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HID Real-Time PCR Analysis Software

USER GUIDE

v1.3 and v1.4

for use with:
7500 Real-Time PCR System for Human Identification
QuantStudio™ 5 Real-Time PCR System (with 0.2-mL 96-Well Block)
Quantifiler™ DNA Quantification Kits

Publication Number MAN0009819

Revision G.0

For Research, Forensic, or Paternity Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: MAN0009819 G.0 (English)

Revision	Date	Description
G.0	7 February 2024	Recommended HID Real-Time PCR Analysis Software v1.4 for use with only the QuantStudio™ 5 Real-Time PCR System with firmware v1.5.1 or later.
F.0	9 November 2022	• Added the following sections: "New features in v1.4" on page 7, Appendix A, "Troubleshooting", and Appendix C, "HID Real-Time PCR Analysis Software v1.4 verification". • Updated "Computer and instrument requirements" on page 10 and Chapter 2, "Install the HID Real-Time PCR Analysis Software and calibrate the instruments". • Updated to current publication style (minor grammar and formatting changes).
E.0	27 August 2018	Updated branding and trademarks, no technical changes.
D.0	8 March 2017	Added support for the QuantStudio™ 5 Real-Time PCR System with 96-well (0.2-mL) sample block. Added the Virtual Standard Curve function.
C.0	14 August 2015	Corrected the quencher listed for the Quantifiler™ Duo DNA Quantification Kit, Quantifiler™ Human DNA Quantification Kit, and Quantifiler™ Y Human Male DNA Quantification Kit. Added reference to evaluating the quality indices determined by the HID Real-Time PCR Analysis Software to determine if highly degraded samples can be better analyzed with the Ion Personal Genome Machine™ (PGM™) System. For more information, see the *Quantifiler™ HP and Quantifiler™ Trio DNA Quantification Kits User Guide* (Pub. No. 4485354).
B.0	31 March 2014	Added "HID Real-Time PCR Analysis Software Validation".
A.0	31 January 2014	New document for v1.2 features (support for Quantifiler™ HP and Trio DNA Quantification Kits; Degradation Index). Incorporates all information from the *HID Real-Time PCR Analysis Software User Guide* v1.1 (Pub. No. 4455443)

The information in this guide is subject to change without notice.

Contents

1 Product information

Product description

The HID Real-Time PCR Analysis Software is designed to assist human identification laboratories that perform DNA quantitation by simplifying assay setup, data review, and dilution and reaction setup for downstream STR analysis. For example, the software automatically selects the appropriate Quantifiler™ kit target, reporter, quencher, and thermal profile.

After a run, the software provides an analysis of each well, then allows you to export the following data:

- All results
- STR kit setup instructions
- Sample dilutions calculations

New features in v1.4

HID Real-Time PCR Analysis Software v1.4 includes all v1.3 functionality and the following new features:

- Compatible with the QuantStudio™ 5 Real-Time PCR System with firmware v1.5.1 or later
- Supports the Java™ software upgrade from v6 to v8

Note: We do not recommend using HID Real-Time PCR Analysis Software v1.4 with the 7500 Real-Time PCR System for Human Identification.

Compatible systems

The HID Real-Time PCR Analysis Software is compatible with the following systems:

System	Recommended HID Real-Time PCR Analysis Software version
7500 Real-Time PCR System for Human Identification	v1.3
QuantStudio™ 5 Real-Time PCR System (with 0.2-mL 96-Well Sample Block) with firmware v1.3.x	v1.3
QuantStudio™ 5 Real-Time PCR System (with 0.2-mL 96-Well Sample Block) with firmware v1.5.1 or later	v1.4

Note: To check the firmware version for your QuantStudio™ 5 Real-Time PCR System, see “Computer and instrument requirements” on page 10.

Compatible HID kits

You can use the HID Real-Time PCR Analysis Software with the following HID kits:

- Quantifiler™ HP DNA Quantification Kit
- Quantifiler™ Trio DNA Quantification Kit
- Quantifiler™ Human DNA Quantification Kit
- Quantifiler™ Duo DNA Quantification Kit

Custom experiment option

IMPORTANT! The custom assay option is supported only for the 7500 system.

You can also use the HID Real-Time PCR Analysis Software for more complex experiments by selecting the **Custom Assay** option on the **Home** screen. For instructions, see the user documentation for your instrument.

HID Real-Time PCR Analysis Software workflow

Set up a Quantifiler™ kit plate, then load the plate in the instrument.

Select the experiment and set up a plate:

1. Start the software.
2. Start a new experiment and specify experiment properties.
3. Define samples; assign targets, samples, and standards to wells.
4. Save the experiment.

Run the plate.

Select analysis settings and thresholds:

- HID settings (includes HID flags)
- Flag settings

Review results:

1. View the analysis summary.
2. View the quantitation results.

Export and print results.

Generate dilution and reactions worksheets for STR setup:

1. Configure the STR library and default dilution settings.
2. Add kits to an experiment.
3. Select the unknown samples for amplification.
4. *(As needed)* Edit dilution settings for individual samples.
5. Export dilution and reaction worksheets.

Perform PCR amplification

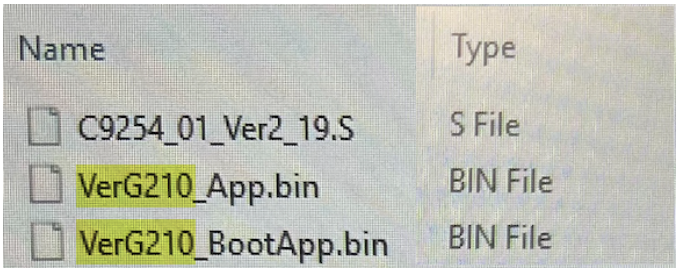
For instructions, see your instrument user guide.

2 Install the HID Real-Time PCR Analysis Software and calibrate the instruments

Computer and instrument requirements

Component	Requirements
Computer	• Processor (minimum: 2.9 GHz): – *(Recommended)* Intel™ Core™ i7 Quad Core™ CPU, 2.9 GHz – Intel™ Core™ i5 Quad Core™ CPU, 2.9 GHz • 16 GB of RAM[1] • One hard drive with at least 10 GB available • 20/48X IDE CD-ROM drive • USB v1.2 • Ethernet network interface adapter (10BASE-T)[2]§ • Microsoft™ Windows™ 10 IoT Enterprise (LTSC 2019 or 2021) **IMPORTANT!** If the computer that performs a run is on a network, avoid excess network use during the run.
Software	• Microsoft™ PowerPoint™ software (for direct export of PowerPoint™ slides) • Microsoft™ Excel™ software (for direct export of data to spreadsheet) **IMPORTANT!** Do not run antivirus applications while the HID Real-Time PCR Analysis Software is running. Antivirus applications may interfere with data collection from the instrument.

(continued)

Component	Requirements
Monitor	• 1280 × 1024 pixel resolution for full screen display[3] • 16-inch • True Color (32 bit) • UL listed
7500 Real-Time PCR Instrument	Instrument firmware vG2.10 (installed on all instruments purchased after 2008) To check the firmware version of your instrument, go to the following location: `<drive>:/Applied Biosystems/7500 system/firmware` Contact Service if your firmware version is not vG2.10. 
QuantStudio™ 5 Real-Time PCR System (with 0.2-mL 96-Well Sample Block)	Instrument firmware v1.3.x To check the firmware version of your instrument: From the **Home** screen, tap ⚙ **Settings** ▸ **About Instrument** ▸ **About Instrument**.
	Instrument firmware v1.5.1 or later To check the firmware version of your instrument: From the **Home** screen, tap ⚙ **Settings** ▸ **About Instrument** ▸ **About Instrument**.

[1] The software may experience communication errors if run on computers with less than 1 GB.
[2] Required only if you plan to connect the computer to a local area network (LAN).
[3] If screen resolution is not set to 1280 × 1024, the **Analysis Summary** screen may not be properly displayed.

7500 Real-Time PCR Systems purchased before February 2008

If your 7500 Real-Time PCR System was purchased before February 2008: Tower and laptop computers require a memory upgrade before the computers can install the HID Real-Time PCR Analysis Software. For more information, see the *7500/7500 Fast Real-Time PCR Systems User Bulletin Memory Upgrade Requirements for 7500 Software v2.0* (Pub. No. 4379705).

Install the HID Real-Time PCR Analysis Software

IMPORTANT! You must have Administrator privileges on the computer to install HID Real-Time PCR Analysis Software.

Install instructions are available in the Release Notes.

1. Obtain the appropriate upgrade version of the HID Real-Time PCR Analysis Software for your system.

Instrument	Recommended HID Real-Time PCR Analysis Software upgrade version
7500 Real-Time PCR System for Human Identification	v1.3
QuantStudio™ 5 Real-Time PCR System (with 0.2-mL 96-Well Sample Block) with firmware v1.3.x	v1.3
QuantStudio™ 5 Real-Time PCR System (with 0.2-mL 96-Well Sample Block) with firmware v1.5.1 or later	v1.4

Note: To check the firmware version for your QuantStudio™ 5 Real-Time PCR System, see "Computer and instrument requirements" on page 10.

2. Insert the HID Real-Time PCR Analysis Software DVD into a computer.

3. Open the Release Notes, then follow the appropriate install instructions for your system.

   Install instructions are provided for the following scenarios:

System	Computer	Scenario
7500 Real-Time PCR System for Human Identification	Instrument computer	Install the HID Real-Time PCR Analysis Software on an instrument computer that is running an earlier version of the HID software
		Install new HID Real-Time PCR Analysis Software on a new instrument computer
QuantStudio™ 5 Real-Time PCR System with firmware v1.3.x	Instrument computer	Install the HID Real-Time PCR Analysis Software on an instrument computer that is running an earlier version of the HID software
		Install new HID Real-Time PCR Analysis Software on a new instrument computer
QuantStudio™ 5 Real-Time PCR System with firmware v1.5.1 or later	Instrument computer	Install the HID Real-Time PCR Analysis Software on an instrument computer that is running an earlier version of the HID software
		Install new HID Real-Time PCR Analysis Software on a new instrument computer

(continued)

System	Computer	Scenario
All systems	Non-instrument computer	Install the HID Real-Time PCR Analysis Software on a non-instrument computer that is running an earlier version of the HID software
		Install new HID Real-Time PCR Analysis Software on a non-instrument computer

Calibrate the 7500 instrument

IMPORTANT! For system layout, electrical, power, safety, and other site requirements, see the *Applied Biosystems™ 7500/7500 Fast Site Preparation Guide* (Pub. No. 4412843).

If you	Perform
Installed new HID Real-Time PCR Analysis Software with a new instrument	Perform all calibrations and run the RNase P plate
Upgraded from an earlier version of the HID Real-Time PCR Analysis Software	After restoring the calibration files from the earlier HID software version (see the Release Notes), perform a custom dye calibration to calibrate ABY™, JUN™, and MUSTANG PURPLE™ (MP) dyes.
Replaced SDS Software v1.2.3	Perform all calibrations and run the RNase P plate

Required materials not supplied

Table 1 7500 Real-Time PCR Instrument—Materials required for calibration

If you	Material	Cat. No.
Replaced SDS Software v1.2.3	7500 Real-Time PCR Systems Spectral Calibration Kit I	4349180
	TaqMan™ RNase P Instrument Verification Plate	4350584
	96-Well Spectral Calibration Plate with ABY™ Dye	4461591
	96-Well Spectral Calibration Plate with JUN™ Dye	4461593
	96-Well Spectral Calibration Plate with MUSTANG PURPLE™ Dye	4461599
Upgraded from an earlier version of the HID Real-Time PCR Analysis Software	96-Well Spectral Calibration Plate with ABY™ Dye	4461591

Table 1 7500 Real-Time PCR Instrument—Materials required for calibration *(continued)*

If you	Material	Cat. No.
Upgraded from an earlier version of the HID Real-Time PCR Analysis Software	96-Well Spectral Calibration Plate with JUN™ Dye	4461593
	96-Well Spectral Calibration Plate with MUSTANG PURPLE™ Dye	4461599

Calibration procedures

The following is an outline of the calibration procedures for the 7500 Real-Time PCR Instrument. For complete instructions, see the *7500/7500 Fast Real-Time PCR Systems System Maintenance Guide* (Pub. No. 4387777).

- Regions of Interest (ROI) calibration
- Background calibration
- Optical calibration
- Dye calibration:
  - Perform dye calibration of the ABY™, JUN™, and MUSTANG PURPLE™ (MP) dyes. Follow the custom dye procedure.
  - Perform dye calibration of all system dyes for new instrument installations, or if replacing SDS Software v1.2.3.
  - Use 60°C as the default temperature for all dye calibrations.
- TaqMan™ RNase P Instrument Verification Plate run

New dye spectra for the 7500 Real-Time PCR Instrument

Figure 1 through Figure 3 show the calibration spectra for ABY™, JUN™, and MUSTANG PURPLE™ (MP) dyes.

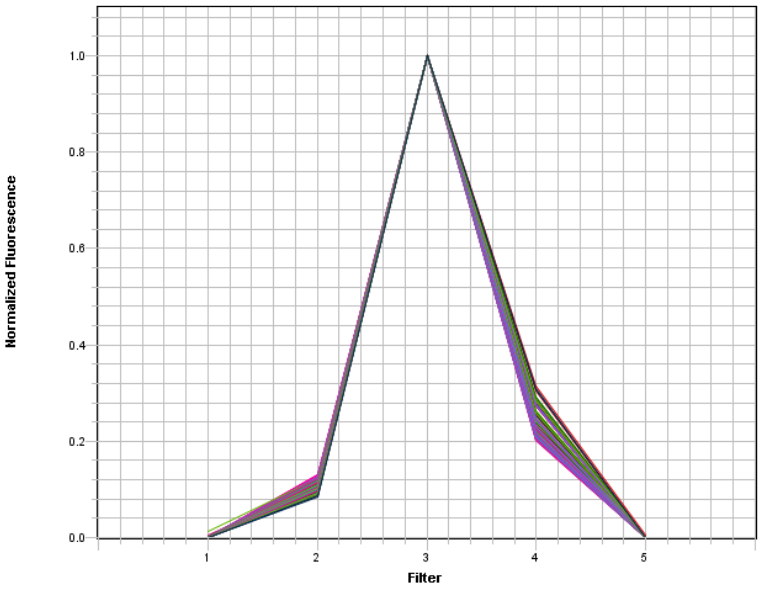


Figure 1 ABY™ dye spectra

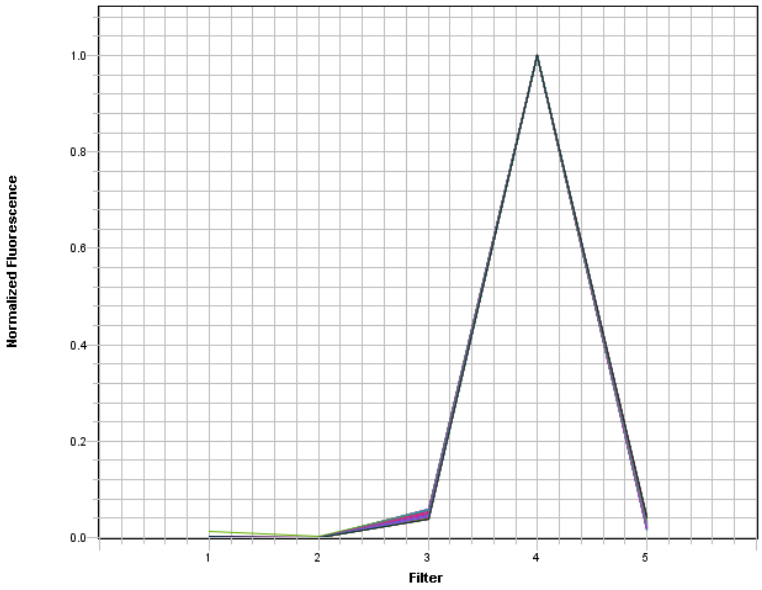


Figure 2 JUN™ dye spectra

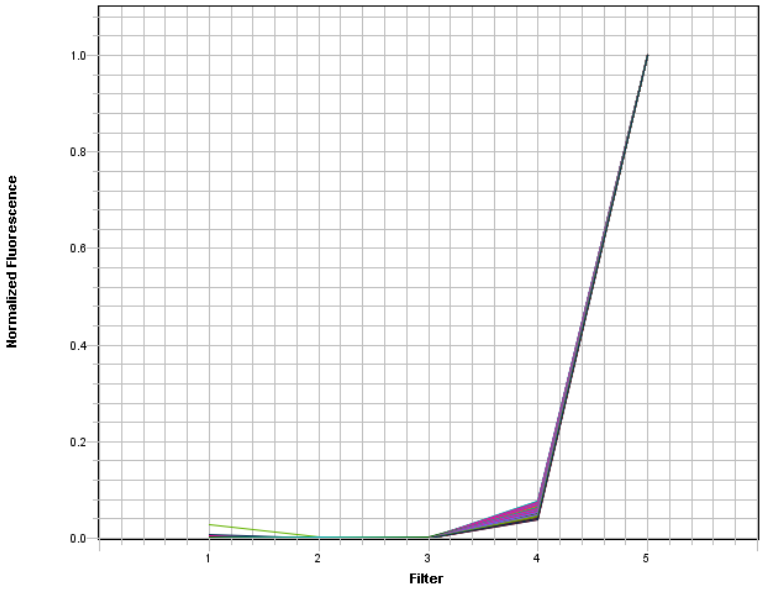


Figure 3 MUSTANG PURPLE™ (MP) dye spectra

Calibrate the QuantStudio™ 5 Instrument

IMPORTANT! For system layout, electrical, power, safety, and other site requirements, see the *QuantStudio™ 5 Real-Time PCR Instrument Site Preparation Guide (for Human Identification)* (Pub. No. MAN0016701).

The QuantStudio™ 5 Real-Time PCR Instrument is calibrated during manufacturing; however, you *must* recalibrate the instrument for the dyes that are used for HID analysis before use. If you installed HID Real-Time PCR Analysis Software with a new instrument, perform custom dye calibrations for the ABY™ and JUN™ dyes.

Required materials not supplied

Table 2 QuantStudio™ 5 Real-Time PCR Instrument—Materials required for calibration

Material	Cat. no.
96-Well Spectral Calibration Plate with ABY™ Dye	4461591
96-Well Spectral Calibration Plate with JUN™ Dye	4461593
TaqMan™ RNase P Instrument Verification Plate, 96-Well 0.2-mL	4432382

Calibration procedures

The following is an outline of the calibration procedures for the QuantStudio™ 5 Real-Time PCR Instrument. For complete instructions, see the *QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide* (Pub. No. MAN0010407).

- Dye calibration:
  - Perform dye calibration of the ABY™ and JUN™ dyes. Follow the custom dye procedure.
  - Use 60°C as the default temperature for all dye calibrations.

  IMPORTANT! You *must* calibrate the ABY™ dye as **ABY-HID** and the JUN™ dye as **JUN-HID**. Calibrating either dye without the "-HID" suffix (as **ABY** and **JUN**) overwrites the existing calibrations for the factory-calibrated system dyes. Doing so potentially creates confusion if the instrument is ever calibrated using the QuantStudio™ 3 and 5 Calibration Kit, which does not have the HID versions of the dyes.

- TaqMan™ RNase P Instrument Verification Plate run

New dye spectra for the QuantStudio™ 5 Real-Time PCR Instrument

Figure 4 through Figure 6 show the calibration spectra for ABY™, JUN™, and MUSTANG PURPLE™ (MP) dyes.

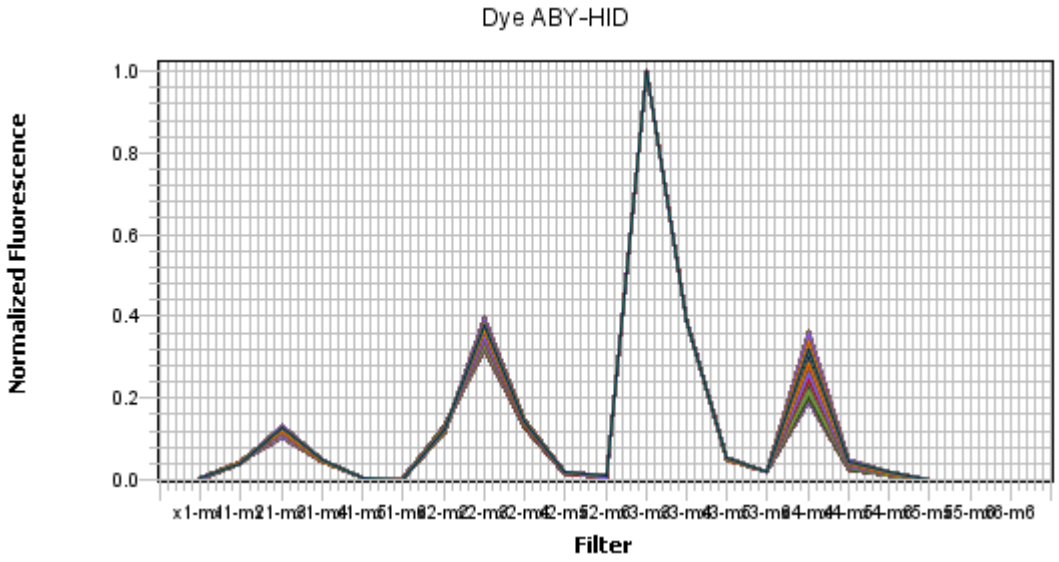


Figure 4 ABY™ dye spectra

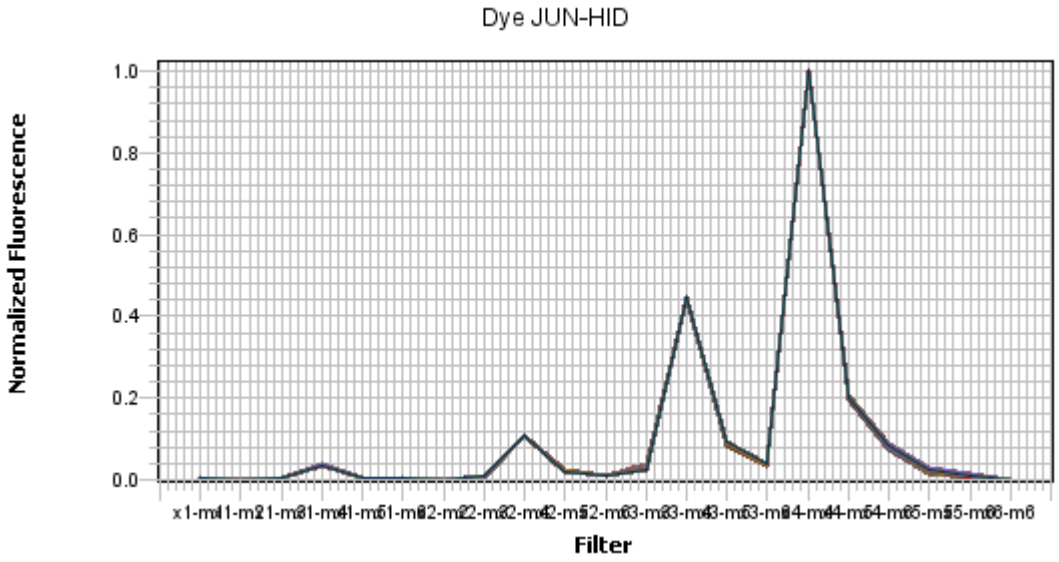


Figure 5 JUN™ dye spectra

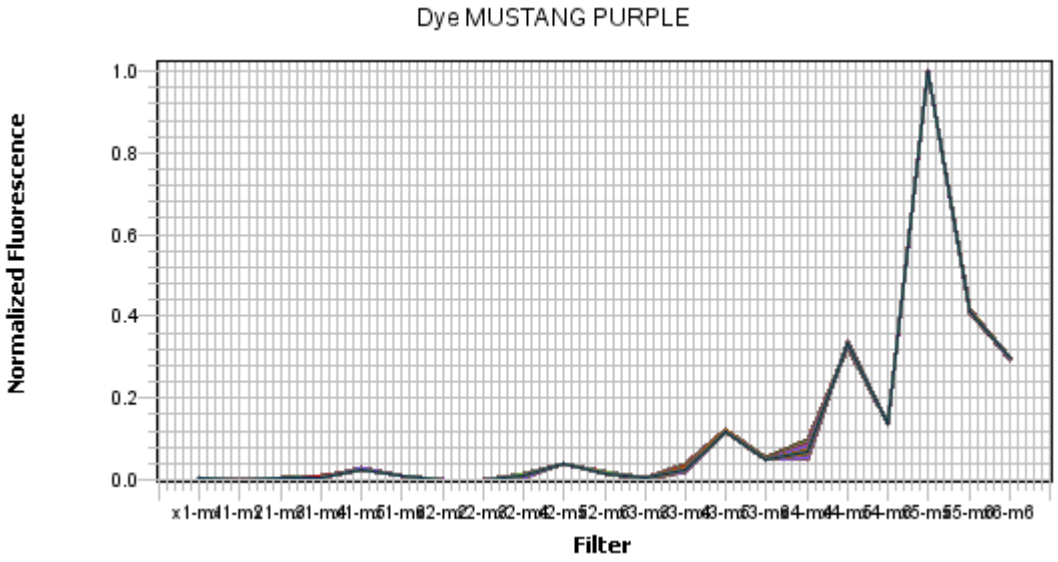


Figure 6 MUSTANG PURPLE™ (MP) dye spectra

3 Customize the software

Modify a default experiment template

You can make changes to the experiment templates provided with the software after making a backup copy of the original templates.

Save a copy of the original template

Before you modify a template, save a copy of the original template:

1. Navigate to: `<drive>:/Applied Biosystems/7500/config/templates`
2. Select **Edit** ▸ **Copy** to copy the templates folder.
3. Navigate to a safe location on your computer.
4. Select **Edit** ▸ **Paste** to insert a copy of the templates folder in the location you selected.

Modify the original template

1. Click the button on the **Home** screen for the experiment type of interest.
2. Modify the template as needed, including:
   - Moving standards and NTCs to different wells
   - Adding samples and/or extraction blanks
   - Setting the plate layout
   - Setting the display defaults for the amplification plot, plate view, and well table
   - Modifying analysis settings (HID, C_T, and flags)
3. In the toolbar, click the down arrow next to **Save**, select **Save as**, then select the name of the original template.

Create an experiment template

1. Set up an experiment with the desired settings, including:
   - Moving standards and NTCs to different wells
   - Adding samples and/or extraction blanks
   - Setting the plate layout
   - Setting the display defaults for the amplification plot, plate view, and well table
   - Modifying analysis settings (HID, C_T, and flags)
2. In the toolbar, click the down arrow next to **Save**, then select **Save as Template**.

To use your template instead of a default template: At the top of the **Home** screen, click **Open**, then select your template.

Link your template to a Home screen button

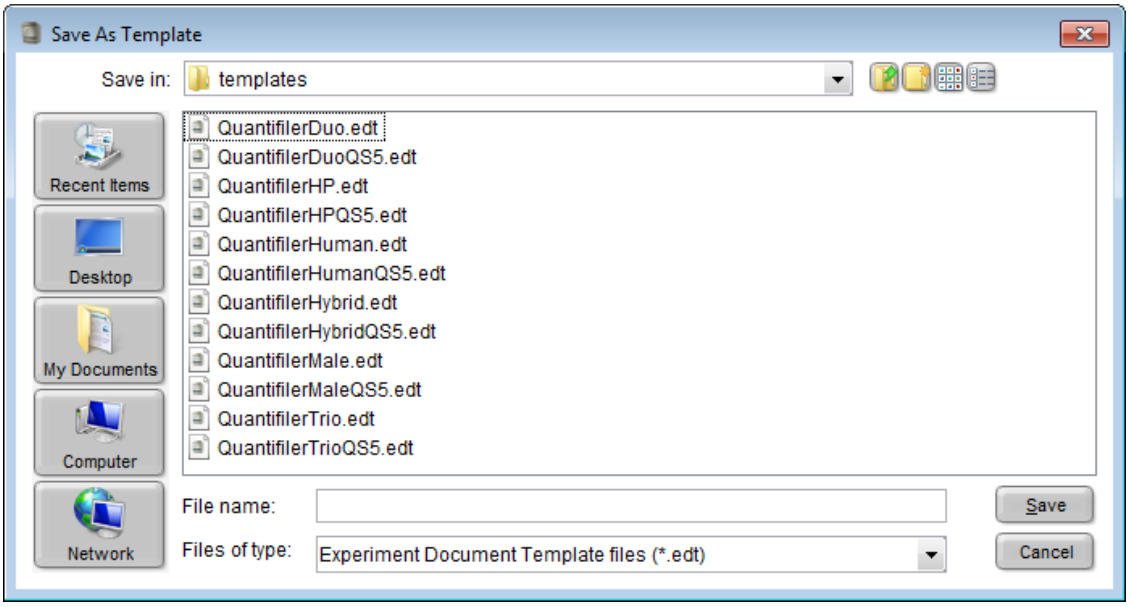
You can link your template to any of the Quantifiler™ assay buttons on the **Home** screen.

The software will automatically use the template as the default experiment when you click the corresponding button. You will still be able to use a different template by opening a different experiment.

1. Before linking, save a copy of the original template.
   a. Navigate to `<drive>:/Applied Biosystems/7500/config/templates`
   b. Select **Edit** ▸ **Copy** to copy the `templates` folder.
   c. Navigate to a safe location on your computer.
   d. Select **Edit** ▸ **Paste** to insert a copy of the `templates` folder in the location that you selected.
2. Link your template to a button on the **Home** screen.
   a. Open the file that you want to link.
   b. In the toolbar, select **Save** ▸ **Save as Template**.
   c. Navigate to `<drive>:/Applied Biosystems/7500/config/templates`

d. Select the file corresponding to the assay button that you want to replace.

IMPORTANT! Files that contain the "QS5" suffix are templates used by the QuantStudio™ 5 Instruments. For example, "QuantifilerTrio.edt" is the template file for the Quantifiler™ Trio DNA Quantification Kit used by 7500 Instruments; "QuantifilerTrioQS5.edt" is the file used by QuantStudio™ 5 Instruments.



IMPORTANT! Ensure that you give the file exactly the same name as the file corresponding to the button that you want to replace.

e. Click **Save**.

Set display defaults

Select data to display in the plate view

1. Click **Show in Wells**.
2. In the dropdown list, select (☑) or deselect (☐) the data to display in the plate view.
3. Click **Set as Default**.

Note: The button is inactive before you change a setting, or if you are logged in as Guest.

Specify the data to display in the well table

1. Click **Show in Table**.
2. In the dropdown list, select (☑) or deselect (☐) the data to display in the well table.
3. Click **Set as Default**.

Note: The button is inactive before you change a setting, or if you are logged in as Guest.

Customize the amplification plot

1. Make changes as described in “Change the appearance of, print, and save plots” on page 58.
2. Select (☑) or deselect (☐) the data to display in the amplification plot: **Threshold** and/or **Baseline Start**.
3. Click **Save Current Settings as Default**.

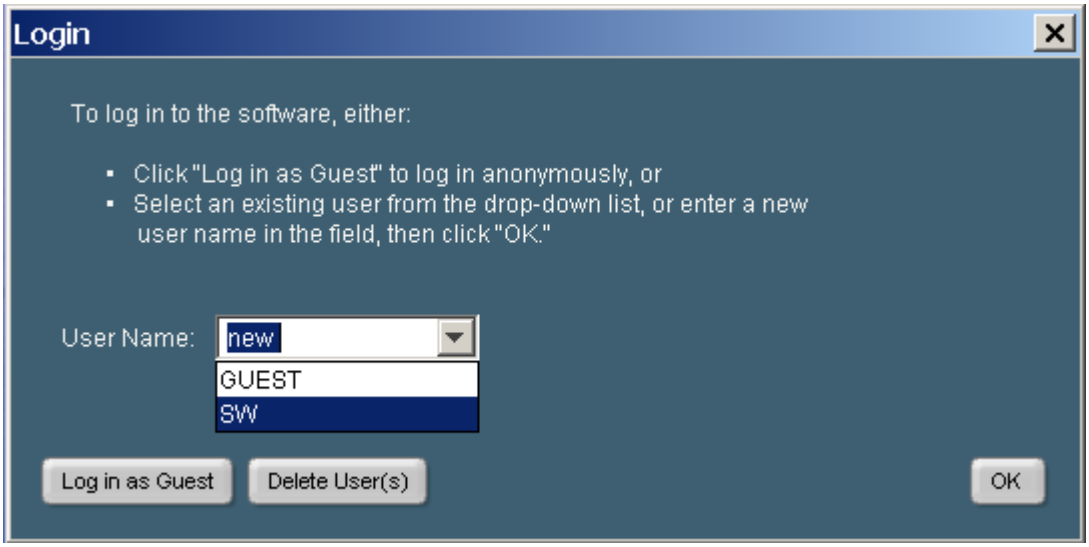
Note: The button is inactive before you change a setting, or if you are logged in as Guest.

4 Select the experiment and set up a plate

This chapter assumes that you have prepared a plate according to the instructions in the user guide for the Quantifiler™ Kit that you are using.

Start the software and select an experiment

1. On the computer desktop, double-click or select **Start** ▸ **All Programs** ▸ **Applied Biosystems™** ▸ **HID Real-Time PCR Analysis Software** ▸ **HID Real-Time PCR Analysis Software**. The **Login** screen should open within 1 minute.



2. In the **User Name** field, enter your user name or select it from the dropdown list.

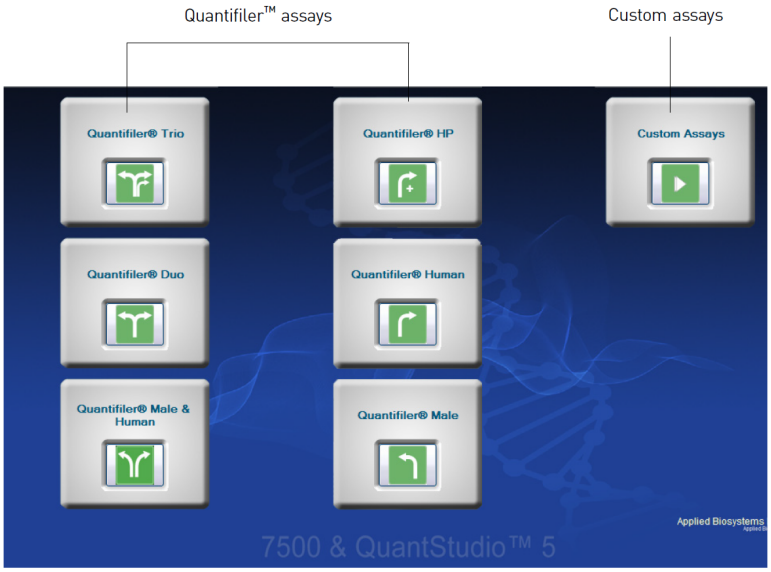
   You can log in as a guest, but only users logged in with a user name can perform the following functions:

   - Edit the names of folders for experiment information import, information export, or data.
   - Enable or disable the requirement to enter a user name to start the software.
   - Set a plate layout as the default layout (see “Link your template to a Home screen button” on page 21).
   - Configure how data is displayed (see Chapter 3, “Customize the software”).

3. Click **OK** to open the **Home** screen.

4. Select an HID experiment.

   - Click one of the Quantifiler™ kit template buttons, or click **Custom Assays**.



   or

   - In the toolbar, click the down arrow next to **New Experiment**, then select the appropriate experiment.

For custom experiments

IMPORTANT! The custom experiments feature is supported for the 7500 system only.

To perform a non-HID experiment, or a modified experiment, click:

- **Custom Assay** on the right side of the Home screen.

  or

- **Assays** in the toolbar, then select **Custom Assays** in the drop-down list.

For information on running custom experiments, see the user documentation for your instrument.

Navigate the software

Each HID Real-Time PCR Analysis Software experiment screen displays instructions for a step in the experiment. Use the **Experiment Menu** at the left of any screen to navigate the software.

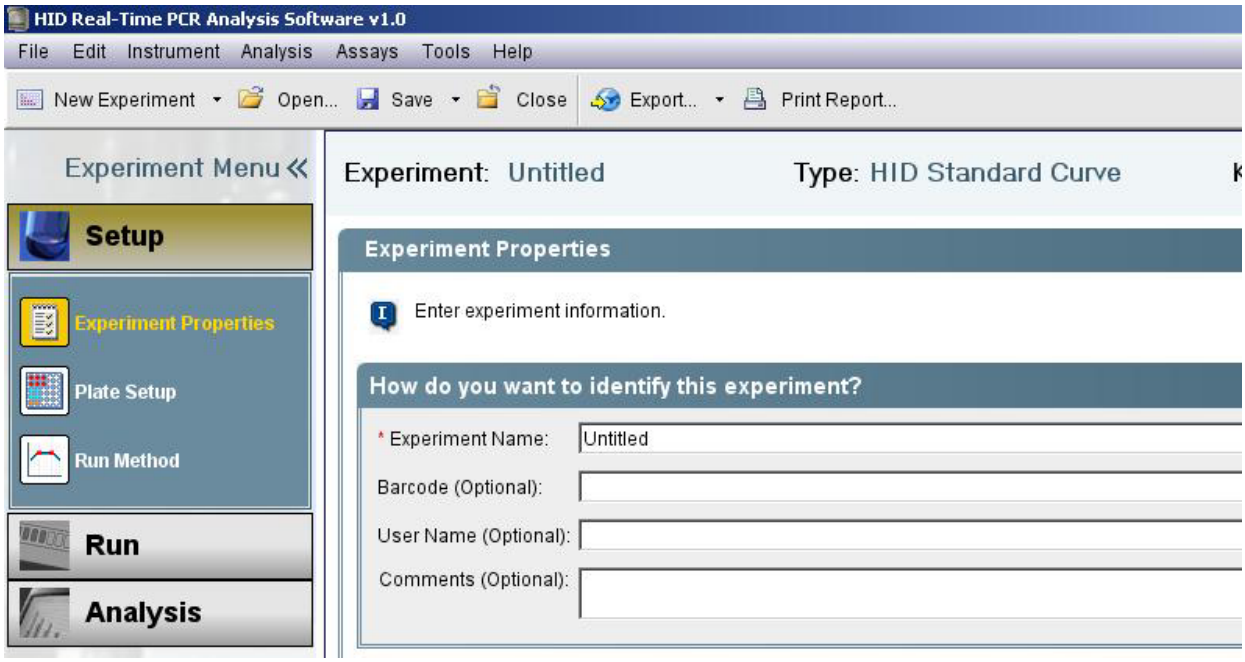
- Click **>> (Expand)** to expand the **Experiment Menu**.
- Click **<< (Collapse)** to collapse the **Experiment Menu**.
- Click **Setup**, **Run**, or **Analysis** to display screens used in the corresponding process.

You can access the software screens in any sequence.

To return to the **Home** screen at any time, click **(Home)** at the bottom left of any screen.

Specify experiment properties

1. In the **Experiment Menu**, select **Setup** ▸ **Experiment Properties**.
2. In the **How do you want to identify this experiment?** pane, enter the name of the plate or experiment information in the **Experiment Name** field. Entries in the other fields are optional.



Note: The name that you enter in the **Experiment Name** field appears on the data report and on XLS spreadsheets that you export. If you do not enter a name, **Untitled** appears on the report and in the exported spreadsheet.

The following parameters are automatically set:

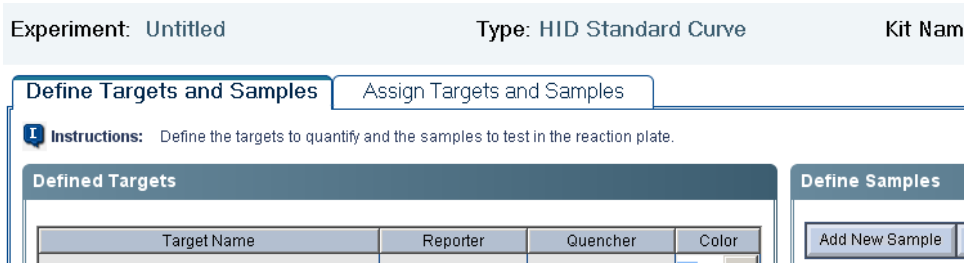
- **Experiment Name**: *Untitled*
- **Instrument**:
  - 7500 (96 wells)
  - QuantStudio™ 5 (96 wells)
- **Experiment Type**: Quantitation-HID Standard Curve
- **Reagents**: TaqMan™ Reagents
- **Ramp Speed**: Standard (~1 hour to complete a run for Quantifiler™ HP™ and Quantifiler™ Trio kits, and ~2 hours for all other Quantifiler™ kits)

Define samples and view targets

Note: Targets are automatically listed and named. Standards dilutions and an NTC sample are listed by default for each Quantifiler™ kit. For information about the standard included in the Quantifiler™ kit, see the kit user guide.

Define samples

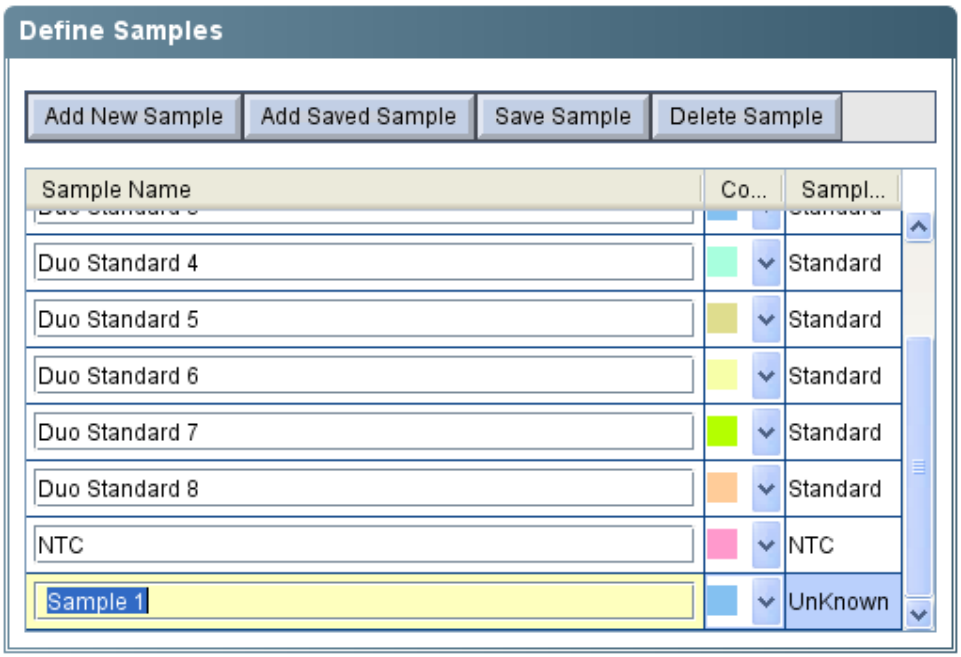
1. In the **Experiment Menu**, select **Setup** ▸ **Plate Setup**, then click the **Define Targets and Samples** tab.



2. In the **Define Samples** pane (right side of the screen), specify sample names.
   - To define a new sample, do one of the following:
     - Click **Add New Sample**. A new line appears in the **Sample Name** field.
     - In the toolbar, select **Tools** ▸ **Sample Library**, then click **New**.

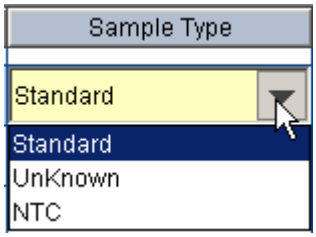
     The default name for the new sample is Sample X (where X=1 or the highest listed Sample # + 1). You can enter a new name for the sample. To save the name of the sample for future experiments, click **OK**.
   - To use a sample from your sample library:
     a. In the **Define Samples** pane, click **Add Saved Sample**.
     b. Select the samples to use, then click **Add Selected Sample(s)**.

Note: You can also add a sample to a single well in the **Plate Setup** screen. See “Assign a new sample to a well” on page 33.



3. Select the sample type: **Standard**, **NTC**, or **Unknown**. Unknown is the default sample type for new samples.

   When you assign the sample type, the software automatically assigns the appropriate task to each target.



4. Repeat steps 2 on page 28 and 3 on page 29 for each sample.

IMPORTANT! List each sample individually. For replicates (identical samples), add the sample name only once. To assign a replicate to a well in the plate, in step 4 on page 32, select the well, then select the checkbox next to the sample name.

View targets

1. In the **Experiment Menu**, select **Setup** ▸ **Plate Setup**.
2. Select the **Define Targets and Samples** tab.
3. In the **Defined Targets** pane, view the targets list to verify that you selected the correct experiment in step 4 on page 25.

Kit	Reporter dyes	Quencher
Quantifiler™ Trio	Small autosomal: VIC™ dye	NFQ-MGB
	Male (Y): FAM™ dye	NFQ-MGB
	Large autosomal: ABY™ dye	QSY™7
	IPC: JUN™ dye	QSY™7
Quantifiler™ HP™	Small autosomal: VIC™ dye	NFQ-MGB
	Large autosomal: ABY™ dye	QSY™7
	IPC: JUN™ dye	QSY™7
Quantifiler™ Duo	Human: VIC™ dye	NFQ-MGB
	Male: FAM™ dye	NFQ-MGB
	IPC: NED™ dye	NFQ-MGB
Quantifiler™ Human	Human: FAM™ dye	NFQ-MGB
	IPC: VIC™ dye	NFQ-MGB

Change a color designation

Perform this procedure to change the color that represents a target in the data analysis.

1. Click (down arrow) in the **Color** column.
2. Select a color in the dropdown list.

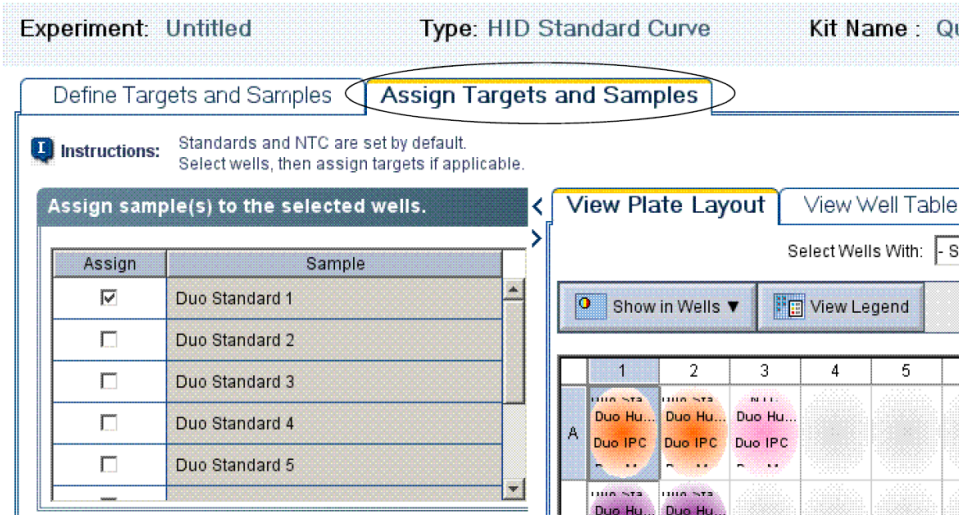
Assign the targets, samples, and standards to wells

Open the Assign Targets and Samples tab

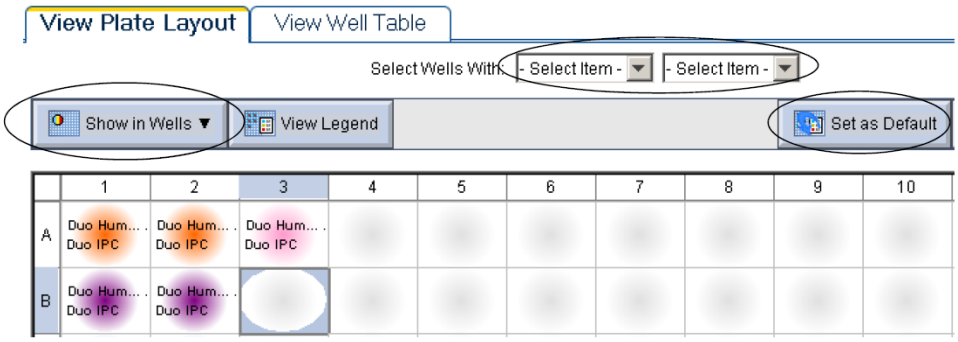
Do one of the following to open the tab.

- In the **Define Targets and Samples** tab, click **Assign Targets and Samples** beneath the **Define Samples** pane.

- In the **Experiment Menu**, select **Setup** ▸ **Plate Setup**, then select the **Assign Targets and Samples** tab.



Assign using the plate layout

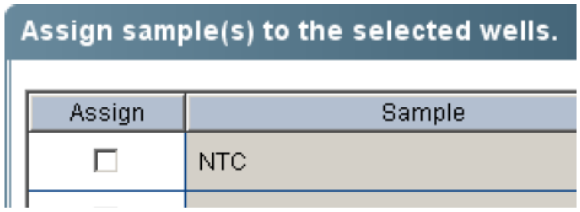


Assign samples, standards, and NTCs to wells

Assign samples, standards, and NTCs using the **View Plate Layout** tab.

1. Select the **View Plate Layout** tab (right side of the screen).
2. *(Optional)* Select wells with specific characteristics.
   a. Click the left **Select Wells With** button above the layout diagram.
   b. Select **Sample**, **Target**, or **Task** in the dropdown list.
   c. Click the right **Select Wells With** button.
   d. Select a specific sample, target, or task.
3. Specify the information to display in the wells:
   a. Click **Show in Wells** to open the dropdown list. Items that are marked with a check (☑) are selected for display.
   b. Click an item to select or deselect it for display.
4. *(Optional)* To save your selections as default settings, click **Set as Default** at the top right of the **View Plate Layout** toolbar.
5. Assign standards, NTCs, and unknown samples to wells.
   a. To select:
      - **Well**—Click the well
      - **Row of wells**—Click a letter on the side of the layout
      - **Column of wells**—Click a number at the top of a column
      - **More than one well, row, or column**—Drag the pointer over the wells, letters, or columns to select

b. In the **Assign sample(s) to the selected wells** pane (left of the plate layout), select the checkbox in the **Assign** column corresponding to the unknown, standard, or NTC sample in the wells. The target for each sample is set by default.



Note: <Sample 1> is automatically assigned to all wells that are not assigned as standards or NTCs.

6. *(Optional)* To change the quantity of standards, enter the quantity in ng/µL in the **Quantity** field in the **Assign targets to the selected wells** pane. The quantity of standard samples is set by default.

7. Repeat steps 4 on page 32 and 5 on page 33 until you assign samples, standards, and NTCs to all wells that you use in the experiment. You can delete empty wells after data analysis.

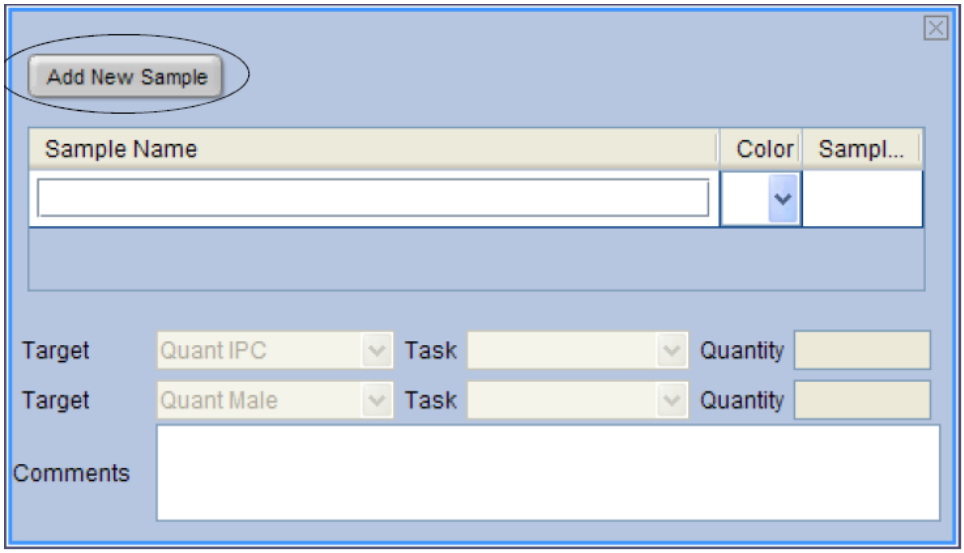
Note: If you delete the samples, standards, or NTCs in a well and then restore them, you must re-enter the well information.

The task for each target/sample combination is set automatically.

8. Clear all wells that do not contain samples or targets.
   a. Select the wells to clear.
   b. Right-click, then select **Clear** from the dropdown list.

Assign a new sample to a well

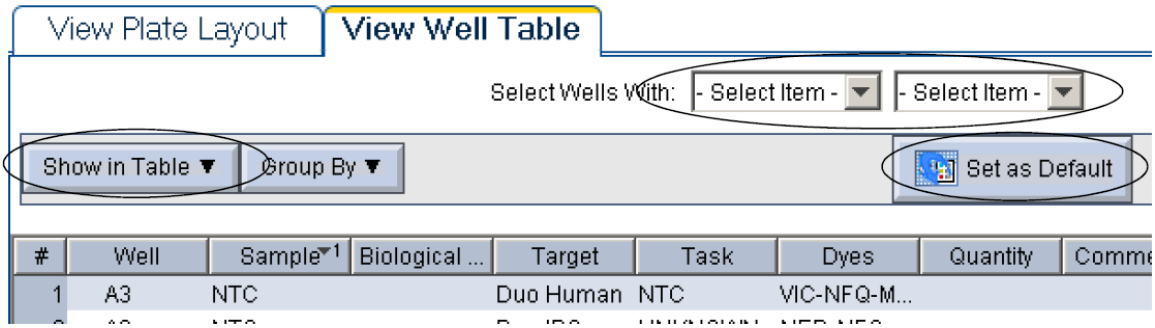
1. Double-click the well to open the **Add New Sample** dialog box.
2. Click **Add New Sample**.
3. The target and task are set by default according to the sample type. To change the sample type, click the down arrow in the **Sample** column header, then select the appropriate sample type from the dropdown list.
4. To change the sample quantity setting for standard samples, perform step 5 on page 33.



Move samples, standards, and NTCs

1. Select the wells for the samples, standards, or NTCs that you want to move.
2. Deselect ( ) the items in the **Assign sample(s) to the selected wells** pane, or right-click the wells and select **Clear**.
3. One at a time, select the new wells for an item that you are moving, then select ( ) the items in the **Assign sample(s) to the selected wells** pane.

Assign using the well table



Assign samples, standards, and NTCs using the **View Well Table** tab.

1. Select the **View Well Table** tab.

   Each row in the table represents one well. To group the rows by a characteristic, click the column header. For example, click **Task** to group rows by task.

2. *(Optional)* Select wells with specific characteristics.
   a. Click the left **Select Wells With** button above the layout diagram.
   b. Select **Sample**, **Target**, or **Task** in the dropdown list.

c. Click the right **Select Wells With** button.

d. Select a specific sample, target, or task.

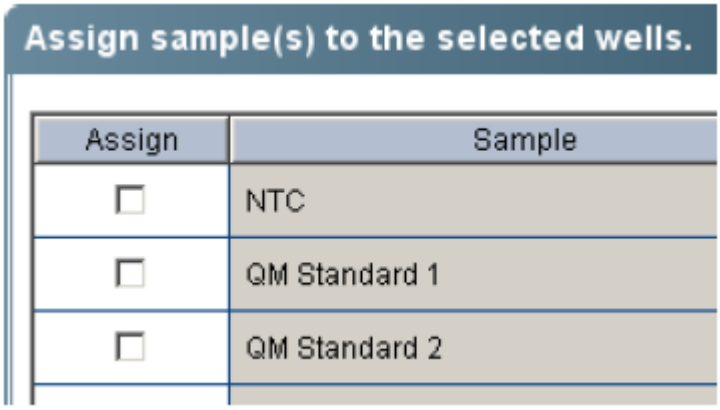
3. Specify the information to display in the table:
   a. Click **Show in table** to open the dropdown list. Items that are checked (☑) are selected for display.

   b. Click an item to select or deselect it for display.

4. *(Optional)* To save your selections as default settings, click **Set as Default** at the top right of the **View Plate Layout** toolbar.

5. Assign samples, standards, and NTCs to wells.
   a. Select the wells.
      - **One well**—Click under one of the column headings in the row next to the well location (for example, to select well A6, click in row A6 under **Sample**).
      - **More than one well**—Drag the pointer over the wells that you want to select, or **Ctrl+Click** the wells that you want to select.

   b. In the **Assign sample(s) to the selected wells** pane, select the checkbox in the **Assign** column corresponding to the unknown, standards, or NTC sample in the wells. The target for each sample is set by default.



Note: <Sample 1> is automatically assigned to all wells that are not assigned as standards or NTCs.

6. *(Optional)* To change the quantity of standards, enter the quantity (in ng/µL) in the **Quantity** field in the **Assign targets to the selected wells** pane. The quantity of samples is set by default.

7. Repeat steps 1 on page 34 and 5 on page 35 until you assign samples, standards, and NTCs to all wells that you use in the experiment. You can delete empty wells after data analysis.

Note: If you delete the samples, standards, or NTCs in a well and then restore them, you must re-enter the well information.

The task for each target/sample combination is set automatically.

8. Clear all wells not assigned.
   a. Click the left **Select Wells With** button at the top of the table.
   b. Select **Sample** from the dropdown list.
   c. In the well table, select the sample names of the wells to clear.
   d. In the **Assign sample(s) to the selected wells** pane, deselect the checkbox in the **Assign** column next to the sample name.

Save the plate layout as EDS or template

IMPORTANT! Do not save the experiment to the network folder until the plate run is completed.

1. In the toolbar, click the down arrow next to **Save**, then select a save option.
   - **Save**—Saves the plate layout as an Experiment Document Single (EDS) file
   - **Save as**—Saves the plate layout as an EDS file with a different name
   - **Save as Template**—Saves the experiment file as a template for future experiments
2. If you want to save the file with a different name, enter the new name in the **File Name** field.
3. Click **Save**.
4. Before you start the run, verify that the plate is loaded in the instrument, as described in your Quantifiler™ kit user guide.

Link your template to a Home screen button

You can link your template to any of the Quantifiler™ assay buttons on the **Home** screen.

The software will automatically use the template as the default experiment when you click the corresponding button. You will still be able to use a different template by opening a different experiment.

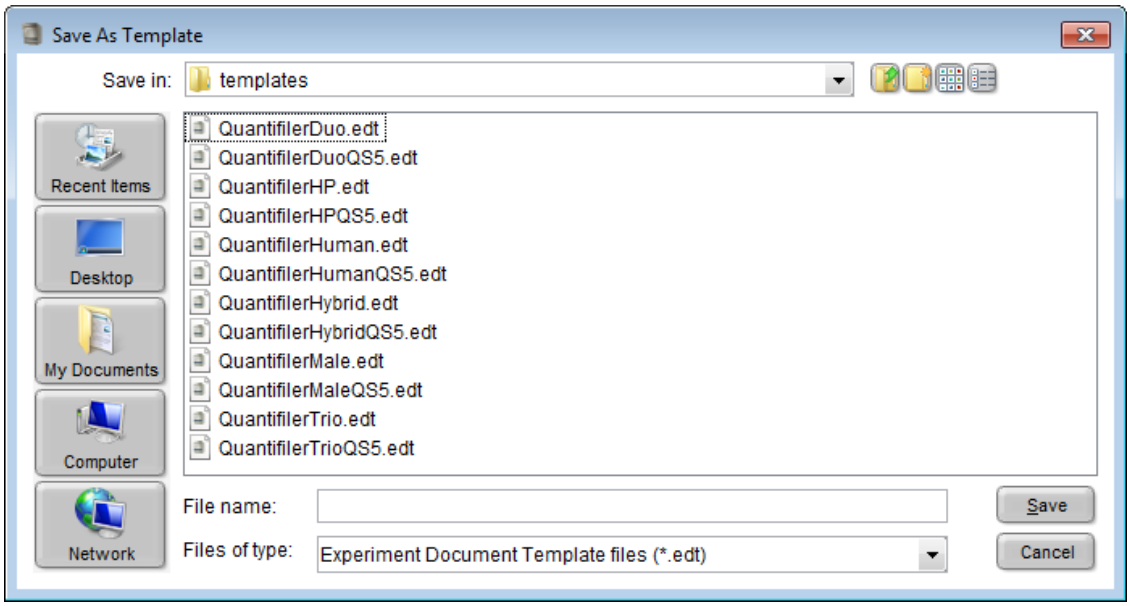
1. Before linking, save a copy of the original template.
   a. Navigate to `<drive>:/Applied Biosystems/7500/config/templates`
   b. Select **Edit** ▸ **Copy** to copy the `templates` folder.
   c. Navigate to a safe location on your computer.
   d. Select **Edit** ▸ **Paste** to insert a copy of the `templates` folder in the location that you selected.
2. Link your template to a button on the **Home** screen.
   a. Open the file that you want to link.

b. In the toolbar, select **Save ▸ Save as Template**.

c. Navigate to `<drive>:/Applied Biosystems/7500/config/templates`

d. Select the file corresponding to the assay button that you want to replace.

IMPORTANT! Files that contain the "QS5" suffix are templates used by the QuantStudio™ 5 Instruments. For example, "QuantifilerTrio.edt" is the template file for the Quantifiler™ Trio DNA Quantification Kit used by 7500 Instruments; "QuantifilerTrioQS5.edt" is the file used by QuantStudio™ 5 Instruments.



IMPORTANT! Ensure that you give the file exactly the same name as the file corresponding to the button that you want to replace.

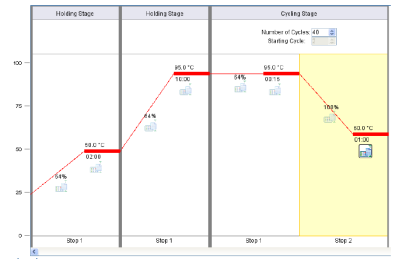
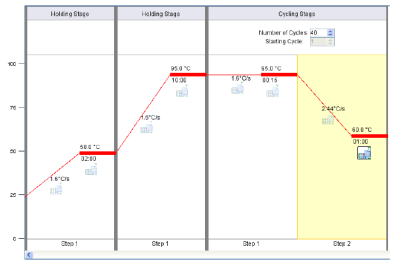
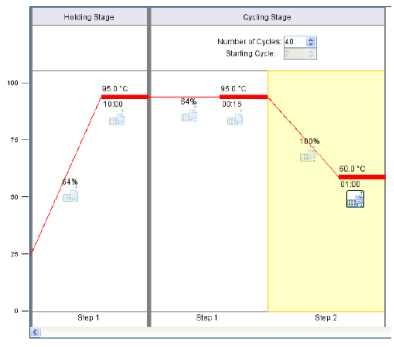
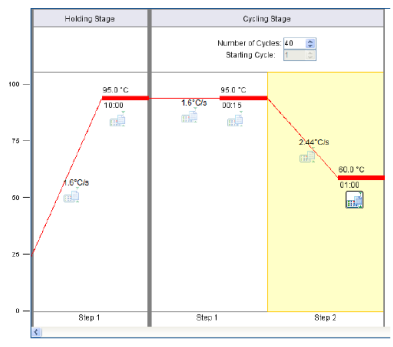
e. Click **Save**.

5 Run the plate

View the run method

1. In the **Experiment Menu**, select **Setup** ▸ **Run Method** to open the **Run Method** screen.
2. Select the **Graphical View** tab to open the thermal profile for the assay.

Note: The **Graphical View** tab displays the run method ramp rate as a percentage when using a 7500 instrument and in degrees Celsius (°C) when using a QuantStudio™ 5 Instrument.

Quantifiler™ kit	Thermal profile	
	7500 instrument	QuantStudio™ 5 instrument
Duo		
Human		

3. Verify the value in the **Reaction Volume** field.
   - 25 µL for Quantifiler™ Human and Duo kits
   - 20 µL for Quantifiler™ HP™ and Trio kits

   For more information on run parameters, see the user guide for your Quantifiler™ kit.

(7500 system only) Set notifications

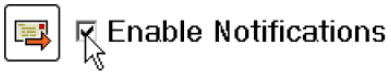
You can set the software to send e-mail notifications for selected events to e-mail addresses that you specify.

Note: This procedure applies only to the 7500 Real-Time PCR Instrument. It does not apply to the QuantStudio™ 5 Real-Time PCR Instrument.

IMPORTANT! Notifications cannot be sent unless the computer that performs the run is on an e-mail network.

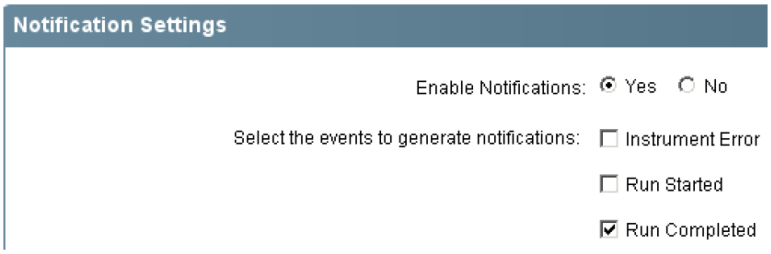
1. In the **Experiment Menu**, select **Run** ▸ **Notification Settings**.

2. Enable or disable notifications.
   - **Enable notifications**—In the **Run Status** pane, select the **Enable Notifications** checkbox.

     or

     In the **Notifications Settings** pane, select **Yes** next to **Enable Notifications**.

   - **Disable notifications**—In the **Notifications Settings** pane, select **No** next to **Enable Notifications**.

3. Select or deselect the events to generate notifications.
   - **Instrument Error**—When selected, notifies addressees that a run stopped before it was completed.
   - **Run Started**—When selected, notifies addressees that a run started.
   - **Run Completed**—When selected, notifies addressees that a run is completed.

4. In the **Enter email addresses for notifications** field, enter the e-mail addresses that notifications will be sent to. Follow the format shown on the screen. Enter a comma between addresses.

5. Define the outgoing server. If you need information about the server, contact your network system administrator.
   a. **Outgoing Server (SMTP)** field—Enter the name of the outgoing server.
      For example: ***smtp.mycompany.com***
   b. **Server requires an encrypted connection?**—If the outgoing server requires an encrypted connection, select **Yes**.
   c. **Server requires authentication**—If the outgoing server requires authentication to receive the e-mail from the instrument, select **Yes**, then enter the authentication user name and password in the dialog box.

Start or stop the run

IMPORTANT! If the computer that performs the run is on a network, avoid excess use of the network during a run.

Note: You can set analysis parameters before or after you run a plate. To set parameters before you run a plate, see Chapter 6, “Select analysis settings and thresholds”.

Start a run

In the **Experiment Menu**, select **Setup**, select any screen, then click **START RUN** at the top-right corner.

Alternatively, click **Run**, select any screen, then carefully click **START RUN** at the top-left corner.

The green **START RUN** button becomes a red **STOP RUN** button, and the run begins.

Note: If you double-click the **START RUN** button, it may not become a **STOP RUN** button, but the run proceeds normally.

Stop a run

When you start a run, the green **START RUN** button becomes a red **STOP RUN** button.

In the **Experiment Menu**, select **Setup**, select any screen, then click **STOP RUN** at the top-right corner.

Alternatively, click **Run**, select any screen, then carefully click **STOP RUN** at the top-left corner.

The run immediately stops.

(7500 system only) Monitor a run

Note: This procedure applies only to the 7500 Real-Time PCR Instrument. It does not apply to the QuantStudio™ 5 Real-Time PCR Instrument.

During a run, you can access the amplification plot, temperature plot, and run method.

In the **Experiment Menu**, select **Run**, then click the data to view.

- **Amplification Plot**—Displays amplification plots of reactions
- **Temperature Plot**—Displays temperature plots of reactions
- **Run Method**—Displays the run method and allows you to edit the run method during the run

Save the results

After a run is complete, the HID Real-Time PCR Analysis Software automatically performs analysis and saves the initial results file.

If you modify the plate (for example, if you remove a well from analysis and reanalyze the results), the software does not automatically save the changes. After reanalysis, the HID Real-Time PCR Analysis Software prompts you to save the results.

After the run, see Chapter 7, “Enhance data analysis” to view and manage the results.

6 Select analysis settings and thresholds

IMPORTANT! All default settings shown in this guide and in the software screens are for illustration only. For your experiments, set the parameters and thresholds according to your laboratory protocol.

Before analyzing data from a completed run, you can edit values for the analysis parameters:

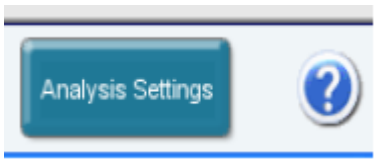
- C_T threshold, baseline start cycle, and end cycle
- HID flag thresholds
- QC flag thresholds

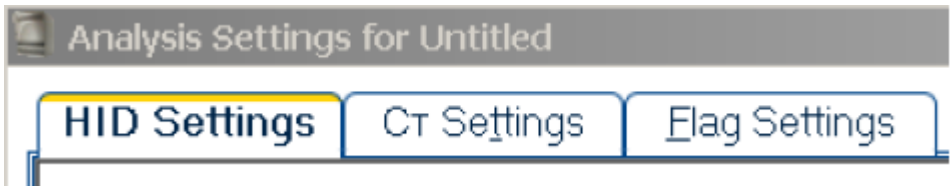
The **Analysis Settings** screen also contains the area where you set the parameters for the Dilution Calculation tool that is used to calculate a dilution scheme for downstream amplification. For more information about the **Dilution Scheme** pane, see “Edit dilution settings for individual samples” on page 66.

Open analysis settings

1. In the **Experiment Menu**, select **Analysis**, then select any one of the data displays.
   - **Amplification Plot**
   - **Standard Curve**
   - **Virtual Standard Curve**
   - **Multicomponent Plot**
   - **Raw Data Plot**
   - **QC Summary**

2. Click **Analysis Settings** at the top-right corner of the screen to open the **Analysis Settings** dialog box.





View and edit C_T settings

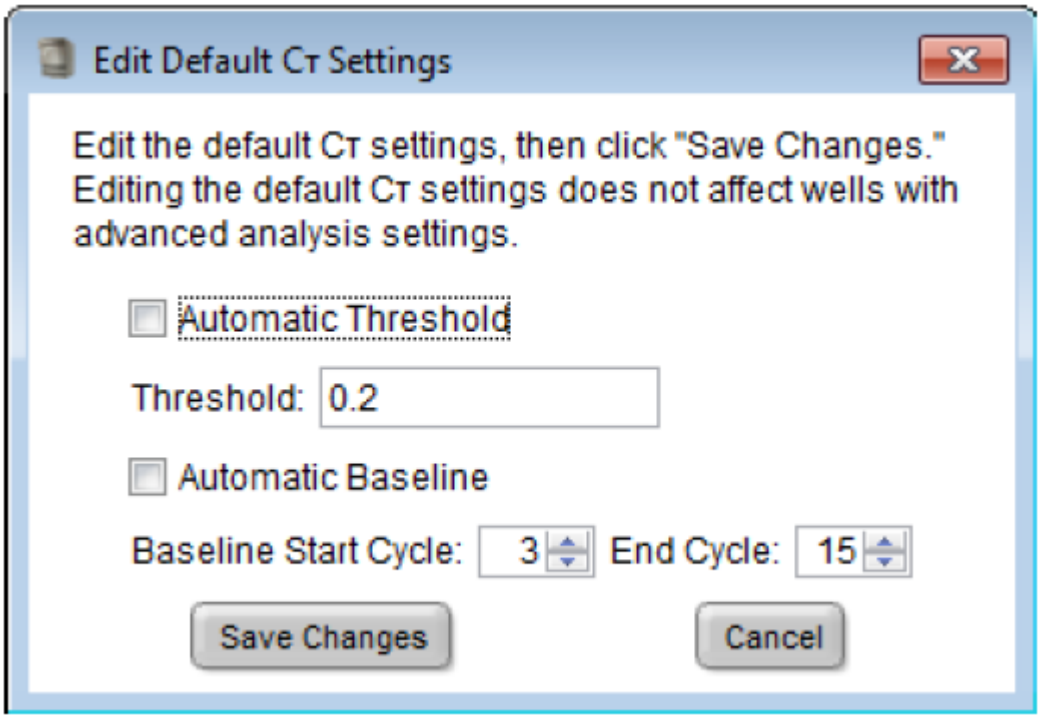
Note: The recommended C_T settings for each Quantifiler™ kit are included in the experiment templates provided with the software and in the kit user guides. The recommended settings are those that were used in the validation experiments performed for each kit by Thermo Fisher Scientific.

1. Select the **C_T Settings** tab to view the settings for C_T.

   The default system settings are:

   - Manual C_T Threshold = 0.2
   - Manual Baseline Start Cycle = 3
   - Manual Baseline End Cycle = 15

2. To change the settings, click **Edit Default Settings**, enter the new values, then click **Save Changes**.



3. To analyze the data with new settings, click **Apply Analysis Settings** at the bottom of the **Analysis Settings** dialog box.

Enter HID settings

1. Select the **HID Settings** tab to view the **Dilution Scheme**, **HID Flags**, and **HID Flag Settings** panes.

   For more information about settings in the **Dilution Scheme** pane, see “Edit dilution settings for individual samples” on page 66.

2. In the **Use** column in the **HID Flags** pane, select the checkbox for each flag that you want to include in the analysis.

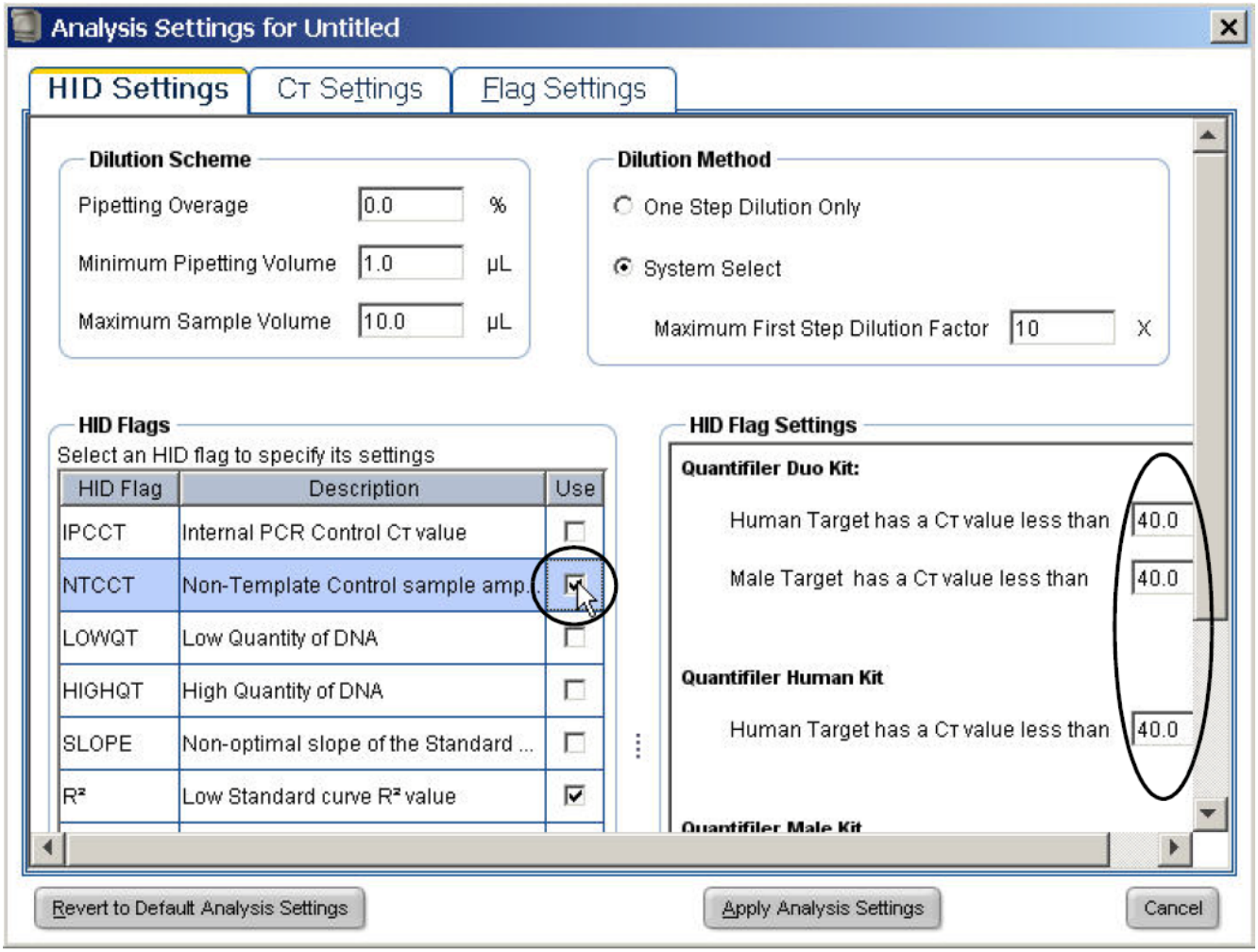
   You can use a flag to identify quality issues and help to interpret results for wells. Flags can indicate samples that may require further attention. You can exclude wells from data analysis (see “Omit wells from analysis” on page 56).

3. Enter threshold settings for the flags that you select.
   a. In the **HID Flags** pane, select the flag of interest.
   b. In the **HID Flag Settings** pane, enter in the corresponding fields the values that you want to use.

Repeat steps 2 on page 44 and 3 on page 44 until you enter settings (or view the default settings), for all the flags that you select.

Note: To save your HID flag settings for future use, save the experiment as a template before you start the run (see "Start or stop the run" on page 40).

4. To analyze the data with new settings, click **Apply Analysis Settings** at the bottom of the **Analysis Settings** dialog box.



HIGHQT

The HIGHQT flag indicates that the quantity, or mean quantity of sample replicates, is above a threshold that you set.

IPCCT

The IPCCT flag indicates one of the following:

Well contents	Cause	Comment
Unknown sample	The IPC (Internal PCR Control) C_T value is greater than the average of the IPC C_T values for all the standards plus the threshold that you set.	We strongly recommend that you base the threshold setting on validation data produced by your laboratory. For information on interpreting the IPCCT flag for Quantifiler™ kit experiments, see your kit user guide.
Standard or NTC	The IPC (Internal PCR Control) C_T value is above the maximum or below the minimum that you set.	In Quantifiler™ kit experiments, the IPC target amplification should be within an expected range. Low or no IPC amplification can indicate the presence of PCR inhibitors, incorrect experiment setup, or reagent or instrument failure.

LOWQT

The LOWQT flag indicates that the quantity, or mean quantity of sample replicates, is below a threshold that you set.

NTCCT

The NTCCT flag refers to the C_T value of the NTC (non-template control). No amplification of human and/or male targets should occur in NTC wells.

MTFR flag and M:F ratio display

The MTFR (Male to Female Ratio) is expressed as 1:X. A well is flagged if X is greater than the threshold that you set. For example, if you set the MTFR flag threshold at 1:10, then a sample that contains 5 ng/µL of male DNA and >55 ng/µL of human DNA generates an MTFR flag. The flag for this condition is a yellow triangle (⚠) in the **Plate Layout** or **Well Table** tab, and a red octagon (🛑) in the **Analysis Summary** pane (see Chapter 7, “Enhance data analysis”.

Samples that generate the MTFR flag are labeled **Thresholds Not Met** in the **Analysis Summary** pane of the **QC Summary** tab. The MTFR flag indicates samples that might require Yfiler™ kit amplification because of low quantities of male DNA relative to female DNA. Autosomal amplification of these samples may result in partial to no profile for the secondary (male) contributor.

In contrast, the M:F ratio display does not have an associated flag. The M:F ratio is also expressed as 1:X and is displayed in the **M:F ratio** column of the well table only if X is greater than or equal to the threshold that you set for the M:F ratio display.

The M:F ratio display threshold is expressed as 1:X where X must be less than or equal to the X value for the MTFR flag. For example, if you set the M:F ratio display to 1:1, then the MTFR flag must be set to 1:>1. Samples with ratios greater than the MTFR flag display the MTFR flag and display the

calculated M:F ratio. The M:F Ratio Display function alerts you to male and female mixtures before STR analysis.

Table 3 Results of example M:F and MTFR settings

Male DNA	Female DNA	Male:Female ratio	HID setting		M:F ratio display?	MTFR flag?
			M:F Ratio display (1:X) X =	MTFR flag (1:X) X =		
1 ng/µL	1 ng/µL	1:1	1	1	Yes	No
1 ng/µL	2 ng/µL	1:2	1	1	Yes	Yes
1 ng/µL	1 ng/µL	1:1	1	2	Yes	No

SLOPE

The SLOPE flag indicates the PCR amplification efficiency for the experiment. The amplification efficiency is calculated using the slope of the regression line in the standard curve. The standard wells are flagged if the slope is not between the minimum and maximum values that you set.

The standard curve is derived from a serial dilution set of standards that contain a range of known quantities. Amplification results for these standards are used to generate a curve.

A slope of –3.3 indicates 100% amplification efficiency. For more information on the standard curve and slope, see the following kit user guides:

- *Quantifiler™ Human DNA Quantification Kit and Y Human Male DNA Quantification Kit User Guide*
- *Quantifiler™ Duo DNA Quantification Kit User Guide*

R^2

The R^2 flag indicates the regression coefficient calculated from the regression line of the standard curve. The R^2 value indicates the closeness of fit between the standard curve regression line and individual C_T data points from the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points.

YINT

The Y-intercept value of the standard curve indicates the expected C_T value for a sample with a quantity of 1 (for example, 1 ng/µL). The YINT flag can assist in evaluating standard performance and serial dilution preparation. Your laboratory can perform validation studies to determine a range for the Y-intercept. Additionally, you can set the HID flag values for each Quantifiler™ kit and the HID flag values for each target (human and male) in the Quantifiler™ Duo assay. A YINT flag may indicate incorrectly prepared standard concentrations, degraded standard, or other preparation errors.

Enter flag settings

1. Select the **Flag Settings** tab to view and define instrument, sample, and data collection flags. Flags not used in the analysis are gray. Table 4 explains the flags.
2. In the **Use** column, select each flag that you want to include in the analysis.
3. Specify the conditions that generate a flag: In the **Condition** column dropdown lists, select conditions (< > =), then enter the corresponding values in the **Value** column.
4. To omit the wells that have a flag from the analysis, select the corresponding **Reject Well** checkboxes.
5. To analyze the data with new settings, click **Apply Analysis Settings**.

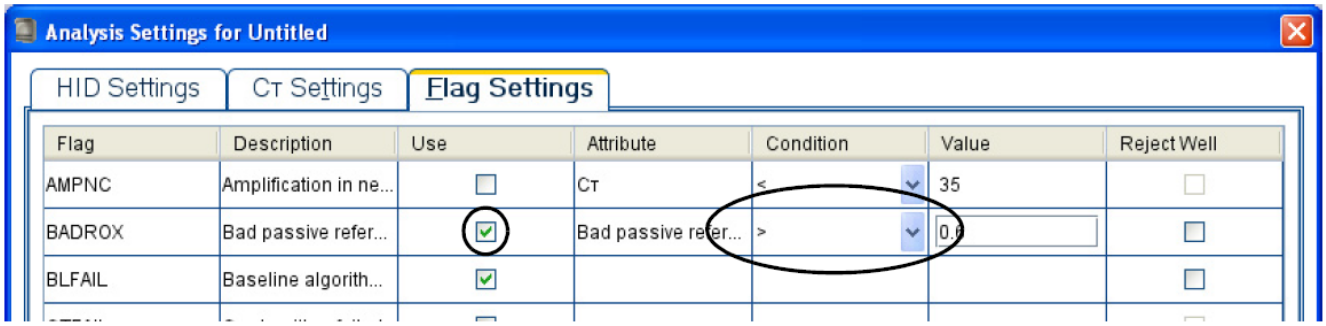


Table 4 QC flags

Flag	Description
AMPNC	Amplification in non-template control
BADROX	Bad passive reference signal
BLFAIL	Baseline algorithm failed
CTFAIL	C_T algorithm failed
DRNMIN	Define acceptable delta Rn based on Ct range
EXPFAIL	Exponential algorithm failed
OFFSCALE	Fluorescence is offscale
HIGHSD	High standard deviation in replicate group
PRFLOW	Low passive reference signal
NOAMP	No amplification
NOISE	Noise higher than others in plate
SPIKE	Noise spikes
NOSIGNAL	No signal in well

Table 4 QC flags *(continued)*

Flag	Description
OUTLIERRG	Outlier in replicate group
PRFDROP	Passive reference signal changes near C_T
THOLDFAIL	Thresholding algorithm failed

Add a virtual standard curve to the experiment

If you are using a virtual standard curve to analyze experiments, use the software to create the virtual standard curve, then assign it to your experiments as needed or export it for further use.

Guidelines for using virtual standard curves

- The software will *not* analyze an experiment using a virtual standard curve if:
  - The plate layout of the experiment contains wells that are configured with the **Standard** task type.
  - The expiration date specified for the virtual standard curve has expired.
  - The Quantifiler™ kit specified for the experiment and the curve do not match.
- When analyzing an experiment using a virtual standard curve, all **Unknown** samples generate the IPCCT (Internal PCR Control C_T) flag by default.
- Laboratories should perform internal validation studies to ensure that implementation of a virtual standard curve is appropriate and generates reliable downstream data. For optimal results, virtual standard curves should be evaluated independently for each real-time PCR instrument. We recommend the re-evaluation of virtual standard curves with each new lot of quantification kit.

Create a virtual standard curve

IMPORTANT! To create the virtual standard curve, you must know the slopes and y-intercepts of the targets for the kit that you are using.

1. In the **Experiment Menu**, select **Analysis ▸ Virtual Standard Curve**.
2. Click **Add Standard Curve to Experiment** in the top-left corner to open the **Virtual Standard Curve Library** dialog box.
3. Click **New** to create a new virtual standard curve.
4. Specify the settings for the virtual standard curve.
   a. Enter a name for the curve.
   b. *(Optional)* Select the **Is Standard Curve Default** checkbox to analyze all new experiments of the same selected kit type (see substep 4d on page 50) using the virtual curve.

c. Select the date on which the curve expires. When the curve expires, the software can no longer use it to analyze data.

d. Select the kit to which the virtual standard curve applies.

e. Enter the slope and y-intercept for each target of the selected kit.

f. Enter any comments for the virtual standard curve, then click **OK** to save it to the library.

Apply a virtual standard curve to an experiment

Before you apply the curve

The software cannot use a virtual standard curve to analyze an experiment that already contains wells that are assigned the **Standard** task. Therefore, before applying a virtual standard curve, you *must* omit or reassign the task of any **Standard** well on the plate layout.

Option	See
Omit wells from the analysis	"Omit wells from analysis" on page 56
Reassign the task assignment of a well	"Assign samples, standards, and NTCs to wells" on page 32

Apply a standard curve

IMPORTANT! Before you apply a virtual standard curve, you *must* omit or reassign the task of any **Standard** well on the plate layout.

To apply a virtual standard curve to the open experiment:

1. In the **Experiment Menu**, select **Analysis** ▸ **Virtual Standard Curve**.
2. Click **Add Standard Curve to Experiment** in the top-left corner of the screen.
3. In the **Virtual Standard Curve Library** dialog box, click **Add selected Virtual Standard Curve**.

Note: When analyzing an experiment using a virtual standard curve, all **Unknown** samples generate the IPCCT (Internal PCR Control C_T) flag by default.

Automatic analysis using a default virtual standard curve

If you select the **Is Standard Curve Default** checkbox in the settings of a virtual standard curve, the software automatically analyzes all new experiments using that default virtual standard curve unless:

- The plate layout of a new experiment contains wells that are configured as standards.
- The expiration date specified for the virtual standard curve has passed.

- You select the **Is Standard Curve Default** checkbox for another virtual standard curve (or the option is deselected for the existing virtual standard curve).

7 Enhance data analysis

View the analysis results

Flagged wells

To view the results of the data analysis:

1. In the **Experiment Menu**, select **Analysis ▸ QC Summary** to open the **QC Summary** screen.
2. Select the **Analysis Summary** tab.

   The tab displays the HID-specific flags that you selected to include in the data analysis and indicates the number of wells that meet or do not meet the threshold that you set. The symbols in the **Analysis Summary** tab are described in Table 5.

   Note: If the screen resolution is not set to 1280 × 1024, the **Analysis Summary** tab may not be properly displayed.

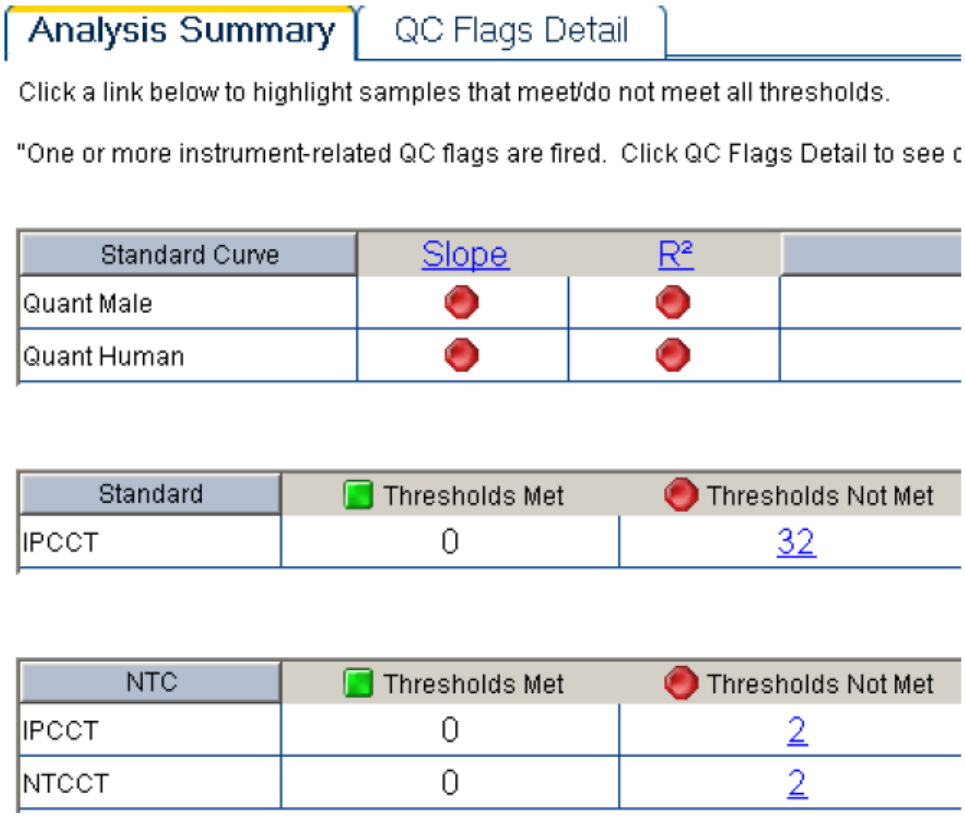


Table 5 Symbols in the Analysis Summary tab

Location	Symbol	Description
Standard Curve bar	Green square ( )	A value for Slope, R2, or Y-Intercept meets the threshold
	Red octagon ( )	A value for Slope, R2, or Y-Intercept does not meet the threshold
Thresholds Met columns	Hyperlinked numbers	The number of wells that meet the thresholds for a flag value
Thresholds Not Met columns	Hyperlinked numbers	The number of wells that do not meet the thresholds for a flag value

Standard Curve bar

The **Standard Curve** bar contains the SLOPE, R^2, and Y-Intercept flags. Click the column heading for a red octagon ( ) to highlight in the plate layout the wells represented in the standard curve. This graphical view simplifies the identification of wells that require further analysis using your laboratory protocol.

Standard bar

The **Standard** bar reports the IPCCT flags for all the wells on the plate that you designated as sample type **Standard**. Click the number in the **Thresholds Not Met** column to view the wells that do not meet the IPCCT threshold in the plate layout or well table format. You can use the amplification, multi-component, or raw data plots to troubleshoot the data for these wells. You can examine the wells that meet the threshold by clicking the number in the **Thresholds Met** column.

NTC bar

The **NTC** bar reports the IPCCT and NTCCT flags for all the wells on the plate that you designated as sample type **NTC** (non-template control). Click the number in the **Thresholds Not Met** column to view the wells that do not meet the IPCCT or NTCCT threshold in the plate layout or well table format. You can use the amplification, multi-component, or raw data plots to troubleshoot the data for these wells. You can examine the wells that meet the threshold by clicking the number in the **Thresholds Met** column.

Unknown bar

The **Unknown** bar reports the IPCCT, HIGHQT, LOWQT, and MTFR flags for all the wells on the plate that you designated as sample type **Unknown**. The HIGHQT, LOWQT, and MTFR (Male to Female Ratio) flags indicate that the quantity of DNA or ratios of male to female DNA in **Unknown** samples may require additional attention. Numbers below the flag indicate the number of wells that do not meet the threshold.

Note: The MTFR flag is not available in Human or HP™ kit experiments.

Click the number in the **Thresholds Not Met** column to view the wells that do not meet a threshold in the plate layout or well table format. You can use the amplification, multi-component, or raw data plots to troubleshoot the data for these wells. You can examine the wells that meet the threshold by clicking the number in the **Thresholds Met** column.

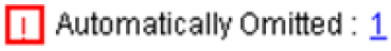
Instrument-related flags

A message may be displayed to indicate that one or more of the instrument-related flags is generated by a potential problem with the instrument. The message prompts you to select the **QC Flags Details** tab to view the flags.

Wells automatically omitted

In certain rare instances, such as assignment of targets to empty wells, the HID Real-Time PCR Analysis Software may automatically omit wells of a Quantifiler™ kit run.

The software automatically omits wells that may prevent the completion of data analysis, so that analysis can continue for the rest of the wells in the plate. These wells are indicated by a red exclamation point above the **Analysis Summary** tables. You can examine the automatically omitted wells by clicking the number next to the exclamation point.



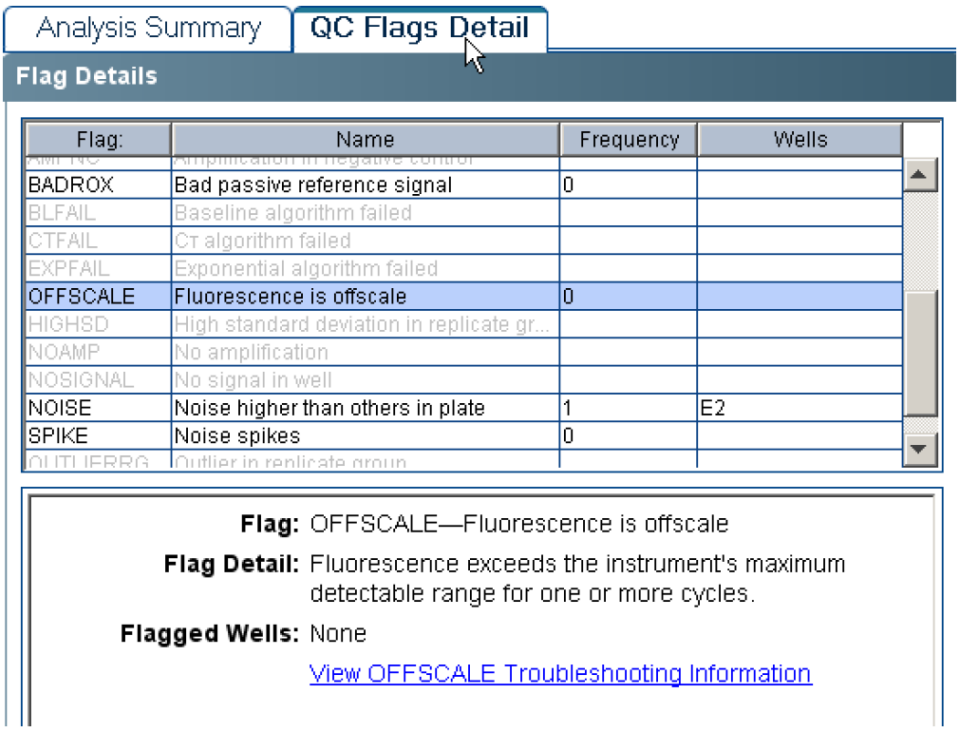
Interpret QC flag information

1. In the **QC Summary** screen, click the **QC Flags Detail** tab to view all QC flags (both general and HID).

2. Click a flag to select all affected wells in the plate layout, and to open a brief description of the flag and wells.

3. *(Optional)* In the **QC Flags Details** description box, click the hyperlink to open the software help system.

   The software help system provides information for troubleshooting the flag and the criteria used for analysis. (For more information about the flags, see Chapter 6, “Select analysis settings and thresholds”.)



For information about how to view and edit sample information, see “Change the appearance of a plot” on page 58.

Omit wells from analysis

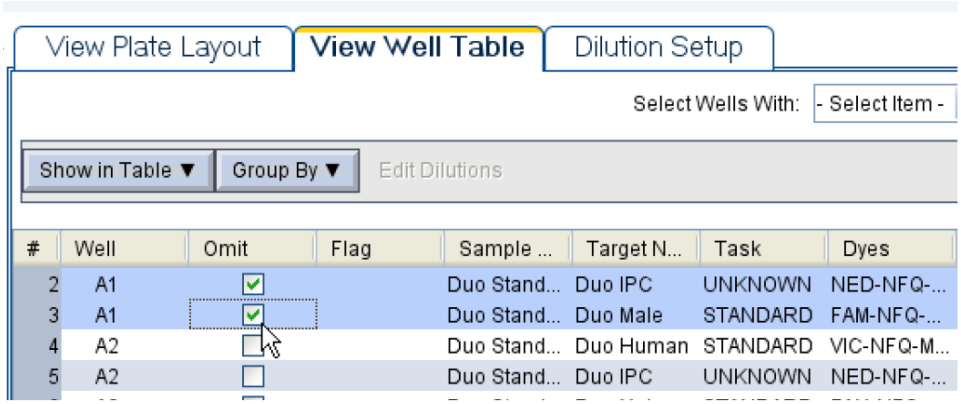
1. In the **Experiment Menu**, select **Analysis**.

2. To view data from individual wells, select any one of the plot screens.
   - **Amplification**—Amplification vs. cycle and amplification vs. well
   - **Standard curve**—C_T vs. quantity of standards, flagged samples, and unflagged samples
   - **Multicomponent plot**—Fluorescence vs. cycle of all reaction components
   - **Raw data plot**—Amplitude vs. filter
   - **Multiple plots view**—Amplification, standard curve, multicomponent, and raw data plots in one pane

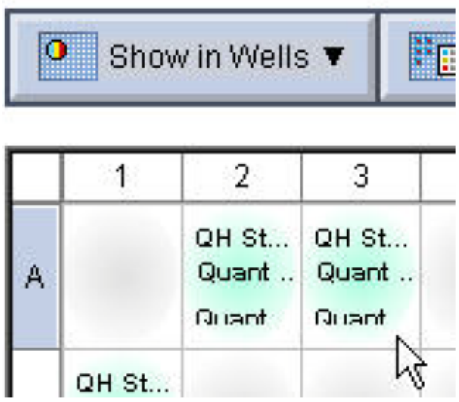
   Note: If no data are displayed, click **Analyze**.

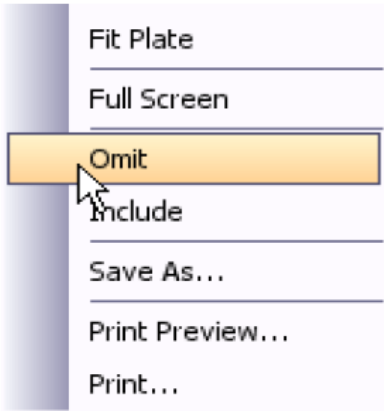
3. Omit wells using the well table or plate layout.
   - **Well table**—Select the **View Well Table** tab, then select the **Omit** checkbox for each well to exclude from the analysis.

     Note: If the **Omit Well** column is not visible in the table, click **Show in Table**, then select **Omit Well** to show the column.



   - **Plate layout**—Select the **View Plate Layout** tab. For each well to omit, right-click the well, then select **Omit** ▸ **Well**.





4. Click **Analyze** to reanalyze the experiment data with the omitted wells excluded from the analysis.
5. Review the data that are analyzed without the omitted wells.

Omit targets in an experiment well

For Duo, HP™, and Trio kit experiments, you can omit one of the standard targets in a well from analysis.

1. Right-click a well with a standard target that you want to omit.

   Note: You can omit only one target from one well at a time.

2. Select **Omit** from the dropdown list, then select a well or an individual target.
   - **Well**—Select a well to omit all targets from the well. The (well omitted) icon appears in the well.
   - **Individual target**—Select the name of the target (for example, **Duo Human**) to omit a specific target from the well. The individual target omitted icon (for example, for Duo Human omitted) appears in the well.
3. Click **Analyze** to reanalyze the experiment data with the omitted targets excluded from the analysis.

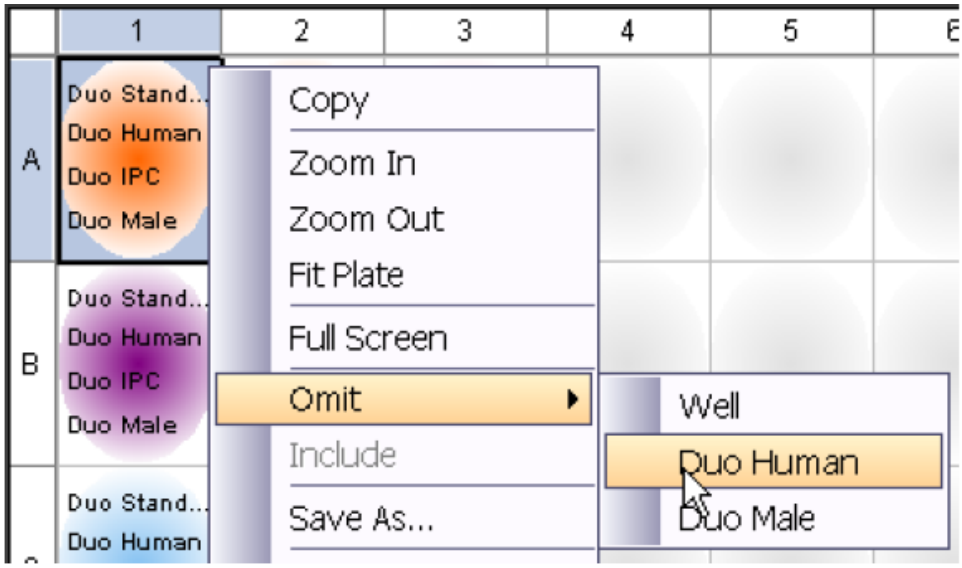


Figure 7 Example—Duo kit experiment

Examine the Degradation Index

The *Degradation Index* refers to the data observed when a sample may be degraded: a decrease in measured amount for large DNA fragments compared to small DNA fragments. While DNA degradation is not the only theoretically possible mechanism for a decrease in amount, it is the predominant mechanism in the absence of inhibitors. The Degradation Index is for use as a general indicator of whether large DNA fragments may perform more poorly in STR reactions. Evaluate the Degradation Index in conjunction with the IPC C_T.

The Degradation Index is automatically calculated by the HID Real-Time PCR Analysis Software using the following formula:

$$\frac{\text{Small autosomal target DNA conc. (ng/µL)}}{\text{Large autosomal target DNA conc. (ng/µL)}}$$

The Degradation Index value is displayed in the **Well Table** view in any of the analysis screens (you may have to scroll to the right to display it.)

See the *Quantifiler™ HP and Quantifiler™ Trio DNA Quantification Kits User Guide* for more information on:

- The Degradation Index
- Evaluating the HID Real-Time PCR Analysis Software quality indices to identify degraded samples

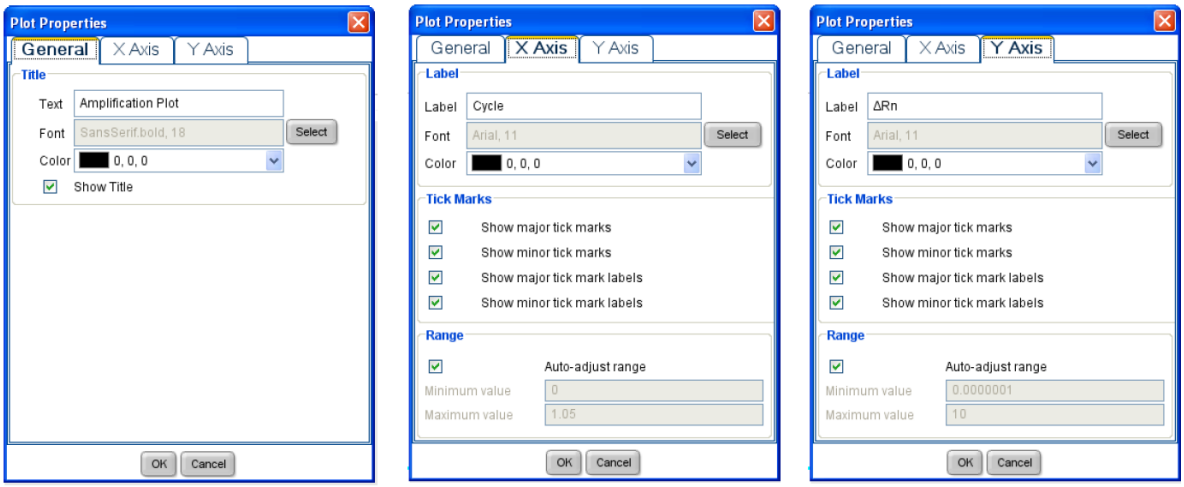
Change the appearance of, print, and save plots

Change the appearance of a plot

1. In the **Experiment Menu**, select **Analysis**, then select any one of the plot screens.
2. In the plot screen, locate the icon bar above the plot.

3. Click (**Hide**) to hide the plot legend.

4. To change the appearance of a plot, click (**Edit Plot Properties**) to open the **Plot Properties** dialog box. Three tabs are available.



5. Select the appropriate tab to enter the values that you want to use to plot the data.

6. Click **OK** to apply the changes.

Select the wells to include in the report

1. In the **Experiment Menu**, select **Analysis**, then select any one of the plot screens.

2. Select the wells to include in the report, using the **View Plate Layout** tab (see step 4 on page 32) or the **View Well Table** tab (see step 4 on page 35).

Print or save a plot

1. To print a plot, click (**Print**) .

2. To save a plot as a JPG file, click (**Save**).

Printed plots and JPG files include the slope, Y-intercept, and R^2.

8 Export and report results

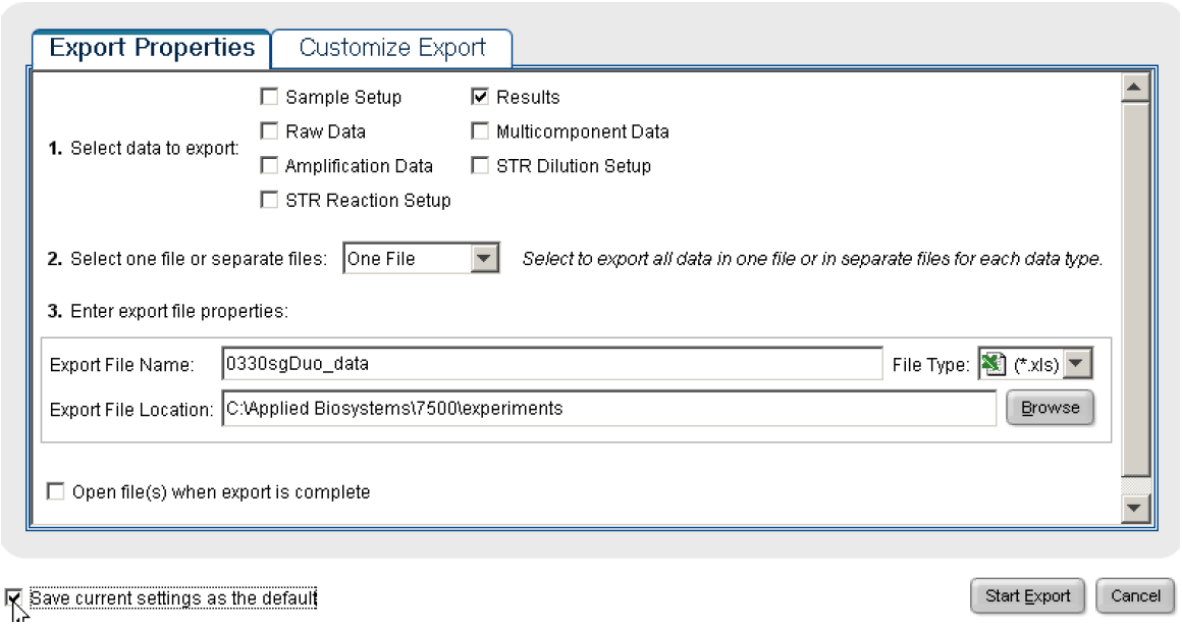
After the HID Real-Time PCR Analysis Software completes analysis and after you review the data, you can generate a customized report (PDF file), then save or print the report.

You can also export and save data in these formats:

- Excel™ (XLS)
- PowerPoint™ (PPT)
- Text (TXT)

Export data

1. In the **Experiment Menu**, select **Analysis**.
2. Select any one of the plot screens, then click **View Plate Layout** or **View Well Table**.
3. Highlight the wells to export.
4. In the toolbar, click (**Export**) to open the **Export Data** screen, then select the **Export Properties** tab.
5. Select one or more checkboxes for the type of data to export.
   - **Amplification Data**—Data that was collected during the cycling or amplification stage.
   - **Multicomponent Data**—Fluorescence data for each dye, for each cycle.
   - **Raw Data**—Raw fluorescence data for each filter, for each cycle.
   - **Results**—Results of the analysis.
   - **Sample Setup**—Setup information such as well, sample name, and sample color.
   - **STR Dilution Setup**—Sample dilution worksheet to prepare samples for amplification. For more information, see Chapter 9, “Generate dilution and reaction worksheets for STR setup”
   - **STR Reaction Setup**—STR reaction setup worksheet to prepare samples for amplification. For more information, see Chapter 9, “Generate dilution and reaction worksheets for STR setup”



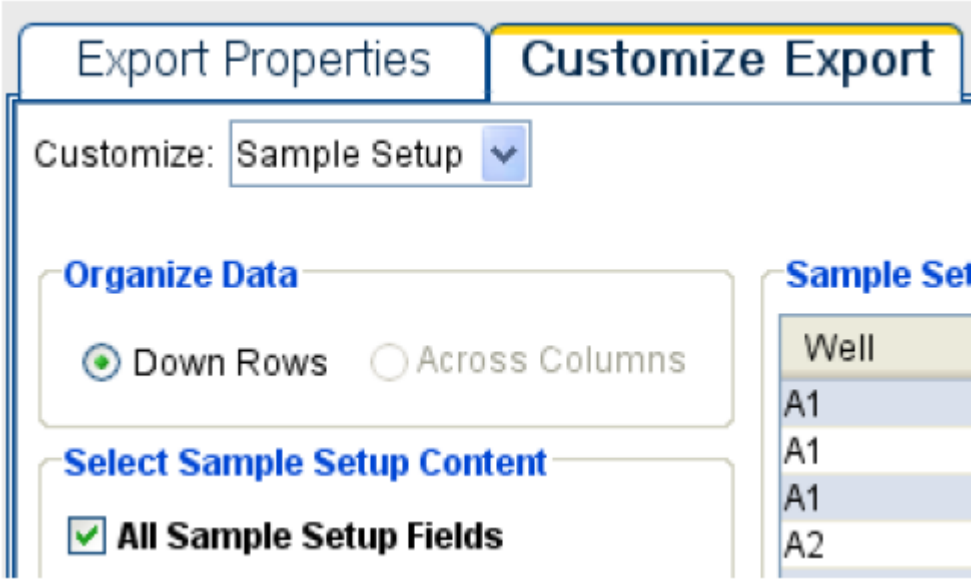
6. From the dropdown list, select **Separate Files** or **One File**.

7. Enter the export file properties.
   - **Export File Name**—Enter the name of the report.
   - **File Type**—Select the type of file. See the online help system for information on creating PPT slides.
   - **Export File Location**—Enter the filepath for the save location.

8. *(Optional)* Customize the data.
   a. Select the **Customize Export** tab.
   b. Select the information that you want to export.

   Note: Sample setup should be exported only as a TXT file.

c. To sort data in the export by column, click the column header (for example, click **Well** to sort the data by well).

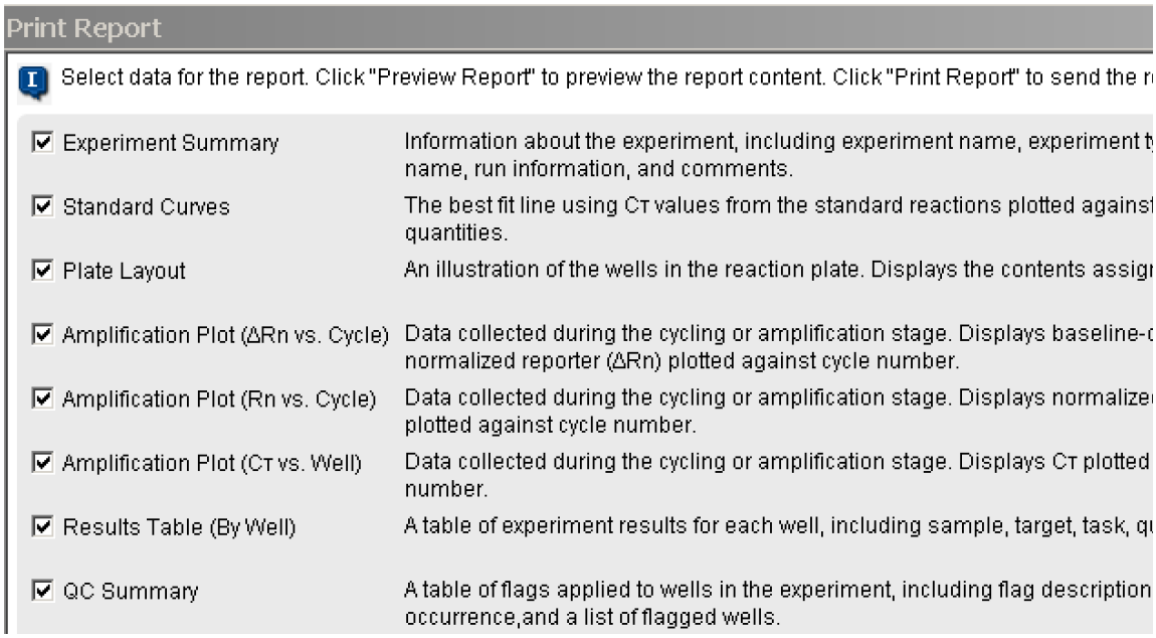


9. Click **Start Export** to export the data to the files that you selected.

Print a report

1. Select **Plate Setup** ▸ **Assign Targets and Samples**.
2. Click **View Plate Layout** or **View Well Table**.
3. Select the wells to include in the report.
4. In the toolbar, click (**Print Report**) to display the **Print Report** screen.
5. Select the checkbox for each data topic that you want to include in the report.

Note: Exported standard curves do not include unknown data points.



6. *(Optional)* Click **Print Preview** to preview the report content.
7. Select a print option.
8. Select **Save** to save the report, or select **Print** to print the report.

Note: If you do not enter a name in the **Experiment Name** field of the **Experiment Properties** screen, the experiment name on the report is “Untitled.”

---

9 Generate dilution and reaction worksheets for STR setup

After a run is complete, you can use the HID Real-Time PCR Analysis Software to generate dilution and reaction worksheets for STR setup.

The software generates dilution and reaction setup worksheets to perform calculations for the kits that you select from the STR Kit Library, and the kit information and default dilution settings that you specify.

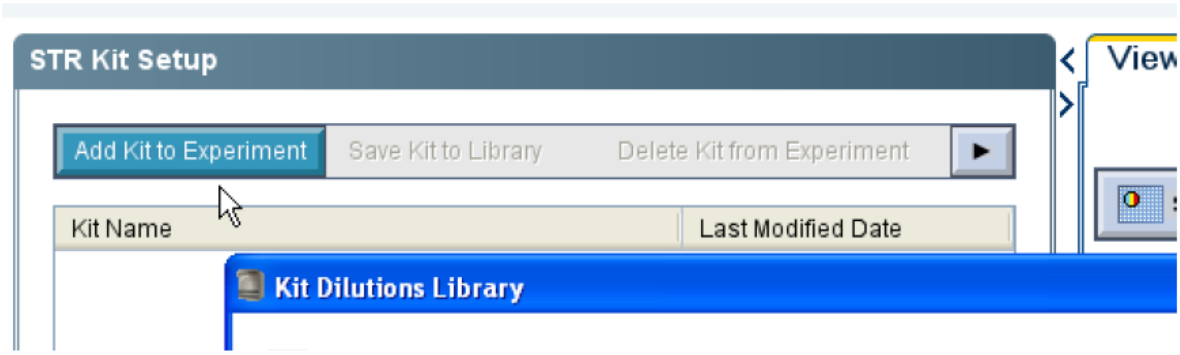
See Appendix D, “Configure STR library and default dilution settings” to:

- Enter, edit, or delete kit information in the STR Kit Library
- Set default dilution settings for the calculations

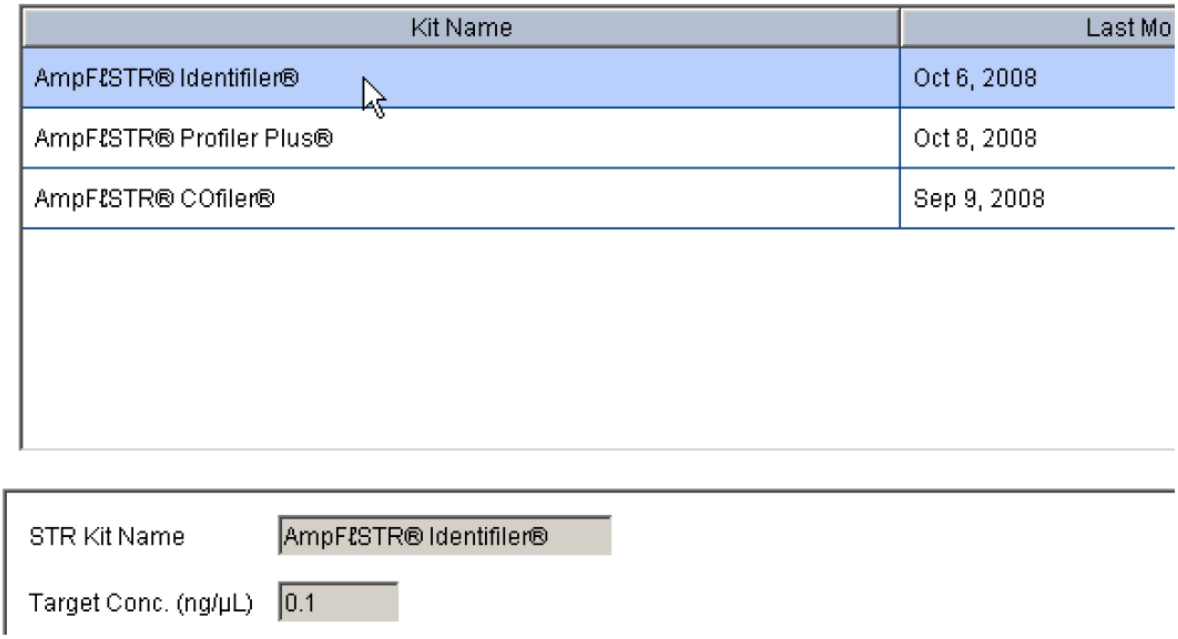
Add kits to an experiment

Before exporting worksheets, add kits to an experiment.

1. Open the experiment of interest.
2. In the **Experiment Menu**, select **Analysis ▸ STR Kit Setup**.
3. In the **STR Kit Setup** pane, click **Add Kit to Experiment** to open the **Kit Dilutions Library** screen.



4. Select the kits to use in the experiment. To edit kit information, see “Configure the STR Kit Library” on page 82.



5. Repeat steps 2 on page 64 through 4 on page 65 until you select all the kits to use in the experiment.

6. To delete a kit from the experiment (not from the Kit **Dilutions** Library), select the kit to delete, then click **Delete Kit from Experiment**.

Select unknown samples for amplification

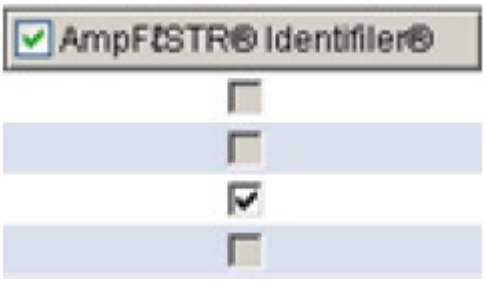
After adding kits to an experiment, select the unknown samples for amplification and associate samples with kits.

1. In the **Experiment Menu**, select **Analysis**, then select any analysis screen.

2. Select the **View Well Table** tab.

Note: If the well table does not display a column for the selected STR kit, click **Show in Table**, then select the kit name from the list of available columns.

3. Select the checkbox for the unknown sample to use and the STR kit with which to use the sample. If a sample is not for amplification (for example, a standard), it is not available for selection.



To select all of the samples for a kit, select the checkbox next to the kit name at the top of the column.

Note: The software automatically assigns the same kit for replicates.

4. Select the **Dilution Setup** tab to view the dilution scheme and the STR kits that you selected for each sample.

5. Repeat steps 2 on page 66 and 3 on page 66 for each unknown sample and kit.

Note: You cannot select an STR kit for standard or NTC sample types. Dilution calculations apply only to the unknown sample (Human or Male) target in the wells, not to standards or NTCs.

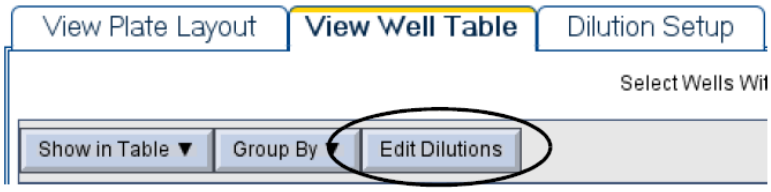
Edit dilution settings for individual samples

If needed, edit the default dilution settings for samples.

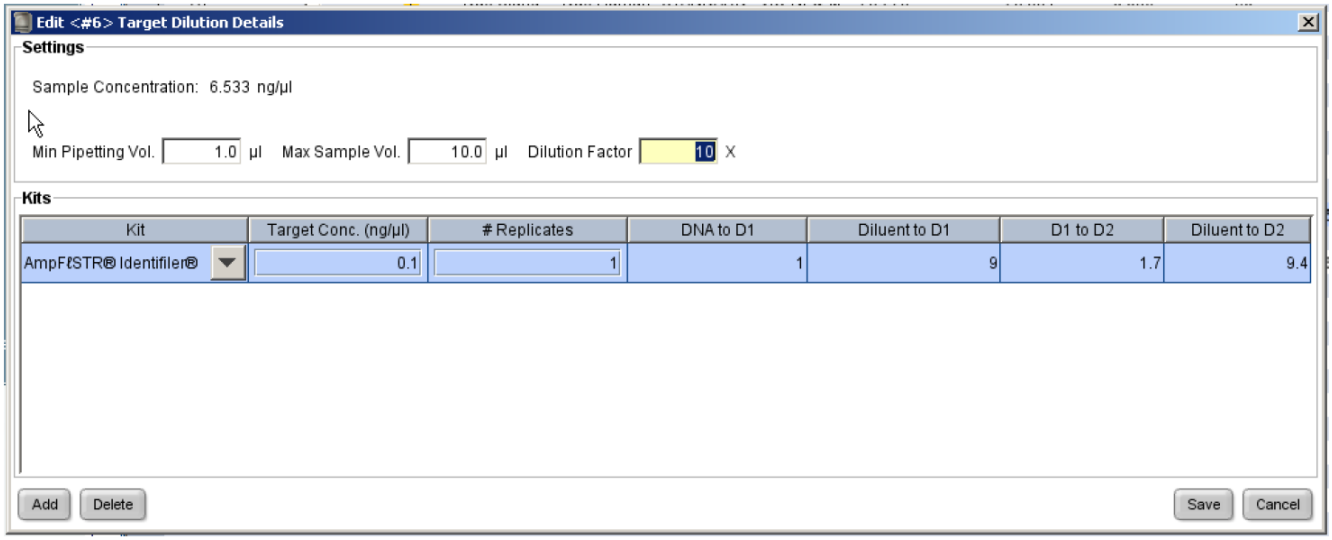
1. Select the **View Well Table** tab.

2. Select the sample of interest.

3. In the toolbar at the top of the well table, click **Edit Dilutions** to open the **Edit Target Dilution Details** screen.



4. View or edit the settings as needed.
   - **Min. Pipetting Vol.**—The minimum quantity to pipette.
   - **Max. Sample Vol.**—The maximum volume of available sample.
   - **Dilution Factor**—For example, enter 10 for 10-fold dilutions.
   - **Target Conc.**—The amount of target DNA that you want to use divided by the total sample volume per STR reaction.
   - **# Replicates**—The number of identical reactions.



Note: If you quantify replicates, the **Edit Target Dilution Details** screen displays the sample concentration or the mean sample concentration.

Note: The software displays target sample concentration based on maximum sample volume, number of replicates, sample volume per STR reaction, and pipetting overage that you set if the desired target concentration cannot be reached.

View the dilution scheme

View the dilution scheme to ensure that the settings are appropriate for your experiment.

1. In the **Experiment Menu**, select **Analysis**.
2. Click any plot to open a plot screen.
3. Select the **Dilution Setup** tab to open the **Dilution Setup** screen.
4. Review the settings for downstream reactions.

View Plate Layout | View Well Table | **Dilution Setup**

STR Kit	Sample Name	Quantity Mean	IPC Ct	STR Target Co...	STR Input Amount (ng)	DNA to D1	Diluent to D1	D1 to D2	Diluent to D2	# of STR Rxn.
AmpFℓSTR® Identifiler®	#6	6.53336906...	26.254...	0.1	1.00	1.0	64.3	10.0	0.0	1
AmpFℓSTR® Identifiler®	740	3.94344282...	26.926...	0.1	1.00	1.0	38.4	10.0	0.0	1
AmpFℓSTR® MiniFiler™	#6	6.53336906...	26.254...	0.1	0.50	1.0	129.7	10.0	0.0	1
AmpFℓSTR® MiniFiler™	740	3.94344282...	26.926...	0.1	0.50	1.0	77.9	10.0	0.0	1
AmpFℓSTR® Yfiler®	#6	6.62583065...	27.185...	0.1	1.00	1.0	65.3	10.0	0.0	1

Export dilution and reaction worksheets

Export the STR Dilution Setup worksheet and the STR Reaction Setup worksheet as described in “Export data” on page 60.

Save new STR kit information from an experiment into the STR kit library

You can save a kit from an experiment into the library (for example, if you import an experiment from a system with a different library setup).

Note: If the STR kit name that you are saving from the experiment is already listed in the library, rename or delete the kit from the library before saving the kit information from the experiment.

1. Open the experiment.
2. In the **STR Kit Setup** screen, select the kit to save.
3. Click **Save Kit to Library**.

A

Troubleshooting

Observation	Possible cause	Recommended action
File integrity warning **Details:** HID Real-Time PCR Analysis Software v1.4 may display a file integrity warning when opening an EDS file that was analyzed in earlier versions of the software.	There is a checksum calculation modification in v1.4.	No action required. The file can be used with no effect on the results obtained.
HID Real-Time PCR Analysis Software v1.4 unexpectedly closes when starting a 7500 instrument run	The instrument server terminated and the server no longer accepts commands.	Restart the 7500 instrument and the computer that is running the HID Real-Time PCR Analysis Software. If the behavior continues, we recommend that you disconnect the computer from the network during runs on the 7500 instrument. **Note:** We do not recommend using HID Real-Time PCR Analysis Software v1.4 with the 7500 Real-Time PCR System for Human Identification.

B

HID Real-Time PCR Analysis Software v1.3 validation

Overview of the software validation for v1.3

HID Real-Time PCR Analysis Software v1.3 is designed for the Quantifiler™ DNA Quantification Kits and the 7500 Real-Time PCR Instrument or the QuantStudio™ 5 Real-Time PCR Instrument with 0.2-mL 96-well sample block. The software enables streamlined quantification run setup, data analysis, and STR reaction setup by providing Quantifiler™-specific templates and quality flags as well as STR sample normalization (dilution) and reaction setup tools. HID Real-Time PCR Analysis Software v1.3 contains the same functionality as the v1.2 software in addition to new features that support the use of virtual standard curves and the use of the QuantStudio™ 5 Real-Time PCR System. For more information, see “Features in v1.3” on page 71.

This appendix describes the results of experiments that Thermo Fisher Scientific performed to validate HID Real-Time PCR Analysis Software v1.3. Data was collected using v1.2 and v1.3 of the HID Real-Time PCR Analysis Software, the 7500 Real-Time PCR System and the QuantStudio™ 5 System, and the Quantifiler™ HP™, Trio, Duo, and Human DNA Quantification Kits.

The data collected from the 7500 and QuantStudio™ 5 instruments were analyzed to verify the following:

- HID Real-Time PCR Analysis Software v1.3 performs as designed to analyze data generated on the 7500 Real-Time PCR System and the QuantStudio™ 5 Real-Time PCR System.
- The new features do not adversely affect the quantification assays or the software functionality carried over from the HID Real-Time PCR Analysis Software v1.2.
- When analyzed with HID Real-Time PCR Analysis Software v1.3, data generated using the 7500 Real-Time PCR System and the QuantStudio™ 5 Real-Time PCR System demonstrate reproducible performance for the respective instrument models.

For validation experiments and results for the Quantifiler™ Trio, Duo, HP™, and Human kits, see the *Quantifiler™ HP and Quantifiler™ Trio DNA Quantification Kits User Guide* (Pub. No. 4485354), the *Quantifiler™ Duo DNA Quantification Kit User Guide* (Pub. No. 4391294), and the *Quantifiler™ Human DNA Quantification Kit and Y Human Male DNA Quantification Kit User Guide* (Pub. No. 4344790).

Features in v1.3

HID Real-Time PCR Analysis Software v1.3 includes all of the v1.2 functionality and includes the following new features:

- Virtual Standard Curve support for Quantifiler™ HP™, Trio, Duo, and Human DNA Quantification Kits.
- Support for the QuantStudio™ 5 Real-Time PCR Instrument with 0.2-mL 96-Well Sample Block.

Materials and methods

Instrument, computer, and software configuration

Instruments:

- 7500 Real-Time PCR System, firmware v2.10 (3)
- QuantStudio™ 5 Real-Time PCR System, 0.2-mL 96-well sample block (3)

	Instrument 1	Instrument 2	Instrument 3	Instrument 4	Instrument 5	Instrument 6
Model	QuantStudio™ 5	QuantStudio™ 5	QuantStudio™ 5	7500	7500	7500
Computer	Laptop	Laptop	Desktop	Laptop	Laptop	Desktop
Microsoft™ OS	Windows™ 7 64-bit	Windows™ 7 64-bit	Windows™ 7 64-bit	Windows™ 7 64-bit	Windows™ 7 32-bit	Windows™ 7 32-bit
HID Real-Time PCR Analysis Software	v1.3	v1.3	v1.3	v1.2 and v1.3	v1.2	v1.2

Chemistries and consumables

Unless otherwise indicated, all materials are available through **thermofisher.com**. Catalog numbers that appear as links open the web pages for those products.

Item	Cat. No.
Quantifiler™ Trio DNA Quantification Kit, 14 kits (from single lot)	4482910
Quantifiler™ HP DNA Quantification Kit, 5 kits (from single lot)	4482911
Quantifiler™ Duo DNA Quantification Kit, 5 kits (from single lot)	4387746
Quantifiler™ Human DNA Quantification Kit, 5 kits (from single lot)	4343895
MicroAmp™ Optical 96-Well Reaction Plate, 10 plates	N8010560
Modified TE Buffer (10/0.1)	300675
MicroAmp™ Optical Adhesive Film, 100 covers	4311971
7500 Real-Time PCR Systems Spectral Calibration Kit I	4349180

(continued)

Item	Cat. No.
ABY™ Spectral Calibration Plate, 96-Well 0.2-mL	4461591
JUN™ Spectral Calibration Plate, 96-Well 0.2-mL	4461593
TaqMan™ RNase P Instrument Verification Plate for 7300/7500 Systems, 96-well	4350584
AmpFℓSTR™ DNA Control 007, Male	N/A
AmpFℓSTR™ Control DNA 9947A, Female	N/A

The following test cases were performed for each chemistry kit:

Quantifiler™ kit	Plates per instrument for the experiment				
	Precision and linearity	Accuracy and reproducibility	Sensitivity	Mixture	Inhibition
Trio Kit	3 plates	1 plate	1 plate	1 plate	1 plate
Duo Kit	3 plates	1 plate	1 plate	1 plate	1 plate
HP™ Kit	1 plate	1 plate	—	—	—
Human Kit	1 plate	1 plate	—	—	—

Samples

Experiment	Sample	Replicates per plate
Precision and linearity	Quantifiler™ Trio, Duo, HP™, and Human Kit DNA Standards	6 standard curve 12 dilution series
Accuracy and reproducibility	One male DNA, 1 ng/µL	96 replicates
Sensitivity	• **Quantifiler™ Trio Kit**—One male and one female DNA diluted to 100, 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng/µL • **Quantifiler™ Duo Kit**—One male DNA diluted to 50, 5, 0.5, 0.05, and 0.005 ng/µL	• 5 dilution series • 5 dilution series
Mixture analysis	• **Quantifiler™ Trio Kit**—One set of male/female DNA mixture at 1:0, 1:1, 1:10, 1:100, 1:1000, 1:4000, 0:1 ratios • **Quantifiler™ Duo Kit**—One set of male/female DNA mixture at 1:0, 1:1, 1:10, 1:100, 1:500, 1:1000, 0:1 ratios	• 3 mixture series • 3 mixture series
Inhibition	• **Quantifiler™ Trio Kit**—One male 007 DNA (0.1 ng/µL) with hematin (550 µM) • **Quantifiler™ Duo Kit**—One male 007 DNA (0.1 ng/µL) with hematin (80 µM)	• 96 replicates • 96 replicates

Data collection and analysis

- Run Method and analysis settings were configured as outlined in the respective Quantifiler™ kit user manuals. The design and execution of all workflows (including instrument calibration, run setup, data analysis, run methods, HID quality flags, dilution calculations import/export and reporting) were identical for both versions of the software.

Note: The 7500 Systems were calibrated as explained in the *Applied Biosystems™ 7500/7500 Fast Real-Time PCR System: Maintenance Guide* (Pub. No. 4387777).

Note: The QuantStudio™ 5 Systems were calibrated as explained in the *QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide* (Pub. No. MAN0010407). The instruments were calibrated for the ABY™-HID and JUN™-HID dyes using the ABY™ Dye Spectral Calibration Plate (Cat. No. 4461591) and the JUN™ Dye Spectral Calibration Plate (Cat. No. 4461593).

- Calibration and experiment (EDS) data files that were previously generated using HID Real-Time PCR Analysis Software v1.2 were imported and reanalyzed using HID Real-Time PCR Analysis Software v1.3. The results from v1.2 and v1.3 were then compared for differences in data output.

Experiments and results

Instrument performance

Test Case	Description	Passing Criteria	Results
Precision and linearity	Using each instrument, set up and run: • Three plates of the standard curve dilution series using the Quantifiler™ Trio and Duo Kits, standard curve dilution series. • One plate using the Quantifiler™ HP™ and Human Kits, standard curve dilution series. Produce a standard curve from each pair of dilution series, so that six curves are generated per plate on each instrument. Statistically evaluate the C_T and R^2 values for variation within an instrument.	For each system (7500 or QuantStudio™ 5): • When running standard curves using the Quantifiler™ Trio, HP™, DUO, and Human Assays, the C_T values shall have a coefficient of variation (CV) ≤20% within an instrument. • When running standard curves using the Quantifiler™ Trio, HP™, DUO, and Human Assays, the system shall have a standard curve R^2 value ≥0.98.	Pass
Accuracy and reproducibility	Using each instrument, set up and run one plate of 007 DNA with 1 ng/µL input using each Quantifiler™ kit, 96 replicates per plate. Statistically evaluate the C_T values and DNA quantity for variation within an instrument.	When running the Quantifiler™ Trio, HP™, DUO, and Human Assays, the within-instrument C_T and quantity values shall have a CV ≤20% within an instrument.	Pass

(continued)

Test Case	Description	Passing Criteria	Results
Sensitivity	Using each instrument, set up and run: • One plate containing seven dilutions of 007 and 9947a DNA (from 0.0001 ng/µL to 100 ng/µL) prepared using the Quantifiler™ Trio Kit. • One plate containing five dilutions of 007 DNA (from 0.005 ng/µL to 50 ng/µL) prepared using the Quantifiler™ Duo Kit. Run five dilution series per sample per plate, providing a total of five replicates for each sample dilution. Statistically evaluate the C_T values and DNA quantity data for variation within an instrument.	When running Quantifiler™ Trio Assays using 0.01 ng/µL to 10 ng/µL DNA input and when running Quantifiler™ Duo Assays using 0.05 ng/µL to 5 ng/µL DNA input, the in-plate C_T and quantity values shall have a CV ≤20% within an instrument.	Pass
Mixture analysis	Using each instrument, set up and run: • One set of male and female DNA mixture samples consisting of seven mixture ratios (1:0, 1:1, 1:10, 1:100, 1:1000, 1:4000, and 0:1) using the Quantifiler™ Trio Kit. • One set of male and female DNA mixture samples with seven mixture ratios (1:0, 1:1, 1:10, 1:100, 1:500, 1:1000, and 0:1) using the Quantifiler™ Duo Kit. Run three replicates for each mixture sample. Statistically evaluate the C_T values and DNA quantity data for variation within an instrument.	When running Quantifiler™ Trio and DUO Assays using mixtures where the male DNA input is 0.02 ng/µL and the female DNA input is between 0.02 and 20 ng/µL, the C_T and quantity values shall have a CV ≤20% within an instrument when results are within range on the standard curve.	Pass
Inhibition	Using each instrument, set up and run: • One plate of 0.1 ng/µL 007 DNA with 550 µM hematin using the Quantifiler™ Trio Kit. • One plate of 0.1 ng/µL 007 DNA with 80 µM hematin using the Quantifiler™ Duo Kit. Run 96 replicates per plate. Statistically evaluate the C_T values and quantity data for variation within an instrument.	When running Quantifiler™ Trio and DUO Assays, the generated C_T and quantity values shall have a CV ≤20% within an instrument.	Pass Exception conditions[1,2]

[1] When tested using the Quantifiler™ DUO Kit and the QuantStudio™ 5 Instrument, samples with extreme hematin concentrations (>40 µM) can produce a biphasic curve that may result in overestimation of DNA concentrations or quantity value CV significantly >20%.

[2] At extreme hematin concentrations (~550 µM), more variation (quantity value CV significantly >20%) may be observed in the large autosomal and Y targets on both 7500 and QuantStudio™ 5 Systems.

Software performance

Test case	Description	Passing criteria	Results
Custom standard curve	Using one 7500 System and one QuantStudio™ 5 System, set up a virtual standard curve using estimated slope and y-intercept values. Perform a Quantifiler™ Trio Kit run without standard curve samples.	The software shall successfully collect the data and automatically apply the virtual standard curve. The generated DNA quantities shall be 100% concordant with the manual calculation.	Pass
	Using three 7500 Systems and three QuantStudio™ 5 Systems, evaluate the virtual standard curve function using the sensitivity of a collected run. Create a virtual standard curve using the parameters (slope and y-intercept) of the standard curve generated from the run. Analyze the data using the actual standard curve and the virtual standard curve, then compare the DNA quantity values.	For each respective system (7500 or QuantStudio™ 5), analyzing the same experiment using the actual and virtual standard curves, the software shall calculate quantification values that match to the third decimal point.	Pass
Workflow and user interface	Using one 7500 System computer and one QuantStudio™ 5 System computer, confirm that the settings match for all run/analysis methods and flag thresholds.	When comparing the v1.2 and v1.3 software, all values for the run/analysis methods and flag thresholds shall be same.	Pass
	Using 7500 and QuantStudio™ 5 Systems, run Quantifiler™ Trio and HP™ assays by following the standard workflow and using the necessary software functions.	The software functions necessary for using the Quantifiler™ assays shall perform without error.	Pass
Backward compatibility	Use the v1.3 software to reanalyze an experiment (EDS) file generated using a 7500 System and the v1.2 software, then compare the quantification values calculated by both versions of the software.	When analyzing the EDS file using the v1.2 and v1.3 software, the calculated quantification values shall match to the third decimal point.	Pass

Conclusions

Based on the validation of HID Real-Time PCR Analysis Software v1.3 and the precision and linearity, accuracy and reproducibility, sensitivity, mixture analysis, and inhibition validation experiments performed using the Quantifiler™ Trio, Duo, HP™, and Human Kits [see the *Quantifiler™ HP and Quantifiler™ Trio DNA Quantification Kits User Guide* (Pub. No. 4485354), *Quantifiler™ Duo DNA Quantification Kit User Guide* (Pub. No. 4391294), and *Quantifiler™ Human DNA Quantification Kit and Y Human Male DNA Quantification Kit User Guide* (Pub. No. 4344790)]:

- All updates to v1.3 were successfully and correctly implemented without negative effects on functionality carried over to v1.3 from v1.2.
- HID Real-Time PCR Analysis Software v1.3 successfully controlled the 7500 Real-Time PCR Systems and QuantStudio™ 5 Real-Time PCR Systems, and reliably and reproducibly set up and collected quantification data using the Quantifiler™ kits.
- The software provided accurate results when used to process Quantifiler™ kits for the analysis of genomic DNA samples.
- The user interface and HID workflows in v1.3 software performed as expected for both instruments when using the Windows™ 7 operating systems.
- The coefficient of variation (CV) for the average values of DNA quantity data and standard curve parameters (C_T and R^2 values) where tested varied <20% within each instrument (7500 and QuantStudio™ 5 Systems) using HID Real-Time PCR Analysis Software v1.2 and v1.3.

Laboratories should determine the appropriate level of testing required before implementation, based on the nature of the changes made to the software, how the software pertains to their laboratory workflow, their internal software validation guidelines, and those of the appropriate governing agencies.

C

HID Real-Time PCR Analysis Software v1.4 verification

Objective of the software verification for v1.4

The objective of this verification is to confirm that the software modifications made in HID Real-Time PCR Analysis Software v1.4 function as expected when using the 7500 Real-Time PCR System for Human Identification, the QuantStudio™ 5 Real-Time PCR System running firmware v1.5.1, and the Quantifiler™ Trio, Duo, HP™, and Human kits.

Testing was performed according to *Quality Assurance Standards (QAS) for Forensic DNA Testing Laboratories* (July 1, 2020), standard 8.8.3.4. Functional, regression, and reliability testing was performed.

Functional testing

Functional tests were conducted to demonstrate that the software changes made in HID Real-Time PCR Analysis Software v1.4 do not affect performance when using the Quantifiler™ kits with the 7500 Real-Time PCR System and the QuantStudio™ 5 Real-Time PCR System.

Note: HID Real-Time PCR Analysis Software v1.4 may unexpectedly close when starting a 7500 instrument run. Therefore, we do not recommend using v1.4 with the 7500 instrument.

Materials and methods

Instrument, computer, and software configuration

Instruments:

- 7500 Real-Time PCR System for Human Identification (2)
- QuantStudio™ 5 Real-Time PCR System, 0.2-mL 96-well sample block (3)

	Instrument 1	Instrument 2	Instrument 3	Instrument 4	Instrument 5
Model	QuantStudio™ 5	QuantStudio™ 5	QuantStudio™ 5	7500	7500
Instrument firmware version	v1.5.1	v1.5.1	Upgraded from v1.3.3 to v1.5.1	v2.10	v2.10
Computer	Dell™ Precision 3551				
Image	LTSC 2021	LTSC 2019	LTSC 2019	LTSC 2021	LTSC 2019
Microsoft™ Windows™ OS	Windows™ 10, 64-bit operating system (IOT Enterprise)				
HID Real-Time PCR Analysis Software	v1.4				

Chemistries and consumables

Unless otherwise indicated, all materials are available through **thermofisher.com**. Catalog numbers that appear as links open the web pages for those products.

Item	Cat. No.
Quantifiler™ Trio DNA Quantification Kit, 1 kit	4482910
Quantifiler™ HP DNA Quantification Kit, 1 kit	4482911
Quantifiler™ Duo DNA Quantification Kit, 1 kit	4387746
Quantifiler™ Human DNA Quantification Kit, 1 kit	4343895
MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 20 plates	4306737
MicroAmp™ Optical Adhesive Film, 100 covers	4311971

(continued)

Item	Cat. No.
ABY™ Spectral Calibration Plate, 96-Well 0.2-mL	4461591
JUN™ Spectral Calibration Plate, 96-Well 0.2-mL	4461593
TaqMan™ RNase P Instrument Verification Plate, 96-Well 0.2-mL	4432382
AmpFℓSTR™ DNA Control 007, Male	4460779, 4478871
Teknova™ DNA Suspension Buffer, pH 8.0	T0227

Data collection and analysis

- Data was collected using HID Real-Time PCR Analysis Software v1.4, the 7500 Real-Time PCR System and the QuantStudio™ 5 Real-Time PCR System (firmware v1.5.1), and the Quantifiler™ Trio, Duo, HP™, and Human DNA Quantification Kits.
- Run Method and analysis settings were configured as outlined in the respective Quantifiler™ kit user manuals.

Results

Test case	Samples	Passing criteria	Result
Linearity	Quantifiler™ Trio, Duo, HP™, and Human Kit DNA standards: • 4 standard curve dilution series for each Quantifiler™ kit	Evaluate the R^2 and slope values for each standard curve: • R^2 value ≥0.985 for the Trio, HP™ and Duo kits; ≥0.980 for the Human kit • The slope values for each target are within the range observed during the developmental validation of each Quantifiler™ kit.	Pass
Accuracy and reproducibility	DNA Control 007, 1 ng: • 4 replicates for each Quantifiler™ kit	The 1 ng/µL 007 DNA concentration is within range 0.60–1.65 ng/µL	Pass
Contamination	DNA Dilution Buffer: • 4 replicates for each Quantifiler™ kit	An undetermined (UND) result	Pass

Regression testing

Regression testing was performed to confirm that the software changes made in HID Real-Time PCR Analysis Software v1.4 do not impact the overall software functionality. The test cases performed were designed to ensure that the features and functionalities of HID Real-Time PCR Analysis Software v1.4 work the same as in v1.3.x.

Materials

Instrument, computer, and software configuration

Instruments:

- 7500 Real-Time PCR System for Human Identification (1)
- QuantStudio™ 5 Real-Time PCR System, 0.2-mL 96-well sample block (2)

	Instrument 1	Instrument 2	Instrument 3
Model	QuantStudio™ 5	QuantStudio™ 5	7500
Instrument firmware version	v1.5.1	Upgraded from v1.3.3 to v1.5.1	v2.10
Computer[1]	Dell™ Precision 3551 and 3571		
Image[2]	LTSC 2019 and 2021		
Microsoft™ Windows™ OS	Windows™ 10, 64-bit operating system (IOT Enterprise)		
HID Real-Time PCR Analysis Software	v1.3.2 and v1.4		

[1] All configurations were tested across each instrument.
[2] All configurations were tested across each instrument.

Results

Test case	Description	Passing criteria	Result
Workflow and user interface	Confirm that the settings match for all run/analysis methods and flag thresholds for each Quantifiler™ kit.	When comparing HID Real-Time PCR Analysis Software v1.3.2 and v1.4, all values for the run/analysis methods and flag thresholds shall be same.	Pass
	Run each Quantifiler™ assay by following the standard workflow and using the necessary software functions.	The software features and functions shall perform without error for each of the Quantifiler™ kits tested.	Pass
Virtual standard curve	Create a virtual standard curve using estimated slope and y-intercept values. Execute a run for each Quantifiler™ kit without adding standards to the experiment.	The software shall successfully collect the data and automatically apply the virtual standard curve.	Pass
Backward compatibility	Use HID Real-Time PCR Analysis Software v1.4 to reanalyze an experiment (EDS) file generated using a 7500 and a QuantStudio™ 5 System with HID Real-Time PCR Analysis Software v1.3.2. Compare the quantification values calculated by both versions of the software.	The calculated quantification values in HID Real-Time PCR Analysis Software v1.4 should be concordant to those obtained in v1.3.2.	Pass

Reliability testing

Reliability testing was performed to evaluate the software performance within and beyond functional aspects. A variety of user scenarios were tested as presented in Table 6. Functional and non-functional tasks from instrument sign-in through uninstallation of the software performed as expected.

Table 6 Test cases used to evaluate the reliability of HID Real-Time PCR Analysis Software v1.4 with the 7500 and QuantStudio™ 5 Systems

Category	Test	Performed as Expected (Y/N)
Install/uninstall	The user can install HID Real-Time PCR Analysis Software v1.4 using a new or an upgrade registration code. The user is able to uninstall HID Real-Time PCR Analysis Software v1.4.	Y
Instrument connectivity	Connection of the Dell™ Precision 3551 and 3571 laptops running HID Real-Time PCR Analysis Software v1.4 to one 7500 System and two QuantStudio™ 5 Systems (firmware v1.5.1).	Y
Calibrations	7500 System calibrations migrated from the Dell™ Precision 3551 to the 3571.	Y
User profiles	The user can create a new profile within HID Real-Time PCR Analysis Software v1.4, then sign in to the software with this new user profile.	Y
Functional testing (One 7500 System and two QuantStudio™ 5 Systems)	The following functions were evaluated to ensure that software changes did not affect functionality: • Import a plate setup • Open files using **File ▸ Open**, or clicking the EDS or EDT file name • Save the EDS files after run completion (7500 and QuantStudio™ 5 Systems) • Export data to a CSV file • Export reports to a PDF file	Y

Conclusions

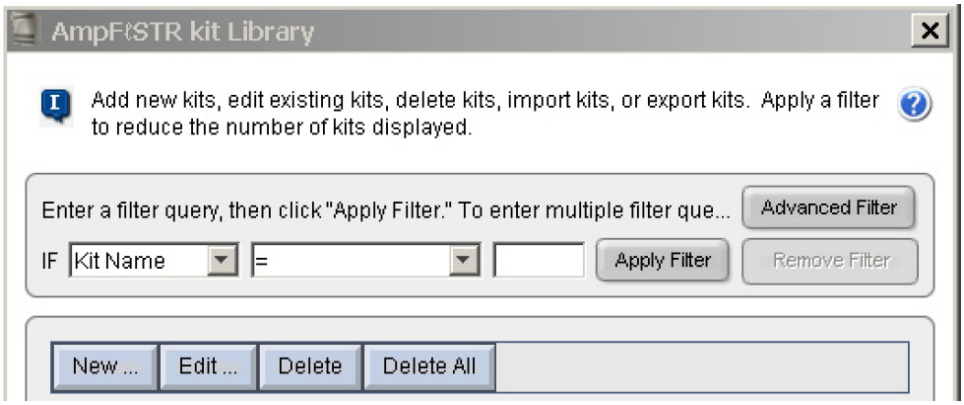
Based on this verification, we can conclude that all updates to HID Real-Time PCR Analysis Software v1.4 did not affect performance when using the Quantifiler™ kits and the 7500 Real-Time PCR System and QuantStudio™ 5 Real-Time PCR System (firmware v1.5.1). This version of software maintains the functionality and reliability of previous versions.

D Configure STR library and default dilution settings

Configure the STR Kit Library

Most AmpFℓSTR™ kits are listed in the STR Kit Library by default. To add or modify amplification kit information, perform the following steps.

1. In the toolbar, select **Tools ▸ AmpFℓSTR™ Kit Library** to open the **Kit Dilutions Library** screen.
2. Add, edit, and/or delete a kit.
   - **Add a kit**—Click **New**. The **Create New STR Kit** dialog box opens.
   - **Edit a kit**—Select the kit, then click **Edit**. The **Create New STR Kit** dialog box opens.
   - **Delete a kit**—Select the kit, then click **Delete**.



3. If you add or edit a kit, enter settings as needed.
   - **STR Kit Name**—The name of the kit that you are adding to the list.

     Note: Kit names must be unique. To use the same kit with different sample types or different input amounts of DNA, add the kit with a different name, such as Identifiler™_1.5 ng.

   - **Target Conc.**—The amount of DNA that you want to use divided by the total sample volume per reaction. Examples:

Total DNA (ng)	Volume/reaction (µL)	Target concentration (ng/µL)
0.5	10	0.05
1.0	10	0.1
1.0	20	0.05
2.0	20	0.1

   - **STR Reaction** pane
     - **PCR Master Mix**—Enter appropriate volumes (µL).
     - **Sample**—Enter appropriate volumes (µL).

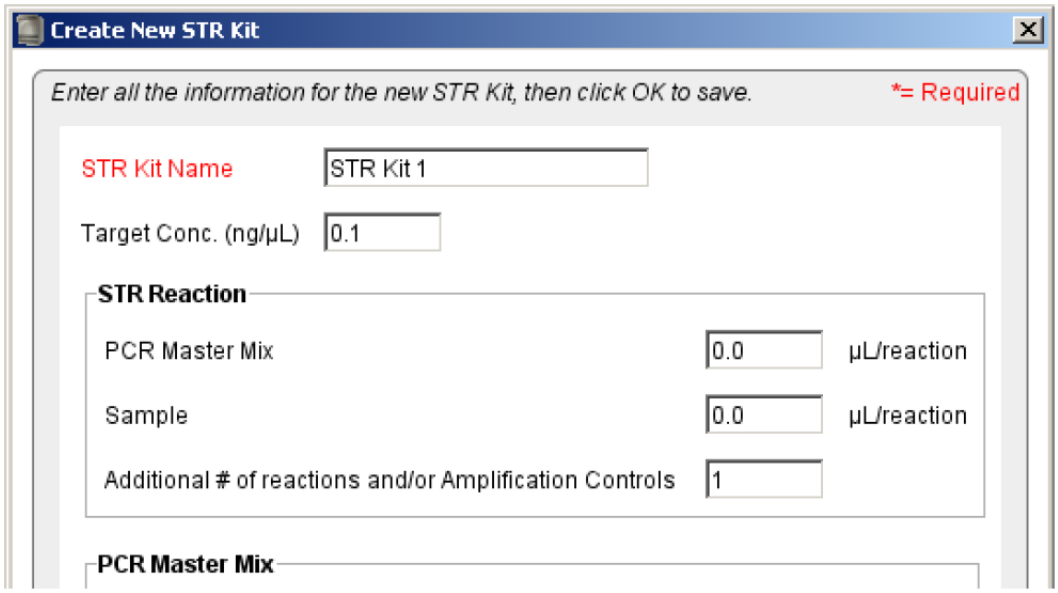
       The volumes of master mix and sample must equal the total volume of the STR reaction:

       ```
       Sample (µL) + PCR master mix (µL) = Reaction volume (µL)
       ```

     - **Additional # of Reactions and/or Amplification Controls**—Enter the number of additional STR reactions per amplifications to allow for pipetting overage.

       IMPORTANT! Because not all kits allow for pipetting overage, you may need to enter more additional reactions to compensate for volume losses that occur during pipetting. For information about pipetting overage, see the user guide for your kit.

   - **PCR Master Mix** pane—List each component of the STR reaction master mix. For more information, see the user guide for your kit.



4. Click **OK.**
5. Repeat steps 2 on page 82 through 4 on page 84 for all needed kits.
6. Verify that the kits to be used in the downstream STR reactions are listed, with correct information.

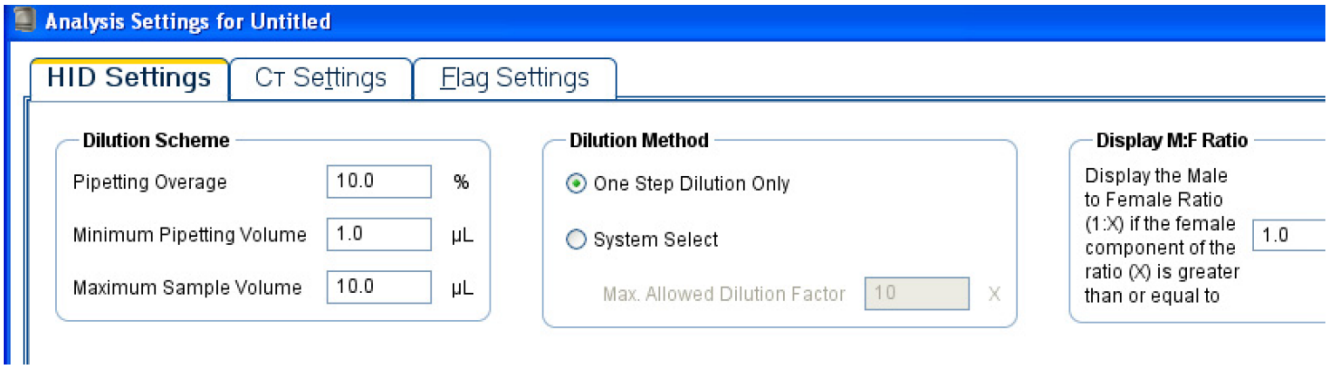
Note: You can also save a kit from an experiment into the library (for example, if you import an experiment from a system with a different library setup). See "Save new STR kit information from an experiment into the STR kit library" on page 68.

Set default dilution settings

In the **Analysis Settings** screen, you can specify default dilution settings to apply to all samples. (You can edit individual sample dilution settings after you associate an STR kit with an experiment.)

1. In the **Experiment Menu**, select any analysis screen, then click **Analysis Settings**.
2. Select the **HID Settings** tab.
3. In the **Dilution Scheme** pane, enter dilution scheme parameters according to your preferences or laboratory protocol.
   - **Pipetting overage**—The percent to add to compensate for error in pipetting. If the sample concentration is less than the target concentration and the sample volume is limited, set the pipetting overage to zero to maximize the amount of DNA in the STR reaction.
   - **Minimum Pipetting Volume**—The minimum volume that you want to pipette.
   - **Maximum Sample Volume**—The maximum quantity of sample that you want to use.
   - **Dilution Factor**—The maximum first dilution that you want to perform with the available DNA. For example, for 10-fold first dilutions, enter **10**.
4. In the **Dilution Method** pane, select one of the following methods.
   - **One Step Dilution Only**—To use a single dilution in all instances.
   - **System Select**—To use a dilution scheme that depends on your preferences, with a maximum of two dilutions.

   The software displays the target sample concentration based on the maximum sample volume, number of replicates, sample volume per STR reaction, and pipetting overage that you set if the desired target concentration cannot be reached.


Analysis Settings for Untitled
HID Settings
CT Settings
Flag Settings
Dilution Scheme
Pipetting Overage
10.0
%
Minimum Pipetting Volume
1.0
µL
Maximum Sample Volume
10.0
µL
Dilution Method
One Step Dilution Only
System Select
Max. Allowed Dilution Factor
10
X
Display M:F Ratio
Display the Male to Female Ratio (1:X) if the female component of the ratio (X) is greater than or equal to
1.0

Documentation and support

Related documentation

Table 7 Documents for HID Real-Time PCR Analysis Software experiments

Document	Pub. No.
Quantifiler™ HP and Quantifiler™ Trio DNA Quantification Kits User Guide	4485354
Quantifiler™ Duo DNA Quantification Kit User Guide	4391294
Quantifiler™ Human DNA Quantification Kit and Y Human Male DNA Quantification Kit User Guide	4344790
Applied Biosystems™ 7500/7500 Fast Real-Time PCR System Maintenance Guide	4412844
7500/7500 Fast Real-Time PCR System Getting Started Guide for Absolute Quantification Experiments	4378658
Applied Biosystems™ 7500/7500 Fast Real-Time PCR System Getting Started Guide: Standard Curve Experiments	4387779
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407
QuantStudio™ Design and Analysis Desktop Software User Guide	MAN0010408

Table 8 Documents for custom experiments

See the following documents for information on performing custom experiments instead of using the HID Real-Time PCR Analysis Software.

Document	Pub. No.
Applied Biosystems™ 7500/7500 Fast Real-Time PCR System Getting Started Guide: Genotyping Experiments	4387784
Applied Biosystems™ 7500/7500 Fast Real-Time PCR System Getting Started Guide: Presence/ Absence Experiments	4387785
Applied Biosystems™ 7500/7500 Fast Real-Time PCR System Getting Started Guide: Relative Standard Curve and Comparative C_t Experiments	4387783
Applied Biosystems™ 7500/7500 Fast Real-Time PCR System Getting Started Guide: Standard Curve Experiments	4387779
7500/7500 Fast Real-Time PCR System Getting Started Guide for Absolute Quantification Experiments	4378658

Customer and technical support

For support:

- **In North America**—Send an email to **HIDTechSupport@thermofisher.com**, or call **888-821-4443 option 1**.
- **Outside North America**—Contact your local support office.

For the latest services and support information for all locations, go to **thermofisher.com/support** to obtain the following information.

- Worldwide contact telephone numbers
- Product support
- Order and web support
- Safety Data Sheets (SDSs; also known as MSDSs)

Additional product documentation, including user guides and Certificates of Analysis, are available by contacting Customer Support.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.