **Negative Controls** – Wells that do not contain known template; that is, the wells are set up to not display amplification signal. (For example, the well may contain a non-target template, include an inhibitor, and so on.)
- **Positive Controls** – Wells that contain known template to generate a specific genotype call for one or both assays. In the software, positive controls are classified according to the reporter dye(s) of the assay(s) that will amplify in the presence of the control. Possible combinations include VIC/VIC, VIC/FAM, or FAM/FAM.

Analysis workflows

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



System requirements

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

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

Compatible Real-Time PCR System Data

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

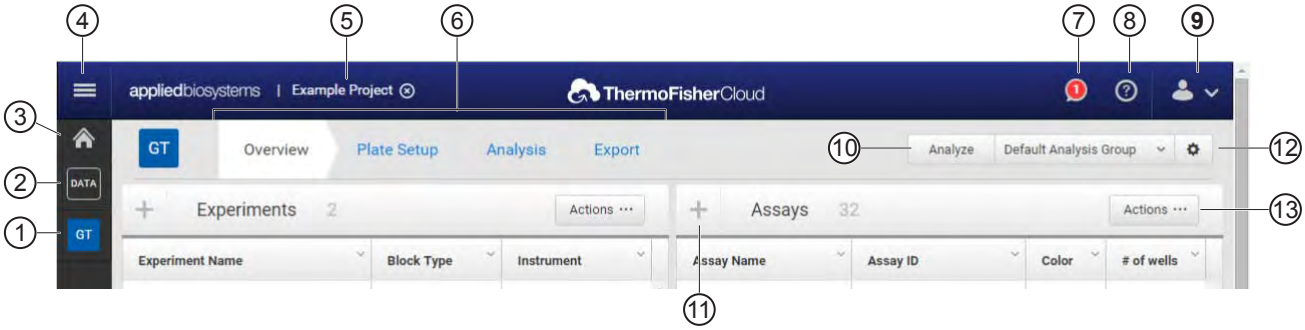
IMPORTANT! The Applied Biosystems™ Analysis Software can import and analyze data from unsupported versions of the instrument software; however, we cannot guarantee the performance of the software or provide technical support for the analyses.

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

Real-Time PCR System	Supported software version(s)	File extension
Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System	v1.1.1 or later	.eds
Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System	v1.0 or later	
Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System		
Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System	v1.0 or later	
Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System		

About the software interface

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



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

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

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

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

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

Note: Click ⊗ to close the project.

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

⑦ 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 **Settings** – Click to edit the analysis settings for the project.

⑬ **Actions** – Click to select from a list of actions that pertain to the related table or plot.

Best practices and tips for using the software

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

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

- Refresh your browser regularly
- Clear your browser cache

2 Manage experiment data

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

Create a project and add experiment data

1. Click (Manage Projects) to view the Dashboard.
2. Create the project:
   a. Click **New Project**.
   b. In the Create Project dialog box, enter a name for the project, select the folder within which you want to place the project, then click **OK**.

      Note: The project name cannot exceed 50 characters and cannot include any of the following characters: / \ < > * ? " | : ; & % $ @ ^ ( ) !

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

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

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

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

Manage projects and experiment data

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

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

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

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

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

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

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

Share experiments, folders, and projects

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

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

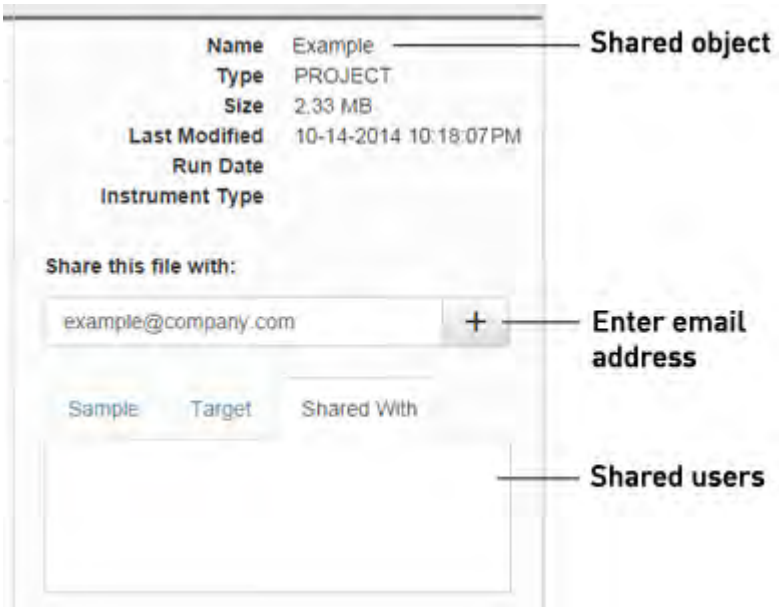
  IMPORTANT! A project is locked (preventing access) when it is open (in use) by any user with shared access to the project. For example, User A shares a project with two colleagues (User B and User C), User B opens the project and begins data analysis (the project is locked and unavailable to Users A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

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

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

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

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



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

IMPORTANT! To share multiple files:

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

- **Un-share** a file, folder, or project:
  a. Click (Home), then click **All Files** to view your data.
  b. Select the shared object, then click the display details icon.
  c. In the details pane, select the **Shared With** tab, then click **un-share** adjacent to the email address of the user from which you want to remove sharing privileges.
  The selected users are notified via email that you are no longer sharing the specified file with them and the shared file(s) will no longer appear in their Home Folder.

About experiment data/files

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

Experiment files contain the following information:

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

File compatibility

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

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

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

3 Set up the project

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

Create or edit an analysis group

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

1. From the Analysis Groups table in the Overview screen, do one of the following:
   - Click **Actions** ▸ **Add** to create a new group.
   - Select an existing group, then click **Actions** ▸ **Edit Analysis Settings** to edit the settings for the group. Go to step 4.
2. From the General dialog box, enter the following information, then click **Next**.
   - **Name** – Enter a name for the analysis group (up to 50 characters).
   - **Samples** or **Experiments** – Select the option to determine the basis by which the Applied Biosystems™ Software will apply the analysis group.
     For example, by selecting "Sample", the software allows you to apply the analysis group to a subset of the samples within the project. Conversely, by selecting "Experiment", the software allows you to apply the analysis group to only some of the experiments or reaction plates added to the project.
   - (*Optional*) **Description** – Enter a description for the analysis group (up to 256 characters).
3. From the Content dialog box, select the samples or experiments to which the analysis group will apply, then click **Next**.

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

Group	Settings
Call Settings	Specify the settings that you want the Applied Biosystems™ Software to use when analyzing the project. • **Call Method** – Determines how the software will make genotyping calls. If you select: – **Autocalling**, the software algorithm is used to call the data points. – **Classification Scheme**, you define the cluster boundaries that are used to call the data points. • **Analyze Data** – Determines what data from the experiment will be used by the software to perform calls. – **Post-PCR Read** – Select to perform calls using the data collected only during the post-PCR read. – **Pre-PCR and Post-PCR Read** – Select to perform calls using the data collected during both the pre- and post-PCR reads. – **Real-time Rn Data** – Select to perform calls using normalized reporter (Rn) data collected throughout the PCR. • **Multiplate Analysis** – When selected, the software normalizes the data for all plates, enabling data comparison across plates. • **Protect Manual Calls** (Protect column) – When selected, the software protects all manual calls. That is, when the software analyzes the data, it will not modify any data points that have been manually called. • **Use Reference Panels for Autocalling** (Reference column) – (Autocalling only) When selected, the software uses reference sample data (imported from a reference panel file) to bias the calls of Unknown data points. • **Use Hardy-Weinberg for Analysis** (H-W column) – (Autocalling only) When selected, the software uses the Hardy-Weinberg equilibrium statistics to bias the calls of data points. IMPORTANT! Using the Hardy-Weinberg equilibrium to influence calls can lead to incorrect genotypes. Enable this feature only if your sample population was selected using Hardy-Weinberg assumptions. • **Use Positive Controls for Analysis** – (Autocalling only) When selected, the software uses positive control data to bias the calls of Unknown data points. **Note:** You cannot manually call positive controls. • **Heterozygote** (Heterozygotes column) – (Autocalling only) Select one of the following: – **Allow** - The autocaller will make heterozygote genotype calls. – **Disallow** - The autocaller will not make any heterozygote genotype calls. – **Disallow in Males** - For samples from males, the autocaller will not make any heterozygote genotype calls. **Note:** In order for the autocaller to perform the Disallow in Males function, your samples must be labeled as male or female in the Gender column of the Samples screen. • **Baseline for Real-time Data** (Baseline column) – (Real-time only) When selected, the software subtracts the baseline fluorescent signal at early PCR cycles from the final fluorescent signal. The final fluorescent signal is generated at the default or user-selected cycle number (see"Cycle #" below). **Note:** This option is disabled for real-time experiment types if the project already contains wells that were imported using current settings.

Group	Settings
	• **Manual Baseline** (Baseline column) – (Real-time only) Instead of using the default range of cycles to calculate a baseline for experiments with real-time data, you can specify a custom range of cycles by entering values in the Start and End fields. The Start cycle must be greater than the End cycle. • **Cycle Number** (Cycle # column) – (Real-time only) By default, the software uses the fluorescent signal extracted from the final cycle in the protocol to create genotyping scatter plots from real-time experiment files. You can change the cycle number that the software uses. The cycle number you enter must be between cycle number 20 and cycle number 60. If left empty (blank), the software uses data throughout all PCR cycles.
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 a **Value**, **Condition**, and **Threshold** are listed for a flag, you can specify the setting for applying the flag. For example, with the default setting for the Genotype Quality Low flag, wells are flagged if the quality metric is less than 0.95. **Note:** If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.
Reference Panels	If you choose to use a reference panel file to analyze your data, select the appropriate reference panel from the list of imported files. **Note:** Reference panels can be generated and imported from the Overview screen. See "Generate a reference panel file" on page 22 or "Import and apply a reference panel file" on page 23 for more information on creating or importing reference panels.

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

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

Manage samples and assays

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

- **Create** a new sample or assay:
  a. From the Samples or Assays table in the Overview screen, click **Actions ▸ Add**.

  b. In the New Sample/Assay dialog box, enter a name for the new sample or assay (up to 256 characters), enter any supporting data.

  c. Click OK to save the sample or assay.

- **Update** an existing sample or assay:
  - Click + to expand the Samples or Assays table in the Overview screen, then edit the entry directly in the table.
  - Select a sample or assay from the table in the Overview screen, click **Actions** ▸ **Update**, then edit the data for the assay or sample.
- **Delete** a sample or assay:
  a. From the Samples or Assays table in the Overview screen, select the sample or assay of interest, then click **Actions** ▸ **Delete**.
  b. In the confirmation dialog box, click **OK** to delete the sample or assay.

Add control identifiers

The Applied Biosystems™ Analysis Software allows you to manually identify the controls used in the experiments added to your project. The control identifiers can be either project-wide (affecting all assays in the project) or assay-specific (applying only to an individual assay).

1. From the Assays list of the Overview screen, click **Actions** ▸ **Control Identifiers**.
2. In the Control Identifiers dialog box, select **Override Control Settings from Experiments**.

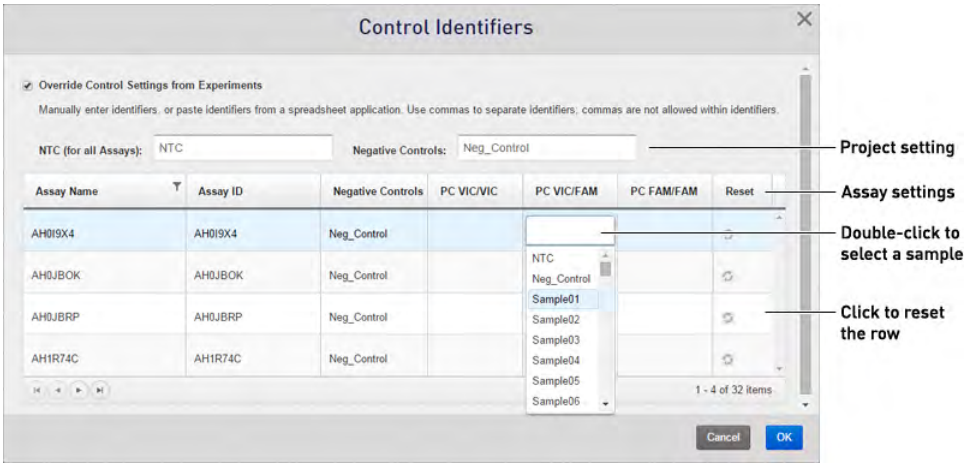
   When this checkbox is:
   - **Selected** – The software uses the control identifiers that you set in the Control Identifiers tab (steps 3 and 4 below), and overrides any control identifiers (tasks) set in the original experiment file. If a sample ID does not match a control identifier, the software assumes the sample is an Unknown.
   - **Deselected** – The software uses the control identifiers (tasks) that were set in the original experiments.
3. In the **NTC** (no template control) and **Negative Controls** fields, enter the project-wide identifiers that you want to use to identify the associated controls for all assays.

4. In the Assays table, enter or select the samples used as controls for each individual assay. For each field, you can either enter the sample name manually, or double-click the field to select the sample from a list.

Option	Enter or select the sample(s) used as...
Negative Controls	Negative controls for the specified assay.
PC VIC/VIC	Homozygous positive controls for the sequence targeted by the VIC™ dye-labeled probe of the specified assay.
PC VIC/FAM	Heterozygous positive controls for the specified assay.
PC FAM/FAM	Homozygous positive controls for the sequence targeted by the FAM™ dye-labeled probe of the specified assay.

Note: By default, the settings in the Assays table are the same as the project-wide settings; however, if you modify the assay settings, the changes apply only to the selected assay (overriding the project settings for the selected assay).

Note: If needed, click the Reset symbol to restore an assay's settings to the project-level setting.



5. Click **OK** to save the changes.

Import target information from AIF files

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

1. From the Targets table in the Overview screen, click **Actions ▸ Import AIF File**.

2. Locate the .aif file with the target information, then click **Open**.

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

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

Import sample information from SSI files

For convenience, the Applied Biosystems™ Software can import sample information directly from supplementary sample information files (.ssi), which you can create to supply detailed supplementary information about your samples (for example, gender and population). The .ssi files are user-created, tab-delimited text files that can be imported from the Overview screen.

1. Create the .ssi file using a spreadsheet application (such as Microsoft™ Excel™ Software):
   a. Open a spreadsheet application.
   b. In each row, enter the following information for each sample. The sample ID (column A, Sample ID) is required; all other information is optional.

	A (required)	B	C	D	E
1	Sample ID	Gender	Population	Non-Consent Assay IDs	Concentration
2	Samp1	F	African-American	a1,a4	0.5 ng/µL
3	Samp3	F	Caucasian	a4	0.5 ng/µL
4	Samp4	M	Japanese		0.5 ng/µL

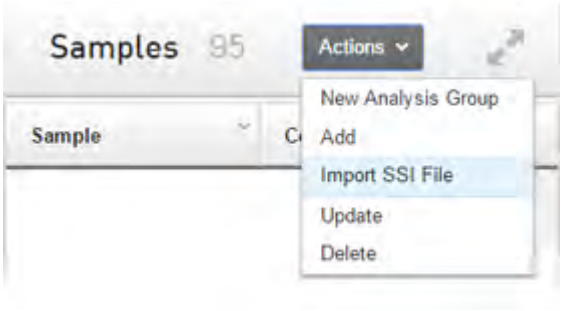
Note: Column D contains Assay IDs for any assays you want to exclude from the sample.

You can list the samples in any order, but you must follow these parameters:

- Enter one sample per row.
- Enter the column headings exactly as shown, including upper- and lowercase letters:
  - **Sample ID**
  - **Gender**
  - **Population**
  - **Non-Consent Assay IDs**
  - **Concentration**
- Enter a sample ID in the **Sample ID** column. In order for the software to correctly correlate sample information in the SSI file with sample information in the imported experiment files, the sample IDs in the SSI file must exactly match the sample IDs in the experiment file, including upper and lowercase letters.
- For any assay that you want to exclude from the sample, enter the assay ID in the **Non-Consent Assay IDs** column; separate multiple assay IDs by commas. All assay IDs must exactly match the assay IDs in the experiment file. If you do not include information in the **Non-Consent Assay IDs** column, all assays are included for that sample.

   c. Save the spreadsheet as a text (*.txt file).

2. From the Samples list of the Overview screen, click **Actions** ▸ **Import SSI File**.



3. From the Open dialog box, select the desired .ssi file with the target information, then click **Open**.

   Note: If the .ssi file lists any assays in the Non-Consent Assay IDs column, the software excludes these assays from the analysis.

Once imported, you can view the sample information in the Samples list of the Overview screen. If the .ssi file contained incorrect information, you can:

- Use the Edit Sample dialog box to edit the sample information directly in the software
- Edit the sample information in the .ssi file, then re-import the file (or import another .ssi file to replace the incorrect information)

Generate a reference panel file

Before you can import a reference panel file into a project, you must first generate the file in the Applied Biosystems™ Analysis Software.

1. Open a project that contains data points that you want to use as reference samples.

2. Click **Analyze** to analyze the project using the default analysis settings.
   If necessary, click **Actions** ▸ **Add** in the Analysis Groups list to create a new analysis group to process the project data.

3. Open the scatterplot for the assay assigned to samples that you want to use as references:
   a. Click **Analysis** to view the results of the analysis.

   b. Click a scatterplot for the assay assigned to samples that you want to use as references.
      The software displays the data points for the selected assay in the scatter plot.

4. Select the samples that you want to use as reference samples:
   a. From the Well Table or the scatterplot, select one or more samples to use as reference samples.

b. Do one of the following:
   - From Well Table, click **Actions** ▸ **Well Level** ▸ **Tag for Ref Panel**.
   - From scatter plot. click **Actions** ▸ **Tag for Ref Panel** to add the selected sample(s) to the reference panel.

5. If necessary, click **Analyze** to analyze the project.

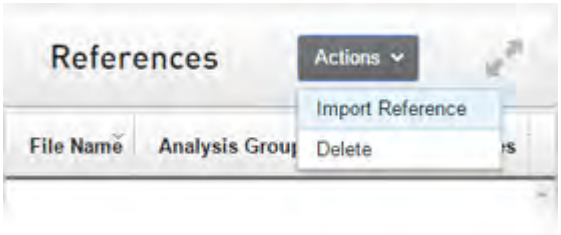
6. Export the reference panel:
   a. From the Analysis screen, click **Export**.
   b. Click **Export** to view the Export screen, then click .
   c. In the Export Reference Panel screen, enter a name for the reference panel, select the appropriate analysis group from the list, then click **Start Export**.
   d. (Optional) Click the entry in the Comments column, then enter any additional information for the exported reference panel.
   e. Click **Download**, select the location for the reference panel file, then click **Save**.

Note: See "Import and apply a reference panel file" on page 23 for more information on using the exported reference panel file.

Import and apply a reference panel file

Once a reference panel file is created, you can import and use it to analyze your project.

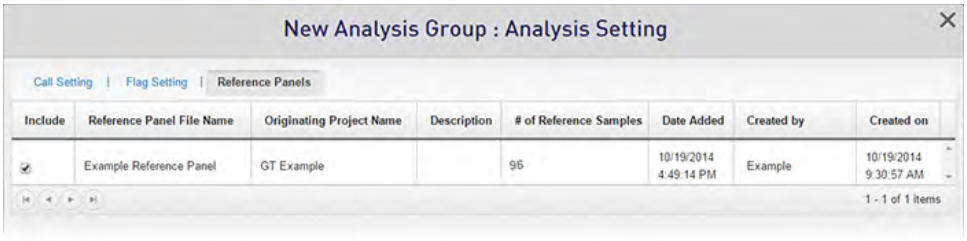
1. In the Overview screen, click **Actions** ▸ **Import Reference** in the References list.



2. In the Open dialog box, select the reference panel file of interest, then click **Open**.
   When imported, the software automatically populates the References list with the information from the imported file.

3. From the Analysis Groups list, select an analysis group, then click **Actions** ▸ **Edit Analysis Settings**.

4. From the Analysis Setting dialog box, click **Reference Panels**, select the appropriate reference panel from the list of imported files. then click **Finish**.



5. Click **Analyze** to reanalyze your project.

4 Edit experiment properties

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

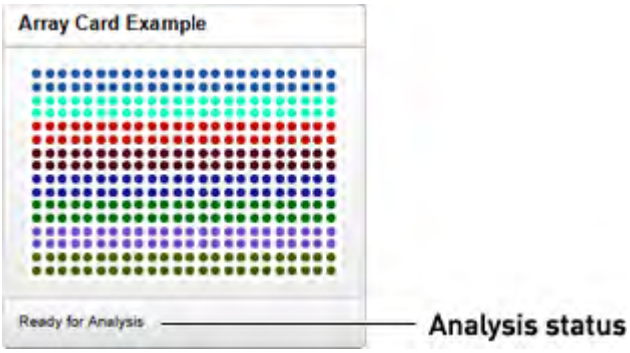
Review and edit the plate setups

After configuring your project with all necessary samples, assays, and reference panels, 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.

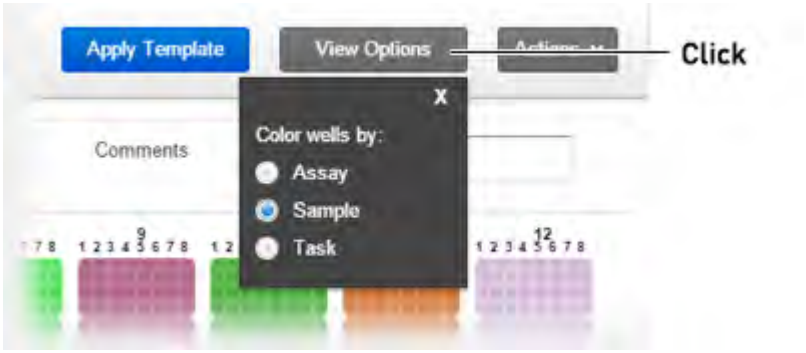
   Note: The software displays plate configuration problems that will prevent analysis of an experiment beneath the image of the related plate.



Apply samples and assays

If the sample or assay assignments within 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.

1. From the Plate Setup screen, select the experiment record that you want to modify.
2. From the Edit Plate screen, click **View Options**, then select either **Assay** or **Sample** to color the plate setup according to the element that you intend to modify.



3. Select the wells of the plate layout that contain the sample or assay that you want to apply.
4. When the wells are selected, click the Sample or Assay field, then select the appropriate item from the list.
5. Once you have completed 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.
   - **NTC** (No-template control) - Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells. The software automatically identifies any well in the project that has a sample ID of NTC as a no-template control. We strongly recommend that you run two NTC wells with every assay in a project.
   - **Negative Controls** - Wells that do not contain known template; that is, the wells are set up to not display amplification signal. (For example, the well may contain a non-target template, include an inhibitor, and so on.)
   - **Positive Controls** - Wells that contain a template known to generate a specific genotype call for one or both assays. In the software, positive controls are classified according to the reporter dye(s) of the assay(s) that will amplify in the presence of the control. Possible combinations include VIC/VIC, VIC/FAM, or FAM/FAM.

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.

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 29.

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 29 for detailed information on constructing template files.

b. Open the project that includes the experiment to which you want to apply the template, then click **Plate Setup**.

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

d. From the Edit Plate screen, click **Actions ▸ Download Template**.

e. Select the template file that contains the desired plate setup, then click **Open**.

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

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

Template files

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

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

		A	B	C	D	E	F
Experiment data (do not edit):	**1**	* Block Type = OpenArray Block					
	2	* Experiment Type = Genotyping					
	3	* Instrument Type = QuantStudio 12K Flex Real-Time PCR System					
	4	* No. Of Wells = 3072					
Column headings (do not edit):	**5**	* Set Up Well Section Info					
	6	Well	Well Position	Sample Name	Assay Name	Task	Comments
Plate setup content (add well data in any order):	**7**	0	A1a1	Sample01	AH70YX8	UNKNOWN	
	8	1378	B10e3	Sample22	AHABB99	UNKNOWN	
	9	3008	D12a1	NTC	AH70YX8	NTC	
	...	...	...	...	...	...	...

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
  - Assay Name
  - Comments

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

  For each well the file contains the following information:

  - **Column A (Well)** – The numerical position of the well on the plate, where wells are numbered left to right and top to bottom. For example, on a 96-well plate, the number of well A1 is "0" and the number of well G12 is "95".
  - **Column B (Well Position)** – The coordinates of the well on the plate.

    Note: For OpenArray™ plates, wells are identified through the combination of the sector coordinates on the plate, and the coordinates of the well within the sector. For example, the position "b2d10" refers to the through-hole at position D10 within sector B2 on the plate.
  - **Column C (Sample Name)** – The name of the sample within the well (up to 256-characters).
  - **Column D (Task)** – The task of the sample within the well, where acceptable values include **UNKNOWN**, **NTC**, **POSITIVE CONTROL VIC/VIC**, **POSITIVE CONTROL FAM/VIC**, **POSITIVE CONTROL FAM/FAM**, and **NEGATIVE CONTROL**.
  - **Column E (Assay Name)** – The name of the assay added to the well, or the identity of the target sequence (up to 256-characters).
  - **Column F (Comments)** – Additional comments describing 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 Review the quality data and results

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

Analyze the data using Autocalling

1. Configure the analysis settings for autocalling:
   a. From the Analysis Groups table in the Overview screen, select an existing group, then click **Actions** ▸ **Edit Analysis Settings** to edit it.

   b. From the Call Method setting of the Analysis Setting dialog box, select **Autocalling**.

   c. Modify the analysis settings as desired. then click **Finish**.

2. Click **Analyze** to analyze your project.

3. When the analysis is complete, click **Analysis** to view the results.

4. View the data in the scatter plot:
   a. In the Analysis screen, select **View** ▸ **Assay**, then select the assay of interest. The software displays the data points for the selected assay in the scatter plot.

   b. From the scatter plot, click (View Options), then select the items to display in the plot (**Flagged data points**, **References**, **Omitted Wells**, **Legend**, and **Traces**).

5. Review the calls and, if necessary, perform manual calling:
   a. When viewing the scatter plot, click (View Options), then select **Show Legend**. The legend appears at the bottom of the scatter plot.
   b. Review the genotype calls.
   c. If any of the calls are incorrect, perform manual calling in the Results table or the scatter plot.

Option	Action
Results table	1. Select the sample in the Results table. 2. Click **Actions** ▸ **Well Level**, then select the appropriate call.
Scatter plot	1. Click and drag a box around one or more data points. 2. Click **Actions**, then select the appropriate call.

   Note: To remove a manual call, select the sample, then click **Actions**, then select **Clear Manual Call**.

   d. Click **Analyze** to view the new results.

   For all manually called wells/data points, in the Results table:
   - Checkmarks appear in the Manual column
   - N/A appears in the Quality column

6. View and modify the data in the Results table:

Tool	Use this tool to...
Mouse/cursor	Select wells. To select: • An individual well, select the well in the Results table. • More than one well at a time, press the **Ctrl** key or **Shift** key when you select the wells in the Results table. When you select wells in the Results table, the corresponding data points are selected in the scatter plot.
Group by drop-down menu	Select how to group the samples in the Results table. For example, if you select **Experiment**, the samples are grouped according the experiment to which they belong. To collapse or expand the groups, click the arrow in the Results table above to each group.
Actions (Change drop-down menu	**View Real Time Plots** to review the amplification and multicomponent data for the wells.
	Set/clear bookmark for wells in the project. The bookmarks persist in the Results screen, so you can easily find bookmarked wells.

Tool	Use this tool to...
Actions (Change drop-down 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 calls in the Results table (the Call column is empty/blank). • Does not include the omitted wells in the analysis. For un-omitted wells, the software: • Reassigns the tasks based on the settings in the Analysis Settings dialog box. • (For control wells) Assigns the call as **N/A**. • (For non-control wells) Assigns the call as **Undetermined**.
	Tag for Ref Panel/Un-tag selected well as a reference sample (for generating a reference panel file).
	Attach a **Comment** to a well.
	Perform manual calling.
or	Expand or collapse the Results table.

7. Use the Results table columns as needed:

Column	Use this column to...
(Omit)	Omit/Un-omit a well from the analysis.
(Bookmark)	Determine whether the well has been bookmarked.
Sample ID	View the ID (a unique name or number) of the sample.
Call	View the genotype call assigned to the well.
Manual	Determine whether the sample has been manually called.
Task	View the task assigned to the well. A task is the function that a sample performs: • Unknown • No template control (NTC) (control identifier) • Positive control (control identifier) for VIC/VIC, FAM/FAM or VIC/FAM • Negative control (control identifier)
Allele 1	View the normalized fluorescent value of the dye associated with the probe used to detect the allele. TaqMan^{fi} OpenArray$^{™}$ Genotyping System fluorescent values are not normalized by the ROX$^{™}$ passive reference dye.
Allele 2	
Allele 1 Amp Score	View the C_T or C_{RT} value calculated for the related well. **Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Allele 2 Amp Score	
Allele 1 CT/CRT values	View the C_T or C_{RT} calculated for the probe targeting the associated allele in the related well.

Column	Use this column to...
Allele 2 CT/CRT values	**Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Passive Reference	View the fluorescent value of the passive reference dye. FAM™ and VIC™ dye fluorescent values are normalized by the passive reference dye (usually the ROX™ dye). TaqMan® OpenArray™ Genotyping System fluorescent values are not normalized by the ROX™ passive reference dye.
Quality	View the estimated quality value of the call made by the autocaller algorithm. The algorithm outputs a quality value for each data point with the following properties: • The quality value is a number between 0 and 1. • The quality value is always 0 for Invalid data points. • The quality value is always 1 for No Amplification data points. • For FAM/FAM, FAM/VIC, and VIC/VIC calls, the Quality Value is a higher value for calls more likely to be correct and a lower value for calls more likely to be incorrect. The quality value is N/A if a well is manually called, is a control, or is called using the Classification Scheme call method.
Well	View the location of the well in the reaction plate. For example, P18 indicates that the sample is located in row P, column 18.
Experiment Filename	View the name of the experiment file to which the well belongs.
Gender	View the gender assigned to the sample.
Population	View the population name(s) assigned to the samples in the project. By default, the population of Unknown samples is named All. You can edit the population of some or all of the samples in a project using the Edit Sample dialog box. Each population name that you assign appears in the Population Statistics tab.
Plate Barcode	View the name or barcode of the reaction plate in which the experiment was run.
Comment	View any comments applied to the well.

8. (If needed) Click **Analyze** to reanalyze and view the new results.

9. Select **View ▸ References**, then view the data in the References Samples table.

Column	Use this column to...
	Indicates that the sample is part of the reference panel.
Sample ID	View the ID (a unique name or number) of the sample.
Call	View the genotype call assigned to the well.
VIC™	View the normalized fluorescence value of the VIC™ and FAM™ dyes.
FAM™	

Column	Use this column to...
Well	View the location of the well in the reaction plate. For example, P18 indicates that the sample is located in row P, column 18.
Experiment Filename	View the name of the experiment file to which the well belongs.
Reference Panel File Name	View the name of the file that contains the reference panel.
Plate Barcode	View the name or barcode of the reaction plate in which the experiment was run.
Originating Project Name	View the name of the project from which the reference panel file was created.

10. Select **View ▸ Population**, then view the data in the Population Statistics table.

Column	Use this column to...
Population	View the population name(s) assigned to the samples in the project. By default, the population of Unknown samples is named "All". You can edit the population of some or all of the samples in a project using the Edit Sample dialog box. Each population name that you assign appears in the Population Statistics tab.
Allele 1 Freq	View the frequency of allele 1 determined for each population in the project.
Allele 2 Freq	View the frequency of allele 2 determined for each population in the project.
1/1 Freq	View the frequency of genotype 1/1 determined for each population in the project.
1/2 Freq	View the frequency of genotype 1/2 determined for each population in the project.
2/2 Freq	View the frequency of genotype 2/2 determined for each population in the project.
Chi-Squared	View the Chi-Squared value calculated for each population in the project. The calculated Chi-Squared value is used to determine if the experimental data is in Hardy-Weinberg equilibrium based on the observed and expected number of genotype calls, assuming 1 degree of freedom.
p-Value	View the P-value calculated for each population in the project. The calculated P-value is the probability of the differences in observed and expected genotype calls accounted for by chance alone. **Note:** Life Technologies recommends that you review Hardy-Weinberg equilibrium fundamentals for application of this P-value.

Analyze the data using Classification Schemes

1. Configure the analysis settings for classification scheme-based analysis:
   a. From the Analysis Groups table in the Overview screen, select an existing analysis group, then click **Actions** ▸ **Edit Analysis Settings** to edit it.

   b. From the Call Method setting of the Analysis Setting dialog box, select **Classification Scheme**.

   c. Modify the analysis settings as desired. then click **Finish**.

2. Click **Analyze** to reanalyze your project.

3. When the analysis is complete, click **Analysis** to view the results.

4. View the data in the scatter plot:
   a. In the Analysis screen, select **View** ▸ **Assay**, then select the assay of interest. The software displays the data points for the selected assay in the scatter plot.

   b. From the scatter plot, click and select the items to display in the plot (**Flagged data points**, **References**, **Omitted Wells**, **Legend**, **Traces**, and **Classification Scheme**).

5. In the scatter plot, click (Select New Scheme), select the appropriate classification scheme, then click **Analyze** to view the new results.

Scheme	Graphic	Regions defined
Standard Diploid Genotypes		Five regions are defined: • Homozygous FAM/FAM • Heterozygous FAM/VIC • Homozygous VIC/VIC • No amplification • Undetermined (white, unlabeled regions) **Note:** The software assigns this scheme as the default.
Rare VIC Allele		Five regions are defined: • Homozygous FAM/FAM • Heterozygous FAM/VIC • Possible Rare Allele (PRA) • No amplification • Undetermined (white, unlabeled region)
Rare FAM Allele		Five regions are defined: • Possible Rare Allele (PRA) • Heterozygous FAM/VIC • Homozygous VIC/VIC • No amplification • Undetermined (white, unlabeled region)
Extremely Rare VIC Allele		Three regions are defined: • Homozygous FAM/FAM • Possible Rare Allele (PRA) • No amplification
Extremely Rare FAM Allele		Three regions are defined: • Possible Rare Allele (PRA) • Homozygous VIC/VIC • No amplification

6. Edit the classification boundaries to adjust the classification regions for the current scheme:
   a. Click (Edit Classification Boundaries).
   b. Click and drag the boundary lines for each region you want to adjust.

      Note: The boundary lines should not intersect outside the NO AMP region (ellipse).

   c. Click **Analyze** to view the new results.

Review the analyzed data in the Plates view

Once analyzed, you can use the Plate view to review the genotyping results of a project summarized per reaction plate. The Plates view provides multiple data views including the:

- **Plates preview** – For each experiment added to the project, the software generates a thumbnail of the reaction plate and lists the pass/fail status of each QC setting (flag). From the preview, you can review the details of any reaction plate by clicking the appropriate record.
- **Plate layout** – Displayed after selecting a thumbnail in the Plate preview. The plate layout displays an illustration of the reaction plate for the selected experiment. The graphic can be configured to color the wells according to experiment function.
- **Results table** – Displayed after selecting a thumbnail in the Plate preview. The results table displays the detailed QC information about all samples/assays in the selected experiment.

1. Click **Analyze** to reanalyze your project, then click **Analysis** to view the results.
2. In the Analysis screen, select **View** ▸ **Plates**.
3. (Optional) In the Plate view, click  to view a summary of the reaction plates associated with the project, look for any experiments that have a:
   - Low Experiment Call Rate flag ( appears in the column) in either the **%E** (Experiment Call Rate) column or the **%R** (Maximum Percent with Low ROX) column.
   - Value in a flag column, where the value indicates the number of wells with the flag.
4. Select the plate of interest to view the detailed data for the related experiment.
5. Review the data in the plate layout:
   a. Click **View Options**, then select **Show Plate Legend** to display the legend for the plate layout.

b. One at a time, select the desired options to view the data as needed (click **View Options** and select the data view):

- **Assay** – Displays the location of each assay in the reaction plate. In this view, each color represents a different assay and each boxed area represents one assay.
- **Sample** – Displays the location of each sample in the reaction plate. In this view, each color represents a different sample and each boxed area represents one sample set.
- **Task** – Displays the tasks/control identifiers assigned each well in the reaction plate. In this view, each color represents a different task/control identifier and each boxed area represents a single well on the reaction plate.
- **Call** – Displays the genotype calls assigned each well in the reaction plate. In this view, each color represents a different call and each boxed area represents a single well on the reaction plate.
- **ROX Range** – Displays the ROX™ dye levels in the reaction plate. In this view, each color represents the signal strength of the passive reference, from Low to Very High.

6. Scroll beneath the Plate Layout to view the data in the Results table data.

Column	Use this column to...
Well	View the location of the well in the reaction plate. For example, P18 indicates that the sample is located in row P, column 18.
(Omitted)	View the omission status of the related well.
(Bookmark)	View whether the well is bookmarked.
Quality data	View any quality flags generated by the associated well.
Call	View the genotype call assigned to the well.
Sample ID	View the ID (a unique name or number) of the sample.
Allele 1 Amp Score	View the C_T or C_{RT} value calculated for the related well. **Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Allele 2 Amp Score	
Allele 1 CT/CRT values	View the C_T or C_{RT} calculated for the probe targeting the associated allele in the related well. **Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Allele 2 CT/CRT values	
Assay name	View the unique name of the assay assigned to the well.
Assay ID	View the ID number of the assay assigned to the well.

Column	Use this column to...
Task	View the task assigned to the well. A task is the function that a sample performs: • Unknown • No template control (NTC) (control identifier) • Positive control (control identifier) for VIC/VIC, FAM/FAM or VIC/FAM • Negative control (control identifier)
Comments	View any comments made by a user about the well.

7. (Optional) Bookmark any wells of interest:
   a. Select one or more wells to bookmark.
   b. Click **Actions ▸ Bookmark**.

   The bookmarks persist in the Results screens, so you can easily find the bookmarked data.

8. (Optional) Apply comment to wells as necessary:
   a. Select one or more wells to annotate.
   b. Click **Actions ▸ Comment**.
   c. In the Input Comment dialog box, enter your comment into the Comment field, then click **OK**.

9. (Optional) Omit wells as necessary:
   a. Select one or more wells to omit.
   b. Click **Actions ▸ Omit Well(s)**.

Review the analyzed data using the Samples view

Once analyzed, you can use the Samples view to review the genotyping results of a project summarized per sample. The Samples view provides multiple data views including the:

- **Samples table** – For each sample in the project, an overview of the pass/fail status of each QC setting (flag).
- **Results table** – Displays the detailed QC information about all samples/assays in the selected sample.

1. Click **Analyze** to reanalyze your project, then click **Analysis** to view the results.
2. In the Analysis screen, select **View ▸ Sample**.

3. Review the summary of samples in the Samples table. Look for any samples that have a:
   - Low Sample Call Rate flag (appears in the column) in either the **Sample Call Rate** column.
   - Value in a flag column, where the number indicates the number of wells with the flag.

4. For each sample of interest, select the sample in the table to view the detailed data in the Results table.

5. Review the data in the Results table data.

Column	Use this column to...
(Omitted)	View the omission status of the related well.
(Bookmark)	View whether the well is bookmarked.
Assay name	View the unique name of the assay assigned to the well.
Assay ID	View the ID number of the assay assigned to the well.
Quality data	View any quality flags generated by the associated well.
Allele 1 Amp Score	View the C_T or C_{RT} value calculated for the related well. **Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Allele 2 Amp Score	
Allele 1 CT/CRT values	View the C_T or C_{RT} calculated for the probe targeting the associated allele in the related well. **Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Allele 2 CT/CRT values	
Call	View the genotype call assigned to the well.
Manual	Determine whether the sample was called manually.
Well	View the location of the well in the reaction plate. For example, P18 indicates that the sample is located in row P, column 18.
Experiment Name	View the experiment associated with the related well.
Task	View the task assigned to the well. A task is the function that a sample performs: • Unknown • No template control (NTC) (control identifier) • Positive control (control identifier) for VIC/VIC, FAM/FAM or VIC/FAM • Negative control (control identifier)
Comments	View any comments made by a user about the well.

If necessary, click **Group By** to select how to group the samples in the Results table. For example, if you select **Experiment Name**, the samples are grouped according the experiment to which they belong.

Note: To collapse or expand the groups, click the arrows in the Results table above to each group.

6. (Optional) Bookmark any wells of interest:
   a. Select one or more wells to bookmark.
   b. Click **Bookmark**, then select **Set Bookmark** from the drop-down list.

   The bookmarks persist in the Results screens, so you can easily find the bookmarked data.

7. (Optional) Apply comment to wells as necessary:
   a. Select one or more wells to annotate.
   b. Click **Tag**, then select **Comment** from the drop-down list.
   c. In the Input Comment dialog box, enter your comment into the Comment field, then click **OK**.

8. (Optional) Omit wells as necessary:
   a. Select one or more wells to omit.
   b. Click **Tag**, then select **Omit** from the drop-down list.

Review the analyzed data in a scatterplot

Perform an initial review of the experiment results in the Allelic Discrimination Plot, which contrasts the normalized reporter dye fluorescence (Rn) for the allele-specific probes of the SNP assay.

1. Click **Analyze** to analyze your project, then click **Analysis** to view the results.
2. View the data in the scatter plot:
   a. From the Analysis screen, select **View ▸ Assay**, then select assays of interest. The software displays the data points for the selected assay in the scatter plot.
   b. From the scatter plot, click (View Options), then select the items to display in the plot (**Flagged data points**, **References**, **Omitted Wells**, **Legend**, **and Traces**).

3. Review the scatterplot data.
   - If the Autocaller is enabled, the plot displays allele symbols for each sample evaluated for the selected SNP.

     The samples are grouped on the plot as follows:

Symbol	Are grouped along the...	The genotypes of the samples are...
red dot	X-axis of the plot	Homozygous for Allele 1 of the selected SNP assay.
blue dot	Y-axis of the plot	Homozygous for Allele 2 of the selected SNP assay.
green dot	Midway between the homozygote clusters	Heterozygous for both alleles of the selected SNP assay (Allele 1 and Allele 2).
yellow dot	Bottom-left corner of the plot	A negative control.
black dot	Anywhere on plot	Undetermined.

   - If the Autocaller is not enabled, the plot displays a cross mark (X – Undetermined) for each sample.

4. For each cluster in the plot:
   a. Click and drag a box around the cluster to select the associated wells in the plate layout and well table.

   b. Confirm that the expected wells are selected in the well table.

      For example, if you select the cluster at the bottom-left corner of the plot, only the negative controls will be selected. The presence of an unknown among the negative controls may indicate that the sample failed to amplify.

   c. Repeat steps 4a and 4b for all other clusters in the plot.

5. If necessary, perform manual genotype calls by selecting one or more data points in the scatterplot, then selecting the appropriate call from the menu.

6. When finished reviewing the data, click ‹ to return to the Analysis screen.

7. Repeat steps 2 to 6 for all assays of interest.

About classification schemes and genotype calls

In contrast to autocalling, where the Applied Biosystems™ Analysis Software makes genotyping calls algorithmically, the software can perform calls based on a classification scheme. The classification schemes broadly divide the scatter plot surface into regions where samples from each genotype are expected. If you select the correct boundaries, then most data points are expected to lie in the center of their appropriate genotype region. Some data points that are on the edge of these regions may be misclassified by chance because of the positioning of the boundaries.

You should carefully review data points that are on the edge of each genotype region. Advanced users may be able to determine the true genotype of these data points. Alternatively, you may choose to label these data points as outliers, or omit them from the analysis.

Scheme	Graphic	Regions defined
Standard Diploid Genotypes		Five regions are defined: • Homozygous FAM/FAM • Heterozygous FAM/VIC • Homozygous VIC/VIC • No amplification • Undetermined (white, unlabeled regions) **Note:** The software assigns this scheme as the default.
Rare VIC Allele		Five regions are defined: • Homozygous FAM/FAM • Heterozygous FAM/VIC • Possible Rare Allele (PRA) • No amplification • Undetermined (white, unlabeled region)
Rare FAM Allele		Five regions are defined: • Possible Rare Allele (PRA) • Heterozygous FAM/VIC • Homozygous VIC/VIC • No amplification • Undetermined (white, unlabeled region)
Extremely Rare VIC Allele		Three regions are defined: • Homozygous FAM/FAM • Possible Rare Allele (PRA) • No amplification
Extremely Rare FAM Allele		Three regions are defined: • Possible Rare Allele (PRA) • Homozygous VIC/VIC • No amplification

Modify the analysis settings for a specific assay

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 Results screen; however, the changes that you make will apply only to the selected assay.

1. From the Analysis screen, select **Assay** from the View drop-down list.
2. From the Assay or Sample view, select the scatterplot that you want to modify.
3. From the Allelic Discrimination screen, click **Analysis Setting** to view the analysis settings associated with the selected assay or sample.

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

Group	Settings
Call Settings	Specify the settings that you want the Applied Biosystems™ Software to use when analyzing the assay data. IMPORTANT! Where noted, settings labeled as "Real-time only" are enabled only for projects that contain experiments with real-time data. Also, settings labeled as "Autocalling only" are enabled only if Autocalling is selected. • **Call Method** – Determines how the software will make genotyping calls. If you select: – **Autocalling**, the software algorithm is used to call the data points. – **Classification Scheme**, you define the cluster boundaries that are used to call the data points. • **Protect Manual Calls** – When selected, the software protects all manual calls. That is, when the software analyzes the data, it will not modify any data points that have been manually called. • **Use Reference Panels for Autocalling** – (Autocalling only) When selected, the software uses reference sample data (imported from a reference panel file) to bias the calls of Unknown data points. • **Use Hardy-Weinberg for Analysis** – (Autocalling only) When selected, the software uses the Hardy-Weinberg equilibrium statistics to bias the calls of data points. IMPORTANT! Using the Hardy-Weinberg equilibrium to influence calls can lead to incorrect genotypes. You should only enable this feature if your sample population was selected following Hardy-Weinberg assumptions. • **Use Positive Controls for Analysis** – (Autocalling only) When selected, the software uses positive control data to bias the calls of Unknown data points. **Note:** You cannot manually call positive controls. • **Heterozygote** – (Autocalling only) Select one of the following: – **Allow** - The autocaller will make heterozygote genotype calls. – **Disallow** - The autocaller will not make any heterozygote genotype calls. – **Disallow in Males** - For samples from males, the autocaller will not make any heterozygote genotype calls. **Note:** In order for the autocaller to perform the Disallow in Males function, your samples must be labeled as male or female in the Gender column of the Samples screen. • **Baseline for Real-time Data** – (Real-time only) When selected, the software subtracts the baseline fluorescent signal at early PCR cycles from the final fluorescent signal. The final fluorescent signal is generated at the default or user-selected cycle number (see"Cycle Number" below). **Note:** This option is disabled for real-time experiment types if the project already contains wells that were imported using current settings. • **Normalize Cluster for Autocalling** – (Autocalling of OpenArray projects only) When selected, the software performs an algorithmic normalization of the cluster data prior to autocalling. The normalization option is available as a global setting. If desired, you can individually enable or disable cluster normalization for specific assays by selecting the appropriate entries in the Normalize Cluster column of the settings table. IMPORTANT! The cluster normalization option assumes that data point distribution in your genotype clusters are approximately similar across the arrays or plates that you are normalizing.

Group	Settings
	IMPORTANT! The cluster normalization feature is designed specifically to optimize the analysis of OpenArray experiments, where genotype clusters typically consist of a large number of samples. Before using cluster normalization, confirm that the clusters generated from your experiments consist of at least 20 samples in the largest cluster. • **Manual Baseline** – (Real-time only) Instead of using the default range of cycles to calculate a baseline for experiments with real-time data, you can specify a custom range of cycles by entering values in the Start and End fields. The Start cycle must be greater than the End cycle. • **Cycle Number** – (Real-time only) By default, the software uses the fluorescent signal extracted from the final cycle in the protocol to create genotyping scatter plots from Real-time experiment files. You can change the cycle number that the software uses. The cycle number you enter must be between cycle number 20 and cycle number 60. If left empty (blank), the software uses data throughout all PCR cycles. IMPORTANT! The cluster normalization feature is designed specifically to optimize the analysis of OpenArray experiments, where genotype clusters typically consist of a large number of samples. Before using cluster normalization, confirm that the clusters generated from your experiments consist of at least 20 samples in the largest cluster.
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 a **Value**, **Condition**, and **Threshold** are listed for a flag, you can specify the setting for applying the flag. For example, with the default setting for the Genotype Quality Low flag, wells are flagged if the quality metric is less than 0.95. **Note:** If you choose to adjust the setting for applying a flag, make minor adjustments as you evaluate the appropriate setting.

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

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

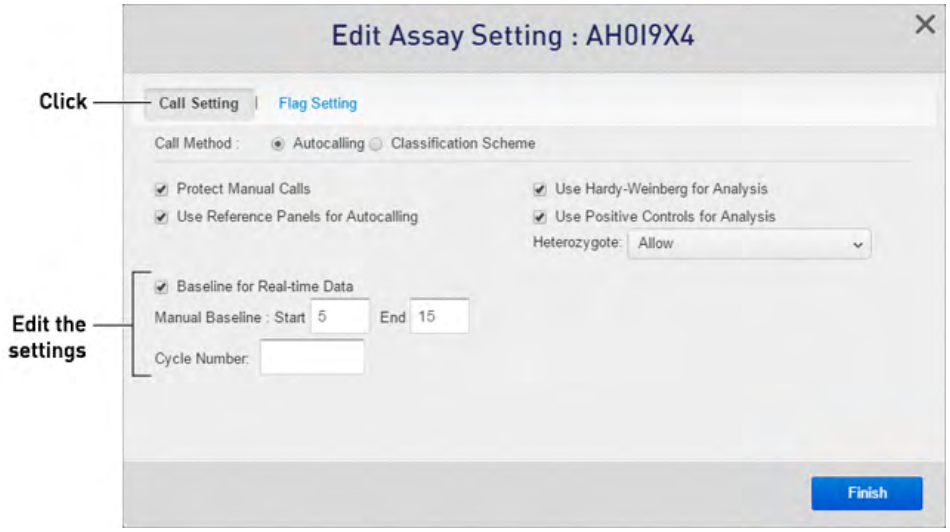
Change the cycle number and baseline normalization

IMPORTANT! Life Technologies recommends that only advanced users optimize the scatter plot data.

Following an analysis, you can modify the cycle number and baseline normalization settings of an individual assay as follows.

Note: Prior to the analysis, you can modify the settings from the Overview screen by modifying the active analysis group.

1. If necessary, click **Analyze** to analyze your project, then click **Analysis** to view the analyzed data.
2. From the Analysis screen, select **View** ▸ **Assay** display the results by assay, then click the scatterplot of interest to adjust the analysis settings for the associated assay.
3. In the Allelic Discrimination view, click **Analysis Setting**.
4. In the Edit Assay Settings dialog box, click **Call Setting**.
5. Select or deselect **Baseline for Real-time Data**.



6. In the **Start** and **End** fields, enter the first and last cycle that the software will use to calculate the baseline.
7. In the **Cycle Number** field, change the cycle number used to generate the endpoint Rn plots.

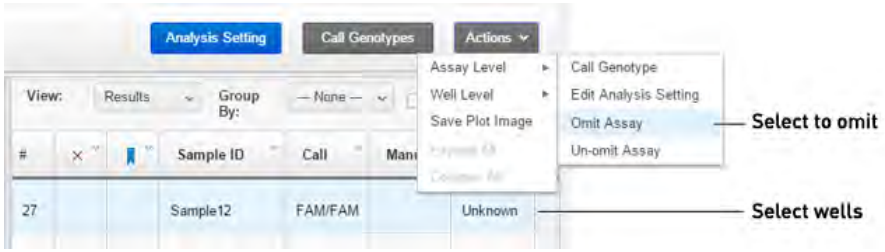
   Note: If you leave the Cycle Number field empty (blank), the software interprets that as the final cycle.

   Note: To apply the change to all assays in the project, edit the analysis settings of the active analysis group.
8. Click **Finish** to save the changes and close the Edit Assay Settings dialog box.

Omit wells from the analysis

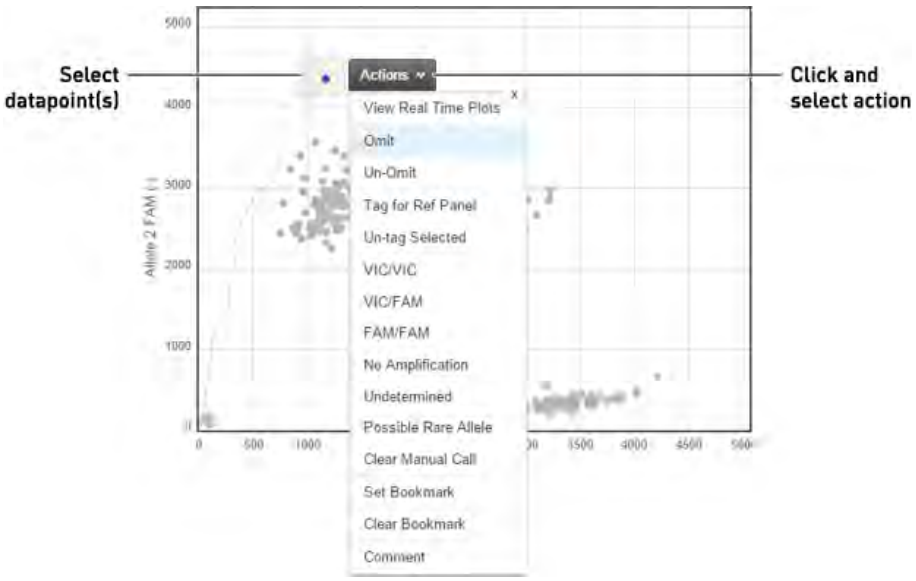
To omit the data from one or more wells that you do not want included in the analysis:

- Omit one or more wells from the **Sample** View:
  a. From the Analysis screen, select **Sample** from the View drop-down list.
  b. From the sample layout, select one or more samples, then click **Actions** ▸ **Omit Sample**.
  c. Click **Analyze** to reanalyze the project without the omitted well(s).
- Omit one or more wells from the **Plate** Layout:
  a. From the Analysis screen, select **Plate** from the View drop-down list.
  b. From the Plate view, select the plate that you want to modify.
  c. From the plate layout, select one or more wells, then click **Actions** ▸ **Omit Well(s)**.
  d. Click **Analyze** to reanalyze the project without the omitted well(s).
- Omit one or more wells from the Well Table:
  a. From the Analysis screen, select **Assay** from the View drop-down list.
  b. From the Assay view, select the scatterplot that you want to modify.
  c. From the Well Table, select the rows that correspond to the wells that you want to omit, then:
     – Select **Actions** ▸ **Well Level** ▸ **Omit** to omit the related well.
     – Select **Actions** ▸ **Assay Level** ▸ **Omit Assay** to omit all data associated with the related assay.



  d. Click **Analyze** to reanalyze the project without the omitted well(s).
- Omit one or more wells from the **Scatterplot**:
  a. From the Analysis screen, select **Assay** or **Sample** from the View drop-down list.
  b. From the Assay or Sample view, select the scatterplot that you want to modify.

c. From the Scatterplot, select one or more data points, then select **Omit** from the floating menu.



d. Click **Analyze** to reanalyze the project without the omitted well(s).

6 Export the results

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

Export the analyzed data from a project

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

1. From the main menu of the project that contains data to export, click **Export**.
2. From the Export screen, 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 analysis group to use to generate the exported data.

   c. 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.

d. Select the check boxes for the data to export.
   - **Analysis Results** - Select to export genotyping analysis results for each well in the project. Select **Basic** or **Advanced** to determine the depth of the exported data, where the Basic option generates abbreviated information about the project and the Advanced option generates detailed information.
   - **Genotype Matrix (No preview)** - Select to export a matrix of the genotypes generated from the analyzed data.
   - **Analysis Settings** - Select to export a summary of the analysis settings used to generate the results.
   - **Populations** - Select to export the populations data.
   - **QC by Samples/Assays/Plates** - Select the appropriate option to export the quality data for the analysis as summarized by sample, assay, or plate.

e. Enter the labels that you want to use for Undetermined, No Amplification, Possible Rare Allele, or Invalid calls.

f. Click **Preview**

3. From the Export:Details screen, select the fields from the data tables to include in the exported file, then click **Start Export**.

   Wait for the Applied Biosystems™ Analysis Software to generate the expored report. The export is complete when the Status column of the exported report displays "Download".

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**.

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

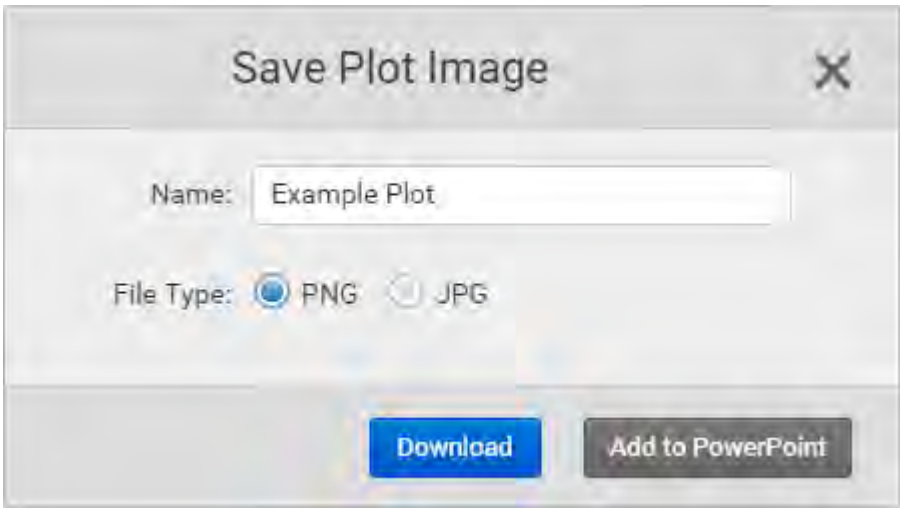
The Applied Biosystems™ Analysis Software allows you to export any plot as a Portable Network Graphics (.png) or Joint Photographic Expert Group (.jpg) file, which can be imported by most spreadsheet and desktop publishing software for presentation.

1. When viewing a plot, click **Actions ▸ Save Plot Image**.

2. Save the image.
   a. Click the File Name field, then enter a name for the exported graphics file.

   b. Select the appropriate file format (.png or .jpg).

c. Click **Download** to download the plot image file, *or* click **Add to PowerPoint** to add the plot to an exported PowerPoint presentation (see "Export project data as a slide presentation" on page 52).



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

Export data for use in other projects

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

- Export a template file

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

  a. Open the project that includes the desired experiment, then select **Plate Setup**.

  b. From the Plate Setup screen, select the experiment record that contains the plate setup information of interest.

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

- Export a reference panel

  A reference panel file contains reference sample data that characterize the clustering of one or more individual assays. Once a reference panel file is exported, you can import the file into other projects to serve as the clustering model for calling Unknown data points.

  a. Open the project that contains data points that you want to use as reference samples.

b. In the Overview screen, select the analysis group that contains the settings that you want to use to generate the reference panel, then click **Analyze** to analyze the project.

c. Click **Export** to view the Export screen, then click  .

d. In the Export Reference Panel screen, enter a name for the reference panel, select the appropriate analysis group from the list, then click **Start Export**.

e. (Optional) Click the entry in the Comments column, then enter any additional information for the exported reference panel.

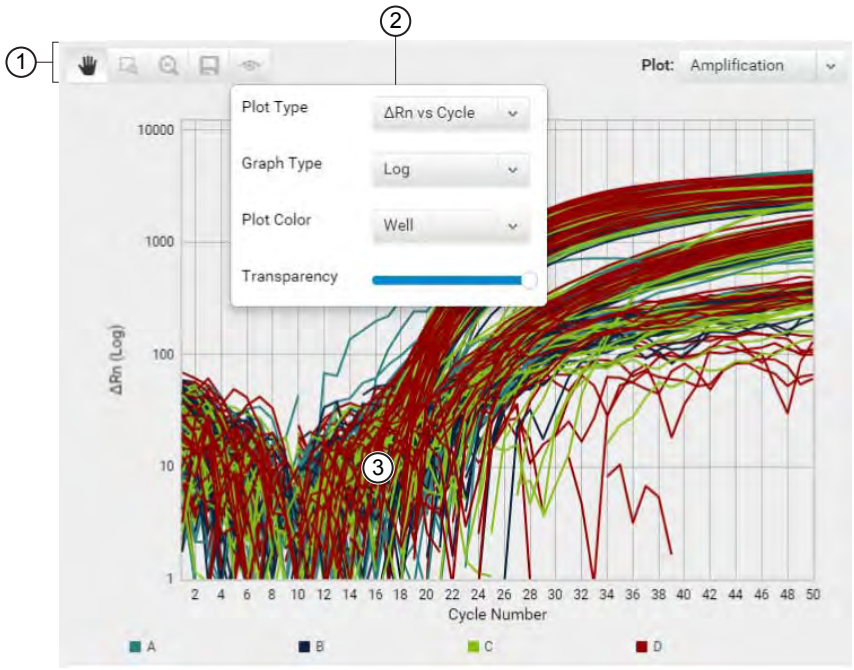
f. Click **Download**, select the location for the reference panel file, then click **Save**.

7 Screens and plots

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

Amplification Plot

The Amplification Plot screen displays post-run amplification of the samples of each experiment added to your project. The plot contrasts ΔRn as a function of cycle number, where ΔRn is the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.



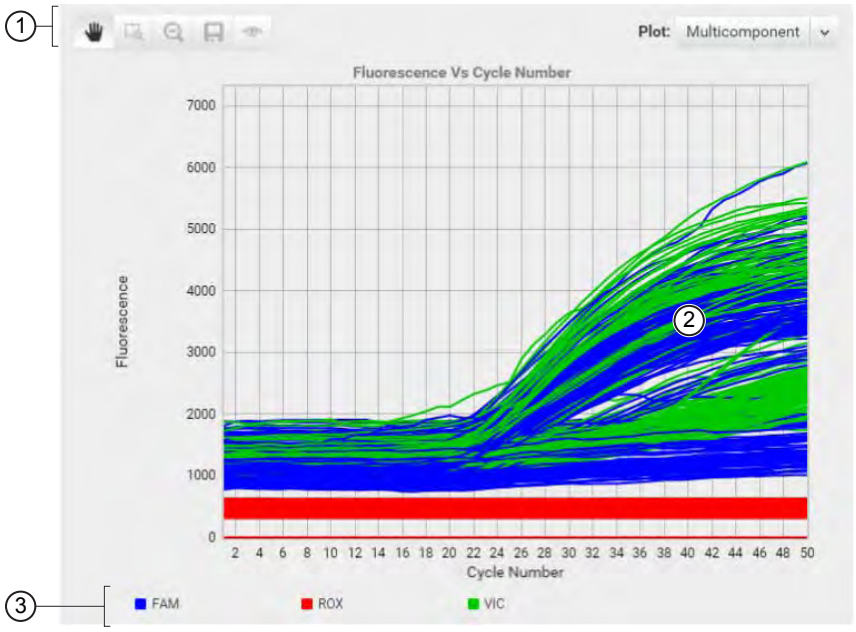
① **Toolbar** – Contains the following tools for controlling the plot:
- – Allows you to click and manually move the position of the plot.
- – Zoom the plot to the selected area.
- – Zooms out the plot to show all data points.
- – Saves the plot as an image (.png or .jpg).
- – Allows you to adjust the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

② **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**), the scale of the y-axis (**log** or **linear**), and the color scheme for the plot.

③ **Amplification curves** – Normalized fluorescence for individual wells throughout the course of the thermal cycling protocol.

Multicomponent Plot

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



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

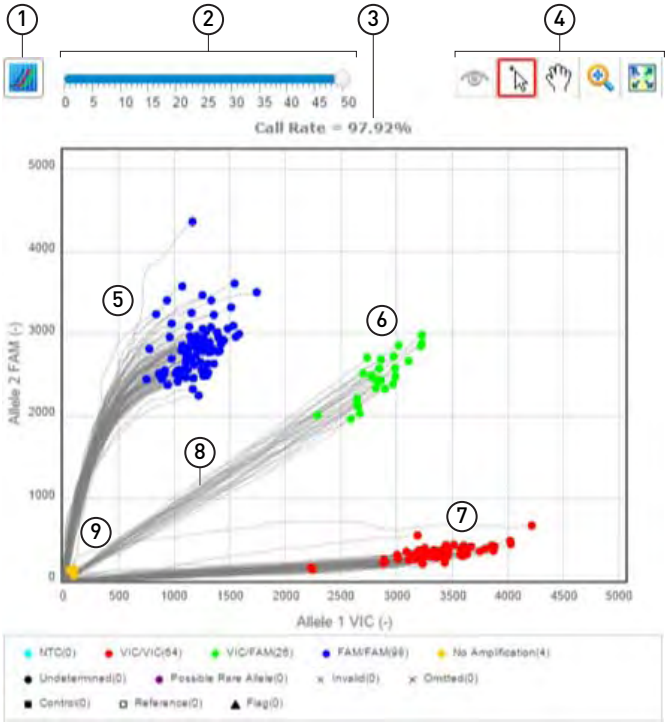
Scatterplot

The Applied Biosystems™ Analysis Software genotypes the DNA samples from the reaction plate simultaneously. First, the software normalizes the fluorescence of the reporter dyes to the fluorescence of the passive reference dye in each well. Next, the software plots the normalized intensities (Rn) of the reporter dyes in each sample well on a scatterplot, which contrasts the reporter dye intensities of the allele-specific probes. Finally, the software algorithmically clusters the sample data, and assigns a genotype call to the samples of each cluster according to its position on the plot.

Note: The clustering algorithm does not call genotypes when only one genotype is present in an experiment.

The clustering of data points can vary along the horizontal axis (Allele 1), vertical axis (Allele 2), or diagonal (Allele 1/Allele 2). This variation results from differences in the extent of reporter dye fluorescent intensity after PCR amplification. The table below shows the correlation between fluorescence signals and sequences in a sample.

A substantial increase in...	Indicates...
VIC™ dye-labeled probe fluorescence only	Homozygosity for Allele 1
FAM™ dye-labeled probe fluorescence only	Homozygosity for Allele 2
Both VIC™ and FAM™ dye-labeled probes fluorescence	Allele 1-Allele 2 heterozygosity



① (View Real-time Plots) – Displays the amplification and multicomponent data for the selected assay.

② **Cycle Number Slider** – For experiments that include real-time data, the slider allows you to display the allelic discrimination data for all samples at each cycle during the PCR amplification.

③ **Call Rate** – The percentage of samples that have successfully been assigned genotype calls.

④ **Toolbar** – Contains the following tools for controlling the plot:

- – Shows/hides the presence of the legend.
- – 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 a image (.png or .jpg).
- – Allows you to adjust the display options for the plot.

⑤ **Homozygote FAM/FAM cluster** – A cluster normally associated with amplification characteristic of samples homozygous for the sequence targeted by the FAM™ dye-labeled probe.

⑥ **Heterozygote FAM/VIC cluster** – A cluster normally associated with amplification characteristic of samples heterozygous for the target sequences.

⑦ **Homozygote VIC/VIC cluster** – A cluster normally associated with amplification characteristic of samples homozygous for the sequence targeted by the VIC™ dye-labeled probe.

⑧ **Amplification trails** – For experiments that include real-time data, the software plots the amplification of each sample throughout the duration of the PCR.

⑨ **No template control cluster** – A cluster normally associated with the lack of amplification characteristic of no template controls.

When you analyze your own experiment:

- Confirm that all controls have the correct genotype.
- 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 data points in the scatterplot.
  b. Check that the data points for the positive controls cluster along the expected axis of the plot. For example, if you select the Positive Control Allele 1/Allele 1, then the controls should cluster along the X-axis.
  c. Repeat the previous steps for the wells containing the other positive controls.

- Screen the negative control cluster for unknown samples that failed to amplify:
  a. Select the data points of the cluster in the lower-left corner of the scatterplot to select the corresponding wells in the well table.
  b. Check that the selected wells in the well table are negative controls, and not unknown samples.
- Samples that clustered with the negative controls may:
  – Contain no DNA
  – Contain PCR inhibitors
  – Be homozygous for a sequence deletion
- Confirm the results by retesting samples that do not cluster tightly or are clustered with negative controls.
- If you select to run replicate reactions, carefully review your data set for outliers to ensure the accuracy of the genotype calls. If outliers are present, confirm the results of the associated samples by retesting them.

Well Table

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

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

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

Table 1 Samples view table

Column	Description
(Omitted)	The omission status of the related well.
(Bookmark)	Indicates whether the well is bookmarked.
Assay name	The unique name of the assay assigned to the well.
Assay ID	The ID number of the assay assigned to the well.
Quality data	The quality flags generated by the associated well.

Column	Description
Allele 1 Amp Score	The C_T or C_{RT} value calculated for the related well. **Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Allele 2 Amp Score	
Allele 1 CT/CRT values	The C_T or C_{RT} calculated for the probe targeting the associated allele in the related well. **Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Allele 2 CT/CRT values	
Call	The genotype call assigned to the well.
Manual	Indicates whether the sample was manually called.
Well	The location of the well on the reaction plate. For example, P18 indicates that the sample is located in row P, column 18.
Experiment Name	The experiment associated with the related well.
Task	The task assigned to the well. A task is the function that a sample performs: • Unknown • No template control (NTC) (control identifier) • Positive control (control identifier) for VIC/VIC, FAM/FAM or VIC/FAM • Negative control (control identifier)
Comments	Comments made by a user about the well.

Table 2 Plates view table

Column	Description
Well	The location of the well on the reaction plate. For example, P18 indicates that the sample is located in row P, column 18.
× (Omitted)	The omission status of the related well.
(Bookmark)	Indicates whether the well is bookmarked.
Quality data	The quality flags generated by the associated well.
Call	The genotype call assigned to the well.
Sample ID	The ID (a unique name or number) of the sample.
Allele 1 Amp Score	The C_T or C_{RT} value calculated for the related well. **Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Allele 2 Amp Score	
Allele 1 CT/CRT values	The C_T or C_{RT} calculated for the probe targeting the associated allele in the related well. **Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Allele 2 CT/CRT values	
Assay name	The unique name of the assay assigned to the well.

Column	Description
Assay ID	The ID number of the assay assigned to the well.
Task	The task assigned to the well. A task is the function that a sample performs: • Unknown • No template control (NTC) (control identifier) • Positive control (control identifier) for VIC/VIC, FAM/FAM or VIC/FAM • Negative control (control identifier)
Comments	Comments made by a user about the well.

Table 3 Results table

Column	Description
× (Omit)	The omission status of the related well.
(Bookmark)	Indicates whether the well is bookmarked.
Sample ID	The ID (a unique name or number) of the sample.
Call	The genotype call assigned to the well.
Manual	Indicates whether the sample was called manually.
Task	The task assigned to the well. A task is the function that a sample performs: • Unknown • No template control (NTC) (control identifier) • Positive control (control identifier) for VIC/VIC, FAM/FAM or VIC/FAM • Negative control (control identifier)
VIC™	The normalized fluorescent value of the VIC™ or FAM™ dyes. TaqMan^{fi} OpenArray™ Genotyping System fluorescent values are not normalized by the ROX™ passive reference dye.
FAM™	
Allele 1 Amp Score	The C_T or C_{RT} value calculated for the related well. **Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Allele 2 Amp Score	
Allele 1 CT/CRT values	The C_T or C_{RT} calculated for the probe targeting the associated allele in the related well. **Note:** C_T and C_{RT} data are available only for experiments that contain real-time PCR data.
Allele 2 CT/CRT values	
ROX™	The fluorescent value of the ROX™ dye. FAM™ and VIC™ dye fluorescent values are normalized by the ROX™ passive reference dye. TaqMan^{fi} OpenArray™ Genotyping System fluorescent values are not normalized by the ROX™ passive reference dye. **Note:** ROX™ dye is in the TaqMan^{fi} master mix used in TaqMan^{fi} assays.

Column	Description
Quality	The estimated quality value of the call made by the autocaller algorithm. The algorithm outputs a quality value for each data point with the following properties: • The quality value is a number between 0 and 1. • The quality value is always 0 for Invalid data points. • The quality value is always 1 for No Amplification data points. • For FAM/FAM, FAM/VIC, and VIC/VIC calls, the Quality Value is a higher value for calls more likely to be correct and a lower value for calls more likely to be incorrect. The quality value is N/A if a well is called manually, is a control, or is called using the Classification Scheme call method.
Well	The location of the well on the reaction plate. For example, P18 indicates that the sample is located in row P, column 18.
Experiment Filename	The name of the experiment file to which the well belongs.
Gender	The gender assigned to the sample.
Population	The population name(s) assigned to the samples in the project. By default, the population of Unknown samples is named "All". You can edit the population of some or all of the samples in a project using the Edit Sample dialog box. Each population name that you assign appears in the Population Statistics tab.
Plate Barcode	The name or barcode of the reaction plate in which the experiment was run.
Comment	Comments applied to the well.

Table 4 References Samples table

Column	Description
	Indicates whether the sample is part of a reference panel.
Sample ID	The ID (a unique name or number) of the sample.
Call	The genotype call assigned to the well.
VIC™	The normalized fluorescence value of the VIC™ and FAM™ dyes.
FAM™	
Well	The location of the well on the reaction plate. For example, P18 indicates that the sample is located in row P, column 18.
Experiment Filename	The name of the experiment file to which the well belongs.
Reference Panel File Name	The name of the file that contains the reference panel.
Plate Barcode	The name or barcode of the reaction plate in which the experiment was run.
Originating Project Name	The name of the project from which the reference panel file was created.

Table 5 Population statistics table

Column	Definition
Population	The population name(s) assigned to the samples in the project. By default, the population of Unknown samples is named "All". You can edit the population of some or all of the samples in a project using the Edit Sample dialog box. Each population name that you assign appears in the Population Statistics tab.
Allele 1 Freq	The frequency of allele 1 determined for each population in the project.
Allele 2 Freq	The frequency of allele 2 determined for each population in the project.
1/1 Freq	The frequency of genotype 1/1 determined for each population in the project.
1/2 Freq	The frequency of genotype 1/2 determined for each population in the project.
2/2 Freq	The frequency of genotype 2/2 determined for each population in the project.
Chi-Squared	The Chi-Squared value calculated for each population in the project. The calculated Chi-Squared value is used to determine if the experimental data is in Hardy-Weinberg equilibrium based on the observed and expected number of genotype calls, assuming 1 degree of freedom.
p-Value	The P-value calculated for each population in the project. The calculated P-value is the probability of the differences in observed and expected genotype calls accounted for by chance alone. **Note:** Life Technologies recommends that you review Hardy-Weinberg equilibrium fundamentals for application of this P-value.

8 Quality flags

The Applied Biosystems™ Analysis Software includes a set of settings that, when activated, cause the software to screen the processed experiment data for qualities that can indicate possible analysis problems. Depending upon the configuration of each quality "flag", the software can either notify you of potential problems, or automatically remove the associated data from the analysis. The use of the quality flags is optional and can be customized to adjust the sensitivity of the associated tests.

Assay Call Rate Low quality flag

An Assay Call Rate Low (%A) quality flag can be raised for any assay. If the percentage of Unknown data points with a genotype call for an assay is less than the threshold, a flag will be raised.

If a well is flagged, review the flagged assay and determine if the issue is isolated to a few data points or throughout. You can omit the data for the assay, perform the experiment with the assay again, or ignore the flag.

Failed Control quality flag

A Failed Control (CTL) quality flag can be raised for any data point that is identified as a control: NTC, Negative Control, or Positive Control. If the user-assigned control identifier (or task) for a data point is inconsistent with the call that would be assigned by the software algorithm to an Unknown with the same FAM™ and VIC™ dye intensities, a flag is raised.

If a well is flagged, review the flagged data point. You can omit the well or ignore the flag if it appears to be inappropriate.

Note: The Failed Control flag can be very important if you selected to use positive controls for analysis and bias the genotype calls.

Experiment Call Rate Low quality flag

An Experiment Call Rate Low (%E) quality flag can be raised for any experiment. If the percentage of Unknown data points with a genotype call in an experiment is less than the threshold, a flag will be raised.

If a well is flagged, review the flagged experiment and determine if the issue is isolated to certain wells of the experiment or throughout. You can remove the experiment from the study, perform the experiment again, or ignore the flag.

Experiment Low ROX™ Rate High quality flag

An Experiment Low ROX™ Rate High (%R) quality flag can be raised for any experiment. If the percentage of data points in an experiment with a low ROX™ dye intensity flag is greater than the threshold, a flag will be raised.

If a well is flagged, review the flagged experiment and determine if the issue is isolated to certain wells of the experiment or throughout. You can remove the experiment from the study and perform the experiment again or ignore the flag.

Genotype Quality Low quality flag

A Genotype Quality Low (GT) quality flag can be raised for any data point that is identified or tasked as an Unknown. If the quality value assigned by the software algorithm for a data point is below the threshold, a flag will be raised.

If a well is flagged, review the flagged data point and determine if the data point is an outlier or located in acceptable coordinates. You can manually assign a call or modify the quality value threshold to include the data point.

LOWROX (Low ROX™ Intensity) quality flag

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

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

NTC FAM™ Intensity High quality flag

An NTC FAM™ Intensity High (NTC) quality flag can be raised for any data point that is identified or tasked as an NTC. If the FAM™ dye signal intensity for a data point tasked as NTC is greater than the threshold, a flag will be raised.

If a well is flagged, review the flagged data point and determine if the high signal is acceptable or not. You can omit the well, raise the threshold to remove the flag, or ignore the flag.

Note: If the coordinates of the NTC data point are located next to Unknown data points, this could indicate experiment cross-contamination, or an amplifying NTC. Track this for further troubleshooting.

NTC VIC™ Intensity High quality flag

An NTC VIC™ Intensity High (NTC) quality flag can be raised for any data point that is identified or tasked as an NTC. If the VIC™ dye signal intensity for a data point tasked as NTC is greater than the threshold, a flag will be raised.

If a well is flagged, review the flagged data point and determine if the high signal is acceptable or not. You can omit the well, raise the threshold to remove the flag, or ignore the flag.

Note: If the coordinates of the NTC data point are located next to Unknown data points, this could indicate experiment cross-contamination, or an amplifying NTC. Track this for further troubleshooting.

Reference Sample Discordance quality flag

A Reference Sample Discordance (REF) quality flag can be raised for any data point that is identified or tasked as an Unknown. If the software algorithm-assigned genotype for a data point is discordant with the genotype of a reference sample data point that has exactly the same sample/assay identification, a flag will be raised.

If a well is flagged, review the flagged data point and determine if the coordinates are located in an acceptable location. You can manually assign a different call, omit the well, or ignore the flag.

Replicate Sample Discordance quality flag

A Replicate Sample Discordance (RPL) quality flag can be raised for any data point that is identified or tasked as an Unknown. If the software algorithm-assigned genotype for a data point is discordant with the genotype of a replicate sample data point that has exactly the same sample/assay identification, a flag will be raised.

A flag will be raised for all data points that have the sample/assay identification, because the software cannot know which data point has the correct genotype.

If a well is flagged, review the flagged data point and determine which of the replicate data points are assigned the correct genotype. You can manually assign a different call, omit the well, or ignore the flag.

Sample Call Rate Low quality flag

A Sample Call Rate Low (%S) quality flag can be raised for any sample identified or tasked as an Unknown. If the percentage of assays with a genotype call for Unknown samples is less than the threshold, a flag will be raised.

If a well is flagged, review the flagged sample and determine if the issue is isolated to a few data points or throughout. You can omit the data for the sample, perform the experiment with the sample again, or ignore the flag.

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Glossary

assay information files

Assay information files are delivered on Information CDs that accompany TaqMan® assay orders. Each assay information file contains reference information about the associated order and technical details of all assays in the shipment.

You can import an assay information file into the Applied Biosystems™ Analysis Software to add supplementary assay information to a project. Assay information files are available in three formats (.html, .txt, and .xml), but the Applied Biosystems™ Analysis Software supports only .txt and .xml files.

IMPORTANT! The assay information file must include an assay ID (in the Assay ID column) for each assay listed in the file. The software matches the assay IDs in the assay information file with the existing assay IDs in the project.

IMPORTANT! When you import an assay information file, information from the file populates the corresponding columns in the Assays list in the Overview screen. All data in the Overview screen are replaced for all assays that are identified in the assay information file. If the assay information file does not contain information for an assay, the existing data in the Overview screen is unaffected.

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amplification efficiency (EFF%)

Calculation of the efficiency of the PCR amplification in a standard curve experiment. EFF% is calculated using the slope of the regression line in the standard curve. A slope close to -3.32 indicates optimal, 100% PCR amplification efficiency. To use amplification efficiency in a gene expression project:

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

amplification plot

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

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

analysis group

An analysis group is a project setting that allows you to create a profile of the analysis and quality settings for the analysis of a project. Analysis groups can be applied either globally to analyze an entire project, or exclusively to a subset of the experiments or samples added to a project. Later in the analysis, the Applied Biosystems™ Analysis Software allows you to switch between analysis groups so that you can compare the effects of changes to the analysis settings on your results.

assays	A PCR reaction mix that contains primers to amplify a target and a reagent to detect the amplified target.
automatic baseline	An analysis setting for the Baseline Threshold algorithm in which the software identifies the start and end cycles for the baseline in the amplification plot.
automatic threshold	An analysis setting for the Baseline Threshold algorithm in which the software calculates the baseline start and end cycles and the threshold in the amplification plot. The software uses the baseline and threshold to calculate the threshold cycle (C_q).
baseline	In the amplification plot, the baseline is a cycle-to-cycle range that defines background fluorescence. This range can be set manually on a target-by-target basis, or automatically, where the software sets the baseline for each individual well.
Baseline Threshold algorithm	Expression estimation algorithm (C_q) which subtracts a baseline component and sets a fluorescent threshold in the exponential region for quantification.
baseline-corrected normalized reporter (ΔRn)	In experiments that contain data from real-time PCR, the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. In the ΔRn vs Cycle amplification plot, ΔRn is calculated at each cycle as: ΔRn (cycle) = Rn (cycle) - Rn (baseline), where Rn = normalized reporter
call rate	The value of the call rate displayed by the Applied Biosystems™ Analysis Software changes depending on where in the software the value is displayed. Specifically, the calculation of the call rate is different if you are viewing the analyzed data within the Plate or Samples data views. **About the plate call rate** When viewed within the Plate data view of the Analysis screen, the call rate is the calculated percentage of successful calls for the wells of the plate (where successful calls are: FAM/FAM, VIC/VIC, and FAM/VIC). The software calculates the plate call rate over all wells on a single reaction plate (regardless of assay or sample). The software: • Does not include NTC, Negative Control, Positive Control, or reference sample wells. • Considers invalid, undetermined, and PRA calls as no-calls. • Recalculates the call rates whenever the data is reanalyzed. The data can be reanalyzed automatically (for example, when you import or delete an experiment) or manually. **About the sample call rate** When viewed within the Sample data view of the Analysis screen, the sample call rate is the percentage of successful calls for each sample in the project (where successful calls are: FAM/FAM, VIC/VIC, and FAM/VIC).

	The software calculates the sample call rate over all assays run on a single sample. The software: • Does not include NTC or reference sample wells. • Does include Negative Controls and Positive Control wells. • Considers invalid, undetermined, and PRA calls as no-calls. • Recalculates the call rates whenever the data is reanalyzed. The data can be reanalyzed automatically (for example, when you import or delete an experiment) or manually.
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.
manual baseline	An analysis setting for the Baseline Threshold algorithm in which you enter the baseline start and end cycles for the amplification plot for a target. If you edit the baseline start and end cycles, the settings are applied to all instances of that target in the project.
manual threshold	An analysis setting for the Baseline Threshold algorithm in which you enter the threshold value and select whether to use automatic baseline or manual baseline values. The software uses the baseline and threshold values to calculate the threshold cycle (C_q).
negative control (NC)	See no template control (NTC).
no template control (NTC)	In the software, the task for targets in wells that contain water or buffer instead of sample. No amplification should occur in negative control wells. Also called negative control (NC).
nonfluorescent quencher-minor groove binder (NFQ-MGB)	Molecules that are attached to the 3′ end of TaqMan® MGB probes. When the probe is intact, the nonfluorescent quencher (NFQ) prevents the reporter dye from emitting fluorescence signal. Because the NFQ does not fluoresce, it produces lower background signals, resulting in improved precision in quantification. The minor groove binder moiety (MGB) increases the melting temperature (T_m) without increasing probe length. It also allows the design of shorter probes.
normalized reporter (Rn)	Fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference (usually ROX™ dye).

omit well	An action that you perform before reanalysis to omit one or more wells from analysis. Because no algorithms are applied to omitted wells, omitted wells contain no results. You can add wells back in to the analysis; no information is permanently discarded.
outlier	A data point that deviates significantly from the values of an associated group (for example, the other technical replicates for a sample).
passive reference	A dye that produces fluorescence signal independent of PCR amplification, and that is added to each reaction at a constant concentration. Because the passive reference signal should be consistent across all wells, it is used to normalize the reporter dye signal to account for non-PCR related fluorescence fluctuations caused by minor well-to-well differences in volume. Normalization to the passive reference signal generally results in data with noticeably high precision among technical replicates.
plate grid (plate layout)	An illustration of the grid of wells and assigned content in the reaction plate, array card, or OpenArray™ plate. The number of rows and columns in the grid depends on the plate or card that you use. In the software, you can use the plate grid to view well assignments and results. The plate grid can be printed, included in a report, exported, and saved as a slide for a presentation.
projects	The Applied Biosystems™ Analysis Software organizes the analysis of experiment data by project, which represents the association of the raw data, all experimental setup information, and any associated settings used to perform the analysis. Once created, projects can be shared with other users and transferred to/from the repository. **Note:** Projects to not contain the data from experiments uploaded to the repository; they link the data for analysis without affecting the original data files.
p-value	The probability of the differences in observed and expected genotype calls accounted for by chance alone. The probability that the differences in observed and expected genotype calls can be accounted for by chance alone. A low p-value indicates there may be evidence against the observed genotype call. **Note:** Life Technologies recommends that you review Hardy-Weinberg equilibrium fundamentals for application of this p-value.
quencher	A molecule attached to the 3' end of TaqMan® probes to prevent the reporter from emitting fluorescence signal while the probe is intact. With TaqMan® probes, a nonfluorescent quencher-minor groove binder (NFQ-MGB) can be used as the quencher.
reference panel files	A reference panel file is a user-generated file that contains reference samples.

reference sample	Reference samples are data points in an experiment that you select to be representative of the clusters for an individual assay. You can identify, collect, and store reference samples for multiple assays in a reference panel file for use in current or future studies. After you import a reference panel file into a project, the software uses the reference samples to bias the calls of Unknown data points. The reference samples cannot be modified and are not calculated in the call rates. **Note:** Reference samples are similar to positive controls in that the software can use both to bias the calls of Unknown data points. However, a positive control represents a well that physically contains known template and is included in one of the experiments in the current project. A reference sample can represent any well from any experiment in any project.
reject well	An action that the software performs during analysis to remove one or more wells from further analysis if a specific flag is applied to the well. Rejected wells contain results calculated up to the point of rejection.
replicates	Identical reactions containing identical components and volumes.
reporter	A fluorescent dye used to detect amplification. With TaqMan® reagents, the reporter dye is attached to the 5' end. With SYBR™ Green reagents, the reporter dye is SYBR™ Green dye.
Rn	See *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.
scatter plot	Display of data collected during the post-PCR read. The allelic discrimination plot is a graph of the normalized reporter signal from the allele 1 probe plotted against the normalized reporter signal from the allele 2 probe.
supplementary sample information (.ssi) file	A Supplementary Sample Information file (.ssi) is a user-created text file (.txt). The .ssi file contains detailed supplementary information about your samples (for example, gender and population). Import an .ssi file into the Applied Biosystems™ Analysis Software to add the supplementary sample information to a project. **IMPORTANT!** The .ssi file must include a sample ID (in the Sample ID column) for each sample listed in the file. The software matches the sample IDs in the .ssi file with the existing sample IDs in the project. **IMPORTANT!** When you import an .ssi file, information from the file populates the corresponding columns in the Samples screen. All data in the Samples screen are replaced for all samples that are identified in the .ssi file. If the file does not contain information for a sample, the existing data in the Samples screen is left as is.

task	In the software, the type of reaction performed in the well for the target. Available tasks include:
technical replicates	Reactions that contain identical components and volumes, and that evaluate the same sample; important for evaluating precision.
thermal profile	The part of the run method that specifies the temperature, time, ramp, number of cycles, and data collection points for all steps and stages of the instrument run.
threshold	In amplification plots, the threshold is the level of fluorescence above the baseline and within the exponential amplification region. For the Baseline Threshold algorithm, the threshold can be determined automatically (see *automatic threshold*), or it can be set manually (see *manual threshold*).
threshold cycle (C_T)	The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.
unknown	In the software, the task for the target in wells that contain the sample being tested. For genotyping experiments, the unknown task is assigned to wells that contain a sample with unknown genotyping.