Presence Absence Analysis Module

USER GUIDE

for use with:

QuantStudio™ Design and Analysis Software v2

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Life Technologies Holdings Pte Ltd | Block 33 | Marsiling Industrial Estate Road 3 | #07-06, Singapore 739256
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Revision	Date	Description
C.0	4 November 2020	Changes for version 2.1: Added sample comment; added sample name in target call rules and invalidation assessment in control rules; added export/import/scan in reagent information.
B.0	30 April 2020	Changes for version 2.0: Removed send to the instrument run queue; updated analysis setting and presence/absence calls.
A.0	26 August 2019	New document.

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1 About the Presence Absence Analysis Module

The Presence Absence Analysis Module for QuantStudio™ Design and Analysis Software v2 is used to determine the presence or absence of a target nucleic acid sequence in a sample.

For more information about presence/absence analysis, see Chapter 5, “About presence/absence 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 (including TaqMan™ Array Card format)
- 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 (including TaqMan™ Array Card format)
- 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: Presence/absence analysis

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3 Set up a plate file for presence/absence 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 Presence Absence 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, hover over the plate file, then click ··· **(Actions)**.

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

Confirm or edit the run method for presence absence analysis

For most analysis, the default run method is appropriate. The following options are compatible with presence absence 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**.

- (*Optional*) Confirm that data collection is turned on in the **Pre Read** stage.
  ΔRn calculation requires pre-PCR read data.

- (*Recommended*) Confirm that data collection is turned on in the **PCR** stage.
  We recommend collecting real-time amplification data during the PCR stage, for troubleshooting purposes.

- Confirm that data collection is turned on in the **Post Read** stage.
  Post-PCR read data is used to determine presence or absence calls.

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

Confirm or edit the plate setup for presence/absence 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]	Presence absence analysis uses the following sample types. • Unknown • Positive Control • Negative Control

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

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

Add targets and assign to wells

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

1. In the **Plate Setup** tab, add targets and assign to wells using the following options.
   - Import an AIF file
   - Import a plate setup file
   - Manually add targets to the **Targets** table
   - Manually add targets to wells in the plate layout
   - Import TaqMan™ assay plate and card files
2. Confirm or edit the target information in the **Target** table.

Column	Description
Name	Target name
Color	Target color

(continued)

Column	Description
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 presence absence analysis. • Unknown • Positive Control • Negative Control • IPC (Internal Positive Control)[2] • Blocked IPC[2]

[1] For more information, see "Sample types for presence/absence analysis" on page 19.
[2] To assign this task type, select the task from the drop-down list.

3. Confirm or edit the target 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)**.

Note:
- Click ··· **(Actions)** ▸ **Export Reagents** to export reagents.
- Click ··· **(Actions)** ▸ **Import Reagents** to import reagents.
- Click ··· **(Actions)** ▸ **Scan Reagents** to scan reagents.

3. Enter the reagent **Name**, **Type**, **Barcode**, **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 presence/absence analysis

Select the Presence Absence Analysis Module

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

Click **Analyze**, then review the results in the **Presence Absence** tab.

Review results in the Amplification Plot

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

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

1. In the **Presence Absence** tab, in the plot pane, review the overall shape of the curves in the **Amplification Plot**.
2. Review the amplification status for each well.

   Table 1 Expected amplification status for control reactions

Control	Target	Expected Result
No template control	IPC	Amplification
	Target of interest	No amplification
No amplification control (NAC; blocked IPC)	IPC	No amplification
	Target of interest	No amplification
Positive control	IPC	Amplification
	Target of interest	Amplification

3. Review or edit threshold settings.
4. Review or edit baseline settings.

Edit Presence Absence Analysis Setting

To enable well calls and sample calls, you must set up sample call rules, in addition to target call rules.

Open the Presence Absence Analysis Module.

1. Click **Actions** ▸ **Presence Absence Analysis Setting**.

   Note:
   - Click **Import** to import settings.
   - Click **Export** to export settings.

2. Once you are finished editing all of the analysis settings, click **Apply**.

The data is reanalyzed using the updated analysis settings.

Edit Target Call Rules

1. In the **Presence Absence Analysis Setting** window, in the **Target Call Rules** tab, select an option from the **Analyze Data** list to determine the method used to calculate ΔRn (Rn = normalized readings).

Option	Description
Post-PCR Read	$\Delta Rn = Rn_{(post\text{-}PCR\ read)}$
Pre-PCR Read and Post-PCR Read	$\Delta Rn = Rn_{(post\text{-}PCR\ read)} - Rn_{(pre\text{-}PCR\ read)}$
Real-time Rn Data	$\Delta Rn = Rn_{(last\ PCR\ cycle)} - Rn_{(first\ PCR\ cycle)}$

   Note: If you did not turn on data collection for a specific stage of the thermal protocol during plate file setup, data analysis for that stage will not be available (see “Confirm or edit the run method for presence absence analysis” on page 9).

2. In the **Target Call Rules** tab, in the table:
   - Click ＋ **(Add)** to add a new target call rule.
   - Click in a cell to edit the following settings if needed.
     - **Sample Name**
     - **Target**
     - **C_q Cutoff**
     - **ΔRn Threshold**
   - Click ⊗ **(Remove)** to delete a target call rule.

3. Once you are finished editing all analysis settings, click **Apply**.

The data is reanalyzed using the updated analysis settings.

Edit Tests settings

In **Presence Absence Analysis Setting**, a test refers to a test for a particular pathogen. If you are doing analysis with multiple tests, define the tests to differentiate for the sample call rules and control rules.

1. In the **Presence Absence Analysis Setting** window, in the **Tests** tab, click + **(Add)** to add a new test.
2. Click in a cell to edit the following settings if needed.
   - **Test Code**
   - **Description**
3. (*Optional*) Click ⊗ **(Remove)** to delete the settings.
4. Once you are finished editing all analysis settings, click **Apply**.

The data is reanalyzed using the updated analysis settings.

Edit Sample Call Rules

For detailed information about sample call rules and example sample call rule settings, see “About sample call rules” on page 21.

1. In the **Presence Absence Analysis Setting** window, in the **Sample Call Rules** tab, click + **(Add)** to add a new sample call rule.
2. In the table, click in a cell to edit the following settings if needed.
   - **Sample Name**
   - **Presence Targets**
   - **Absence Targets**
   - **Test Code** (see “Edit Tests settings” on page 15 for more information)
   - **Call**
   - **Assessment**
3. (*Optional*) In the table, click ⊗ **(Remove)** to delete a sample call rule.
4. Once you are finished editing all analysis settings, click **Apply**.

The data is reanalyzed using the updated analysis settings.

Edit Control Rules

1. In the **Presence Absence Analysis Setting** window, in the **Control Rules** tab, click + **(Add)** to add a new control rule.
2. In the table, click in a cell to edit the following settings if needed.
   - **Sample Name**
   - **Test Code** (see "Edit Tests settings" on page 15 for more information)
   - **Expected Call**
   - **Invalidate Calls**
   - **Invalidation Assessment**

   Note: If you select **Invalidate Calls**, the software will automatically set well calls and sample calls for all other samples to invalid if the control result does not match the **Expected Call**. The well call and sample call for the control will still display as either Presence or Absence to indicate why the control failed. Only well calls and sample calls for the indicated **Test Code** will be affected.
3. Click ⊗ **(Remove)** to delete the settings.
4. Once you are finished editing all analysis settings, click **Apply**.

The data is reanalyzed using the updated analysis settings.

Review presence/absence calls

To enable well calls, you must define sample call rules in the **Presence Absence Analysis Settings** (see "Edit Presence Absence Analysis Setting" on page 14).

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

- In the **Presence Absence** tab, use one of the following options to review target calls:

Option	Description
Plate layout	1. In the plate layout pane, in the **Color By** dropdown list, select **Sample**. 2. Hover over a well to see the individual calls for each target in the well.
Target Call Table	In the table pane, click **Target Call**, then review the call, C_q, and ΔRn for each target in a well. For more information, see "About call types" on page 20.

- In the **Presence Absence** tab, use one of the following options to review well calls:

Option	Description[1]
Plate layout	1. In the plate layout pane, in the **Color By** dropdown list, select **Sample**. 2. Review the call for each well sample, as indicated by the icon displayed in the middle of the well: – + (Presence)—The target nucleic acid sequence is present in the sample – — (Absence)—The target nucleic acid sequence is absent in the sample – ! (Warning)—The sample data needs review for possible errors – ? (Inconclusive)—A well call cannot be made – ✕ (Invalid)— The IPC failed **Note:** • You can also hover over a well to see the call for each well sample. • If there is no icon displayed in the well, the well call is undetermined.
Well Call Table	In the table pane, click **Well Call**, then review the calls. For more information, see "About call types" on page 20.
Sample Call Table	In the table pane, click **Sample Call**, then review the calls. For more information, see "About call types" on page 20.
Control Status Table	In the table pane, click **Control Status**, then review the calls.

[1] For more information about sample call rules, see "About sample call rules" on page 21.

Omit outliers from presence/absence 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 **Presence Absence** 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 **Target Call Table**	Select **Omit** in the row of the outlier well.

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

(*Optional*) Add a sample comment

1. In the **Presence Absence** tab, in the table pane, click **Sample Call**.
2. Click a cell in the **Comment** column to enter a sample comment.
3. Click **Actions** ▸ **Save** or **Actions** ▸ **Save As** to save the sample comment.

(*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.

5 About presence/absence analysis

Overview of presence/absence analysis

Use presence/absence analysis to determine the presence or absence of a target nucleic acid sequence in a sample. The software calls the target present or absent based on an algorithmically determined call threshold. (The call threshold is different from the C_q threshold; the C_q threshold is not used to make calls.)

Presence/absence calls are based on real-time PCR data, or endpoint data (data collected after the PCR stage).

- The data that is collected is the normalized intensity of the reporter dye, or Rn.
- If endpoint experiments include pre-PCR data points, the software calculates the ΔRn value according to the following formula:

  $\Delta Rn = Rn_{\text{(post-PCR read)}} - Rn_{\text{(pre-PCR read)}}$, where Rn = normalized readings.

We recommend collecting real-time amplification data during the PCR stage, for troubleshooting purposes.

Sample types for presence/absence analysis

Presence/absence sample types depend on whether the experiment is set up with or without an internal positive control (IPC).

- **Multiplex presence/absence experiments using IPC (recommended)**—multiplex assays for the target of interest and the IPC target. The IPC is used to confirm that a negative result for the target of interest is not caused by a failed PCR.

Sample type (Type column in Samples table)	Sample description	Target task assignment[1] (Task column in Targets table)
Unknown	– Test sample – IPC template	– Unknown – IPC[2]
Negative control	No template control[3] – Water or buffer – IPC template	– Negative control – IPC[1]

(continued)

Sample type (Type column in Samples table)	Sample description	Target task assignment[1] (Task column in Targets table)
Negative control	No amplification control (NAC; blocked IPC)[3] – Water or buffer plus a blocking agent – IPC template; amplification prevented by blocking agent	– Negative control – Blocked IPC[1]

[1] The software automatically assigns a task to the target in a well based on the sample type in that well.
[2] To edit the automatic target task assignment, select an option from the dropdown list.
[3] Minimum of two replicates is required for this control.

- **Singleplex presence/absence experiments without IPC**

Sample type (Type column in Samples table)	Sample description	Target task assignment[1] (Task column in Targets table)
Unknown	Test sample	Unknown
Negative Control	Water or buffer	Negative Control

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

The software makes calls for individual wells. Running three or more replicates of each reaction can help identify outlier wells that may be present.

About call types

Presence/absence analysis uses the following call types.

Call type	Description
Target Call	• Each target call is for one particular target in a particular well. • A target call can be presence or absence, based on the target call rules.
Well Call	• Each well call is for one particular test in a particular well. • A well call can be derived from multiple target calls from the same well. For example, a well call for one test can be derived from four target calls from the same well. • For multiple tests in the same well, the Well Call Table has multiple rows for the same well, with one row for each test.

(continued)

Call type	Description
Sample Call	• Each sample call is for one particular test of one particular sample. • A sample call is derived from well calls. Since the same sample can be run on multiple wells, either as replicates or for different tests, the Sample Call Table can be very different from the Well Call Table. • If the same sample has different calls in different wells for the same test, the sample call becomes inconclusive for that particular test.

About sample call rules

To enable well calls, you must define sample call rules in the **Presence Absence Analysis Setting** (see “Edit Presence Absence Analysis Setting” on page 14).

For each sample type, create a unique sample call rule for all applicable presence/absence target combinations.

Table 2 Sample call rule table settings

Sample call rule setting	Description
Sample Name	Enter a sample name. Call rules that include a sample name are given priority over call rules that do not include a sample name. For example, if multiple call rules match the results for a given sample, the call rule that matches the sample name will be used. The sample name can contain wildcard characters, such as * for any number of characters and ? for exactly one character. For example: • "PositiveControl*" will match "PositiveControl", "PositiveControl1" and "PositiveControl-A" • "PositiveControl?" will match "PositiveControl1", but not "PositiveControl" or "PositiveControl-A"
Presence Targets	Select all of the targets that are present for a particular call.
Absence Targets	Select all of the targets that are absent for a particular call.
Test Code	(*Optional*) If there are multiple tests in one well, enter or select a test from the dropdown list. To edit test settings, see “Edit Tests settings” on page 15.

Table 2 Sample call rule table settings *(continued)*

Sample call rule setting	Description
Call	Select one of the following call options: • Presence—The target nucleic acid sequence is present in the sample • Absence—The target nucleic acid sequence is absent in the sample • Warning—The sample data needs review for possible errors • Inconclusive—A well call cannot be made • Invalid—The IPC failed
Assessment	(*Optional*) Enter an assessment or recommended action for the sample call.

Two sample call rules are considered conflicting if both can be applied to a sample (the sample name, presence targets and absence targets match) but each give a different call.

If a sample call rule does not exist for a particular sample name/target combination, the software cannot make a call for that well, and the result will be undetermined.

The undetermined call is not included in the Sample Call Table or Well Call Table, only in the Target Call Table.

Example sample call rule settings

In the following example, there is one test that includes four targets per well: Target 1, Target 2, Target 3, and IPC.

Table 3 Example sample call rule settings

Sample Name[1]	Presence Targets	Absence Targets	Test Code[2]	Call	Assessment[3]
—	—	Target 1 Target 2 Target 3 IPC	—	Invalid	Repeat test
—	IPC	Target 1 Target 2 Target 3	—	Absence	Report results
—	Target 1	Target 2 Target 3	—	Inconclusive[4]	Repeat test
—	Target 2	Target 1 Target 3	—	Inconclusive[4]	Repeat test
—	Target 3	Target 1 Target 2	—	Inconclusive[4]	Repeat test

Table 3 Example sample call rule settings *(continued)*

Sample Name[1]	Presence Targets	Absence Targets	Test Code[2]	Call	Assessment[3]
—	Target 1 Target 2	Target 3	—	Presence[4]	Report results
—	Target 2 Target 3	Target 1	—	Presence[4]	Report results
—	Target 1 Target 3	Target 2	—	Presence[4]	Report results
—	Target 1 Target 2 Target 3	—	—	Presence[4]	Report results
Positive Control	Target 1 Target 2 Target 3 IPC	—	—	Presence	—
Negative Control	IPC	Target 1 Target 2 Target 3	—	Absence	—

[1] Call rules that include a sample name are given priority over call rules that do not include a sample name. For example, if multiple call rules match the results for a given sample, the call rule that matches the sample name will be used.

[2] The **Test Code** is not defined because there is only one test.

[3] (*Optional*) Enter an assessment for a sample call rule.

[4] For this sample call, the IPC target call can be Presence or Absence. Therefore, the IPC is not included in the sample call rule.

Documentation and support

Related documentation

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

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  - Safety Data Sheets (SDSs; also known as MSDSs)

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

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