applied biosystems

Genotyping Analysis Module

USER GUIDE

for use with QuantStudio™ Design and Analysis Software v2

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Revision	Date	Description
B.0	15 April 2020	Remove send to the instrument run queue.
A.0	14 October 2019	New document.

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1 About the Genotyping Analysis Module

The Genotyping Analysis Module for QuantStudio™ Design and Analysis Software v2 is used to detect single nucleotide polymorphism (SNP) variants of a target nucleic acid sequence.

For more information about genotyping analysis, see Chapter 5, “About genotyping analysis”.

Compatible data files

The software is compatible with data files for the following real-time PCR instruments:

- QuantStudio™ 7 Pro Real-Time PCR System
- QuantStudio™ 6 Pro Real-Time PCR System
- QuantStudio™ 5 Real-Time PCR System (if the plate file for the instrument run is created using QuantStudio™ Design and Analysis Software v2)
- QuantStudio™ 3 Real-Time PCR System (if the plate file for the instrument run is created using QuantStudio™ Design and Analysis Software v2)
- QuantStudio™ 1 Real-Time PCR System (if the plate file for the instrument run is created using QuantStudio™ Design and Analysis Software v2)

Data files for the following instruments are a legacy file format that can be opened in the software, but can only be saved as the updated file format:

- QuantStudio™ 5 Real-Time PCR System (if the plate file for the instrument run was not created using QuantStudio™ Design and Analysis Software v2)
- QuantStudio™ 3 Real-Time PCR System (if the plate file for the instrument run was not created using QuantStudio™ Design and Analysis Software v2)
- QuantStudio™ 1 Real-Time PCR System (if the plate file for the instrument run was not created using QuantStudio™ Design and Analysis Software v2)
- QuantStudio™ 6 Flex Real-Time PCR System
- QuantStudio™ 7 Flex Real-Time PCR System
- QuantStudio™ 12K Flex Real-Time PCR System (except OpenArray™ format)
- StepOnePlus™ Real-Time PCR System
- ViiA™ 7 Real-Time PCR System
- 7500/7500 Fast Real-Time PCR System
- 7900HT Real-Time PCR System

Note: To convert a legacy data file into the updated file format, open the data file, then click **Actions ▸ Save As**.

2 Workflow: Genotyping analysis

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IMPORTANT! If you omit wells or configure the analysis settings, click **Analyze** to reanalyze the data.

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3 Set up a plate file for genotyping analysis

For detailed instructions about setting up a plate file, see **Help ▸ Help Contents**.

Select a system template or existing plate file to set up a new plate file

A plate file contains the information that is necessary to perform an instrument run, including instrument setup, run method, plate setup, and analysis setting.

A system template is a non-editable plate file that is included with the software.

A new plate file must be created from a system template or a previously created plate file.

For detailed information about system templates and plate files, see **Help ▸ Help Contents**.

1. In the home screen, click **Set Up Plate**.
   The **Plate Gallery** opens to the **System Templates** tab.

2. **IMPORTANT!** Select a system template or a plate file that corresponds to your instrument, block, and run mode. These properties are not editable after the plate file has been created.

   In the left pane, select the appropriate options to filter the system template and plate file lists.

   - **Instrument**
   - **Block**
   - **Run Mode**
   - **Analysis**

     Note: Thermal protocol, plate setup, and post-run analysis options are independent of analysis module selection. Analysis module selection can be changed at any point during plate file set or post-run analysis (see “Select the Genotyping Analysis Module” on page 13).

3. Navigate to, then select a system template or plate file.

Tab	Description
System Templates	Contains system templates, non-editable plate files that are included with the software. Select a system template to automatically generate a new plate file that can be edited, then saved.
My Plate Files	Contains plate files that were previously saved to **My Plate Files**. plate files that are included with the software. Select an existing plate file to edit, then save, or to save as a new plate file.
Recents	Contains plate files that were recently opened. Recently opened plate files from **System Templates** and **My Plate Files** do not populate this tab. Select an existing plate file to edit, then save, or to save as a new plate file.

Note: To view all options for opening the plate file, mouse over the plate file, then click ··· **(Actions)**.

The plate file opens in the **Run Method** tab.

Confirm or edit the run method for genotyping analysis

For most analysis, the default run method is appropriate. The following options are compatible for genotyping analysis.

- PCR
- 1-step RT-PCR
- 2-step RT-PCR

- In a plate file, in the **Run Method** tab, adjust the run method elements as needed.

  For detailed instructions about editing the run method, see **Help ▸ Help Contents**.

- Click ··· **(Actions) ▸ Filter Settings** to confirm or edit filter settings.

Confirm or edit the plate setup for genotyping analysis

For detailed instructions about plate setup, or to download example plate setup files, see **Help ▸ Help Contents**.

Add samples and assign to wells

For detailed instructions about plate setup, see **Help ▸ Help Contents**.

1. In the **Plate Setup** tab, add samples and assign to wells using the following options.
   - Import a plate setup file
   - Manually add samples to the **Samples** table
   - Manually add samples to wells in the plate layout
2. Confirm or edit sample information in the **Samples** table.

Column	Description
Name	Sample name
Color	Sample color
Type[1]	Genotyping analysis uses the following sample types. • Unknown • Positive 1/1 • Positive 1/2 • Positive 2/2 • Negative Control

[1] For more information, see "Sample types for genotyping analysis" on page 19.

3. Confirm or edit sample well assignments in the plate layout.

Add SNP assays and assign to wells

For detailed instructions about plate setup, see **Help ▸ Help Contents**.

1. In the upper-left corner of the **Plate Setup** tab, click **SNP**.
2. Add SNP assays and assign to wells using the following options:
   - Import an Assay Information File (AIF)
   - Import a plate setup file
   - Manually add SNP assays to the **SNP Assays** table
   - Manually add SNP assays to wells in the plate layout
   - Import TaqMan™ assay plate and card files
3. Confirm or edit target information in the **SNP Assays** table.

Column	Description
Name	SNP assay name
Color	SNP assay color
Allele 1 Reporter	Fluorescent reporter dye for allele 1 of the SNP assay
Allele 1 Quencher	Quencher for allele 1 of the SNP assay

Column	Description
Allele 2 Reporter	Fluorescent reporter dye for allele 2 of the SNP assay
Allele 2 Quencher	Quencher for allele 2 of the SNP assay
Task[1]	The software automatically assigns a task to the target in a well based on the sample type in that well. The following tasks are used for standard curve analysis. • Unknown • Positive 1/1 • Positive 1/2 • Positive 2/2 • Negative Control

[1] For more information, see "Sample types for genotyping analysis" on page 19.

4. Confirm or edit SNP assay well assignments in the plate layout.

Edit reagent information

1. In the **Plate Setup** tab, in the **Targets/SNP Assays** table pane, click **Reagents**.
2. In the **Reagents** table, click ＋ **(Add)**.
3. Enter the reagent type, name, part number, lot number, and expiration date.

   Note: If the master mix that you enter is not compatible with the current run method, you have the option to apply the recommended run method for your master mix, instrument, block, and run mode.
4. (*Optional*) Click ⊗ **(Remove)** in the row of a reagent to delete it from the table.

Select a passive reference

1. In the upper-left corner of the **Plate Setup** tab, select a passive reference from the dropdown list.
2. (*Optional*) Save the plate file or data file.

Review and save the plate file

1. In the **Run Summary** tab, review the run method selections, then edit if needed.
2. Review the plate setup, then edit if needed.
3. *(Optional)* Click the barcode field, then scan the plate barcode.
4. *(Optional)* Select **Add to My Plates**.

5. Select an instrument from the list.

   If the instrument does not appear on the list, click ⚙ **System** ▸ **Instruments** to add a new instrument.

6. Save the plate file.

Start the run on an instrument. For specifics on starting an instrument run, see the instrument documentation.

4 Perform genotyping analysis

Review results in the Amplification Plot

For detailed instructions about reviewing results in the **Amplification Plot**, see **Help ▸ Help Contents**.

If no data are displayed in the **Quality Check** tab, or if reanalysis is required, click **Analyze**.

1. In the **Quality Check** tab, in the plot pane, select **Amplification Plot** from the dropdown list.
2. Review the amplification status for each well.
3. Review or edit threshold settings.
4. Review or edit baseline settings.

Select the Genotyping Analysis Module

1. In an open data file, click **Actions ▸ Analysis Modules**.
2. In the **Analysis Modules** window, select **Genotyping**, then click **Ok**.
   The Genotyping Analysis Module opens.

Click **Analyze**, then review the results in the **Genotyping** tab.

Review results in the Allelic Discrimination Plot

For more information about the allelic discrimination plot, see “Allelic Discrimination Plot overview” on page 19.

If no data are displayed in the **Genotyping** tab, or if reanalysis is required, click **Analyze**.

1. In the **Genotyping** tab, in the upper-left corner of the **Allelic Discrimination Plot** pane, select an assay from the **Assays** list (see Figure 1 on page 15).
   The Allelic Discrimination Plot is displayed for the selected assay.

2. Confirm that the data points for each genotype control cluster along the expected axis of the plot.

Table 1 Cluster assignments in an allelic discrimination plot

Content of samples	Location in AD plot
Allele 1 homozygote[1]	Lower right corner
Allele 2 homozygote[2]	Upper left corner
Alleles 1/2 heterozygote[3]	Approximately midway between Allele 1 and Allele 2 clusters
No-template control	Bottom left corner
Undetermined	Anywhere outside the regions described above
No amplification	With no-template control (NTC) cluster in the bottom left corner

[1] Labeled with allele 1 fluorescent dye.
[2] Labeled with allele 2 fluorescent dye.
[3] Labeled with allele 1 and allele 2 fluorescent dyes.

3. Confirm that the cluster at the bottom-left corner of the plot contains only negative control wells.

   Samples can unexpectedly cluster with the negative controls for one of the following reasons.

   - Samples contain no DNA
   - Samples contain PCR inhibitors
   - Samples are homozygous for a sequence deletion

4. Visually evaluate clusters for the three possible genotypes (see Figure 1 on page 15).

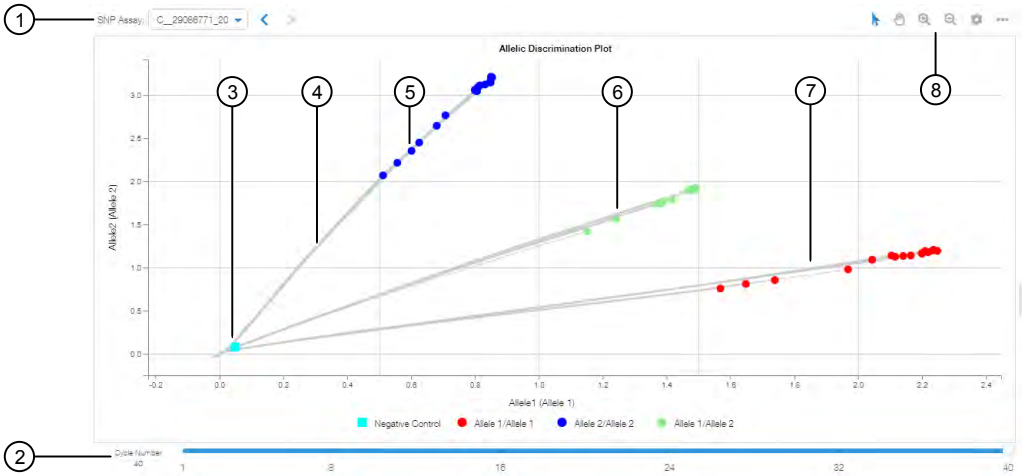

Figure 1 Allelic Discrimination Plot

1. Assay selection
2. **Cycle Number** slider—For analysis that includes real-time data, the slider allows you to display the allelic discrimination data for all samples at each cycle during the PCR amplification.
3. NTC cluster
4. Real-time PCR amplification trails—For analysis that includes real-time data
5. Allele 2 homozygote cluster
6. Allele 1/2 heterozygote cluster
7. Allele 1 homozygote cluster
8. Plot toolbar

To confirm results, retest outliers and samples with no amplification (cluster with negative controls).

Omit outliers from genotyping analysis

Outlier wells have C_q values that differ significantly from the average for the associated replicate wells. To ensure C_q precision, consider omitting the outliers from analysis.

1. In the **Genotyping** tab, select an option to omit wells from analysis.

Option	Description
Omit wells in the **Plate Layout**	Select outlier wells, then click ··· **(Actions)** ▸ **Omit Wells**.
Omit wells in the **Well Table**	Select **Omit** in the row of the outlier well.

2. Click **Analyze** to reanalyze the data with any outliers removed.

(*Optional*) Review dye signal profile in the MultiComponent Plot

For more information about the **MultiComponent Plot**, see **Help ▸ Help Contents**.

If no data are displayed in the **Quality Check** tab, or if reanalysis is required, click **Analyze**.

1. In the **Quality Check** tab, in the plot pane, select **MultiComponent Plot** from the dropdown list.
2. Review the signal profiles for the passive reference dye, reporter dye, and negative control wells.
3. Review the plot to ensure that there are no irregularities in the dye signals.

(*Optional*) Review signal profile in the Raw Data Plot

For detailed instructions about reviewing results in the **Raw Data Plot**, see **Help ▸ Help Contents**.

If no data are displayed in the **Quality Check** tab, or if reanalysis is required, click **Analyze**.

1. In the **Quality Check** tab, in the plot pane, select **Raw Data Plot** from the dropdown list.
2. Click-drag the **Cycle Number** slider through all of the cycles, then confirm that each filter displays the characteristic signal increase.

Edit Genotyping Analysis Setting

Open the Genotyping Analysis Module.

1. Click **Actions ▸ Genotyping Analysis Setting**.
2. Select an option from the **Analysis Data** list to determine the data that used for genotyping analysis.

Analysis Data	Description
Post Read	Only post-PCR read data is used to determine calls.
Pre Read & Post Read[1]	The pre-PCR read is subtracted from the post-PCR read to determine calls.

Analysis Data	Description
Real Time Data[2]	The normalized reporter data (Rn) from the selected cycle of the cycling stage is used to determine calls. Analysis is not restricted to the last cycle. Adjust the analysis cycle using the **Cycle Slider** in the **Allelic Discrimination Plot** pane (see Figure 1 on page 15).

[1] The run method must include a pre-read stage.
[2] Data collection must be on during the PCR stage.

3. (*Optional*) Edit the following settings:
   - **Min Confidence %**: Change the confidence threshold to increase or decrease stringency for making a genotype call.
   - (*Real Time Data Analysis Only*) **Baseline Start** and **Baseline End**: Enter the first and last cycle that the software will use to calculate the baseline.
   - (*Real Time Data Analysis Only*) **Cycle Number**: Change the cycle number used to generate the normalized reporter data (Rn).

Option	Description
Edit default analysis settings	1. In the table, in the **Default Setting** row, click in a cell to edit the setting. 2. To apply the default analysis settings to an assay, in the assay row, select **Use Default**.
Edit analysis settings for an individual assay	In the table, in the assay row, click in a cell to edit the setting.

4. Click **Apply**.

The data is reanalyzed using the updated analysis settings.

5 About genotyping analysis

Overview of genotyping analysis

Genotyping analysis is used to detect single nucleotide polymorphism (SNP) variants of a target nucleic acid sequence.

Genotyping analysis uses preformulated TaqMan™ SNP Genotyping Assays that include the following components:

- Two sequence-specific primers for amplification of sequences containing the SNP of interest
- Two allele-specific TaqMan™ probes for Allele 1 and Allele 2

For genotyping analysis, the software performs the following tasks:

1. The software normalizes the fluorescence of the reporter dyes to the fluorescence of the passive reference dye in each well.
2. The software plots the normalized reporter dye signal of each sample well on an Allelic Discrimination Plot, which contrasts the reporter dye intensities of the allele-specific probes.
3. 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.

Sample types for genotyping analysis

Genotyping analysis includes the following sample types.

Sample type (Type column in Samples table)	Sample description	Automatic target task assignment[1] (Task column in Targets table)
Unknown	Test sample	Unknown
Positive 1/1	Control sample that is heterozygous allele 1/allele 2	Positive 1/1
Positive 1/2	Control sample that is heterozygous allele 1/allele 2	Positive 1/2
Positive 2/2	Control sample that is homozygous for allele 2	Positive 2/2
Negative Control	Water or buffer No amplification of the target should occur in NTC wells.	Negative control

[1] The software automatically assigns a task to the target in a well based on the sample type in that well.

Allelic Discrimination Plot overview

The Allelic Discrimination Plot contrasts the Rn or the ΔRn of the reporter dyes for the allele-specific probes of the SNP assay. It is an intermediary step in the software algorithm for genotyping calls.

A typical Allelic Discrimination Plot shows homozygote clusters, a heterozygote cluster, and the no-template controls. The points in each cluster are grouped closely together and each cluster is located well away from the other clusters. For expected cluster locations in the plot, see Figure 2 on page 20.

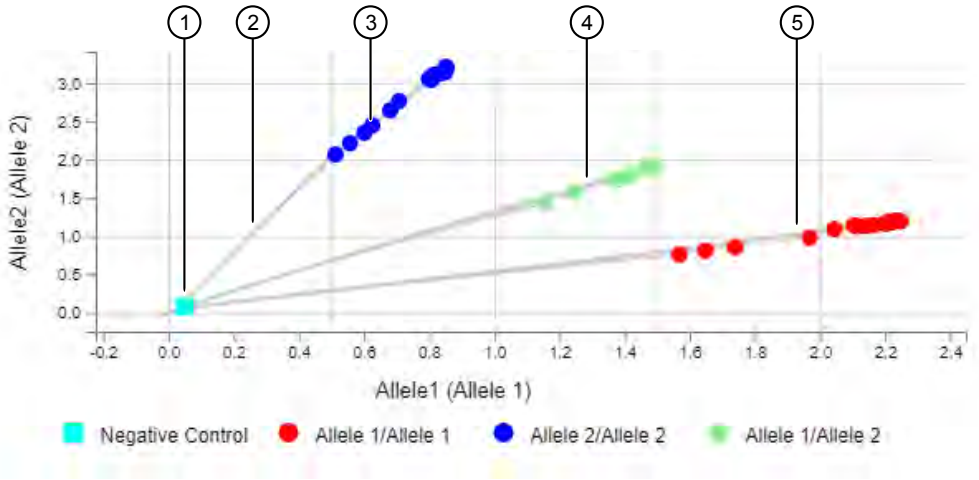

Figure 2 Example allelic discrimination plot

① No-template control cluster
② Real-time PCR amplification trails (for analysis that includes real-time data)
③ Allele 2 homozygote cluster
④ Allele 1/2 heterozygte cluster
⑤ Allele 1 homozygote cluster

Note:

- Undetermined samples may appear anywhere on the plot.
- No-amplification samples cluster with the no-template control samples.

Note: The clustering algorithm does not call genotypes if all the samples are one genotype (form one cluster).

Documentation and support

Related documentation

Document	Publication number
QuantStudio™ Design and Analysis Software v2 User Guide	MAN0018200
QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems User Guide	MAN0018045
QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems Site Preparation Guide	MAN0017992

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