appliedbiosystems

AccuSEQ™ Real-Time PCR Software v3.0

USER GUIDE

Windows™ 10 Operating System

for use with:
QuantStudio™ 5 Real-Time PCR Instrument
SEQ family of detection kits

Catalog Number A40303
Publication Number 100084348
Revision B

For Research Use Only. Not for use in diagnostic procedures.

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Revision history: Pub. No. 100084348

Revision	Date	Description
B	17 December 2019	Update to include the resDNASEQ™ Quantitative HEK293 DNA Kit (Cat. No. A46014).
A	9 September 2019	New user guide for AccuSEQ™ Real-Time PCR Software v3.0.

Contents

1 Product information

Product description

The Applied Biosystems™ AccuSEQ™ Real-Time PCR Software v3.0 is the software component of an integrated workflow for the SEQ family of analytical detection and quantification assays. These assays are used for analytical testing for impurities and contaminants during the biopharmaceutical manufacturing process. The software has templates for Applied Biosystems™ MycoSEQ™ Mycoplasma Real-Time PCR Detection Kit, the Applied Biosystems™ ProteinSEQ™ protein quantitation systems, and the Applied Biosystems™ resDNASEQ™ Quantitative DNA Kits. It also includes a custom template option to support real-time PCR assays without a pre-defined template, such as the Applied Biosystems™ ViralSEQ™ viral detection assays. Security, audit, and e-signature capabilities help enable 21 CFR Part 11 compliance. For more information about the AccuSEQ™ Real-Time PCR Software, see **thermofisher.com/accuseq**.

The AccuSEQ™ Real-Time PCR Software v3.0 is designed to work with the QuantStudio™ 5 Real-Time PCR Instrument using 0.1-mL 96-well plates, with either fast or standard cycling. See the instrument user guide for additional information (*QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide* (Pub. No. A43322).

Minimum computer requirements

Component	Requirement
Computer	Intel Core i5 (Quad Core 2.6Hz) or higher processor, with 16GB of RAM and a minimum of 250 GB hard disk space.
Operating system	Windows™ 10 Pro, 64-bit
Browser	(Recommended) Google™ Chrome™ version 74 or higher
Screen resolution	1280 x 1024

Assay types supported by the AccuSEQ™ Real-Time PCR Software v3.0

Purpose	Description
MycoSEQ™ assays (page 29)	
Detection of more than 90 species of mycoplasma.	The MycoSEQ™ assay is a modified quantitative experiment that uses end-point data to provide a presence/absence call. The assay uses Power SYBR™ Green detection technology, which utilizes multiple parameters, amplification plot (C_t), melting temperature (T_m), and derivative value (D.V.) for interpretation of results. Multiparameter analysis provides highly sensitive and specific detection of fewer than 10 Mycoplasma genome copies per reaction.
ProteinSEQ™ assays (page 43)	
Detection of Protein A contaminants	The ProteinSEQ™ Protein A Quantification Kit is a Protein A quantification solution with single-digit picogram sensitivity and a near 5- log dynamic range. ProteinSEQ™ (Protein A) experiments use a standard curve to determine absolute quantity of the analyte in your sample.
Detection of CHO HCP contaminants	The ProteinSEQ™ CHO HCP Quantification Kit is a HCP protein quantification solution with high sensitivity and a 4-log dynamic range. ProteinSEQ™ (CHO HCP) experiments use a standard curve to determine absolute quantity of the analyte in your sample.
resDNASEQ™ assays (page 57)	
Detection of residual host cell DNA from a variety of expression systems.	resDNASEQ™ assays combine high-recovery PrepSEQ™ sample preparation and TaqMan™ Assay-based quantification of residual DNA. resDNASEQ™ experiments use a standard curve to determine absolute quantity of residual DNA in your sample.
Custom assays (page 70–95)	
Used to design, run, and analyze your own standard curve or melt curve experiment.	The custom workflow allows you to design your own template and experiments, and is meant for experiments not covered by existing factory default templates, such as the ViralSEQ™ assays.

Workflow

Sign in to the AccuSEQ™ Real-Time PCR Software

▼

Create an experiment from a template

See Chapter 6, "Create a custom template" to edit or create a new template.

▼

Assign plate and well attributes

▼

Set up and run the PCR reactions

▼

Load the plate in the instrument

▼

Start the run

▼

Monitor the run

▼

Chapter 8, "Review the results and generate a report"

▼

Export data and print reports

Use security, audit, and e-signature (SAE) functions

The AccuSEQ™ Real-Time PCR Software includes security, audit, and e-signature (SAE) features to help enable 21 CFR Part 11 compliance. These functions are collectively referred to as the SAE module.

Overview of the SAE module components

The Security, Audit, and E-Signature module provides the following SAE functionality in the AccuSEQ™ Software:

- **System security**—Requires users to sign in to the AccuSEQ™ Software and determines user access to functions (permissions).

  If a user account does not have permission to perform a function, the function is grayed out in the software.

  For more information on permissions, see *Security, Audit, and E-Signature (SAE) Administrator Console v1.0 User Guide* (Pub. No. MAN0018760).
- **Auditing**—Automatically audits changes to experiments and other information that is accessible only to an administrator.
- **E-signature** —Allows users to provide an electronic signature (user name and password) after reviewing an experiment. For more information, see "E-sign an experiment" on page 108.

  Note: If the **E-Sign** button in the **Results** screen is grayed out, the signed-in user account does not have the e-signature function enabled.
- **Audit & E-Sign Reports**—Allows any user to view the audit and e-signature records for an experiment. For more information, see "View audit and e-sign reports for an experiment" on page 109.

Configuring the SAE module

For information on configuration, see *Security, Audit, and E-Signature (SAE) Administrator Console v1.0 User Guide* (Pub. No. MAN0018760).

Network and password security requirements

Network configuration and security

The network configuration and security settings of your laboratory or facility (such as firewalls, anti-virus software, network passwords) are the sole responsibility of your facility administrator, IT, and security personnel. This product does not provide any network or security configuration files, utilities, or instructions.

If external or network drives are connected to the software, it is the responsibility of your IT personnel to ensure that such drives are configured and secured correctly to prevent data corruption or loss. It is the responsibility of your facility administrator, IT, and security personnel to prevent the use of any unsecured ports (such as USB, Ethernet) and ensure that the system security is maintained.

Password security

Thermo Fisher Scientific strongly recommends that you maintain unique passwords for all accounts in use on this product. All passwords should be reset upon first sign in to the product. Change passwords according to your organization's password policy.

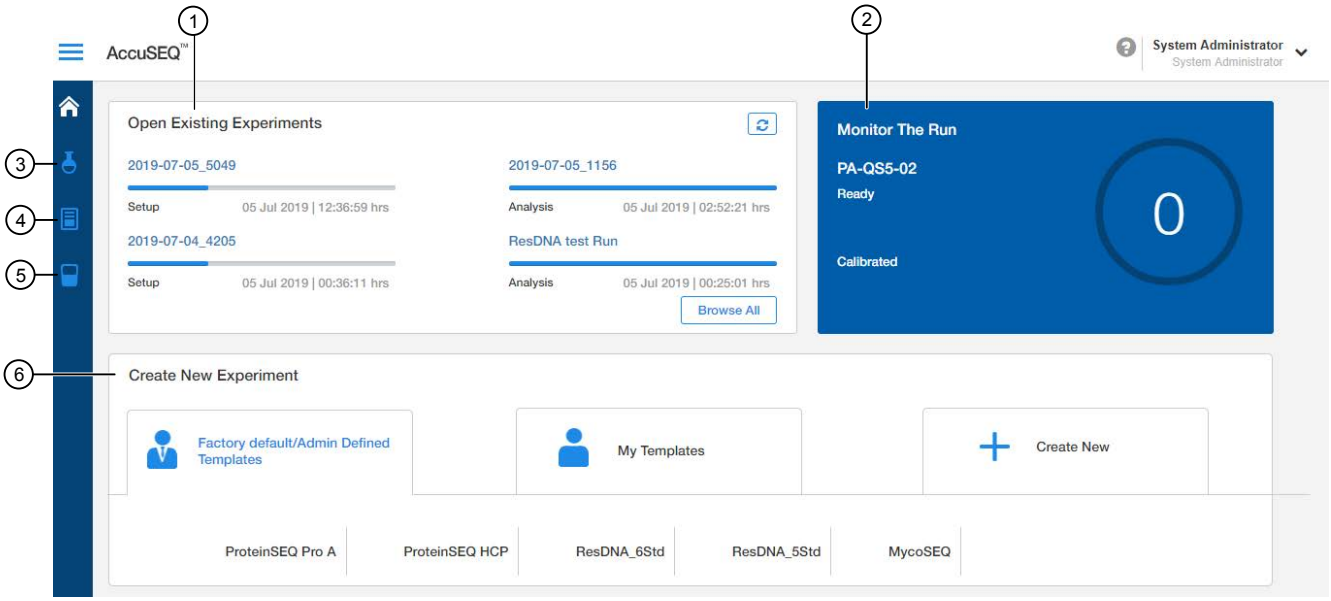
It is the sole responsibility of your IT personnel to develop and enforce secure use of passwords.

Navigate the AccuSEQ™ Real-Time PCR Software

Home screen

Use the **Home** screen to:

- View the last four experiments run
- Monitor a run. See "Monitor the run" on page 35.
- Create a new template. See "Create or open a template" on page 71
- Create an experiment from a template. See "Create an experiment from a template" on page 84
- Create a copy of an existing experiment. See "Create a copy of an existing experiment" on page 85

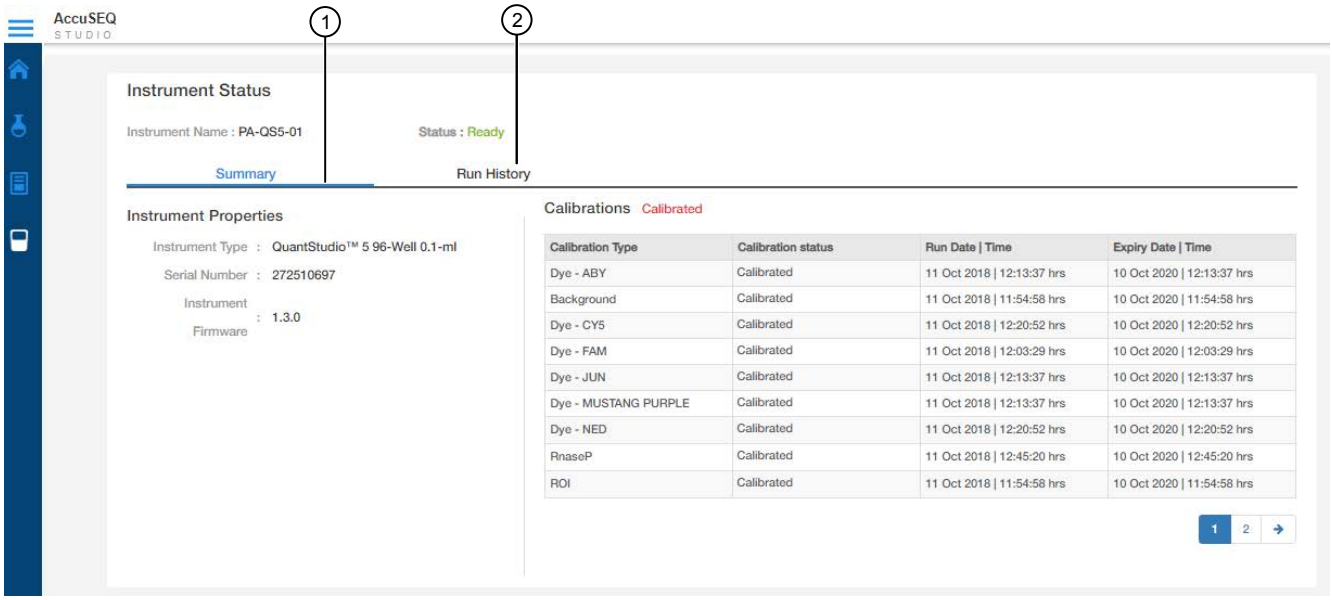


① **Open Experiment**—View the last 4 experiments run on the instrument. Click **Browse All** to open a full list of experiments.
② **Monitor the Run**—View the time remaining for the run; view the instrument calibration status. For information on instrument calibration, see the *QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide* (Pub. No. A43322).
③ **Experiments** list
④ **Templates** list
⑤ **Instrument status**
⑥ **Create Experiment**—Select from existing templates or create a new one.

Instrument status screen

Use the **Instrument status** screen to:

- View the instrument calibration log
- View the instrument Run History



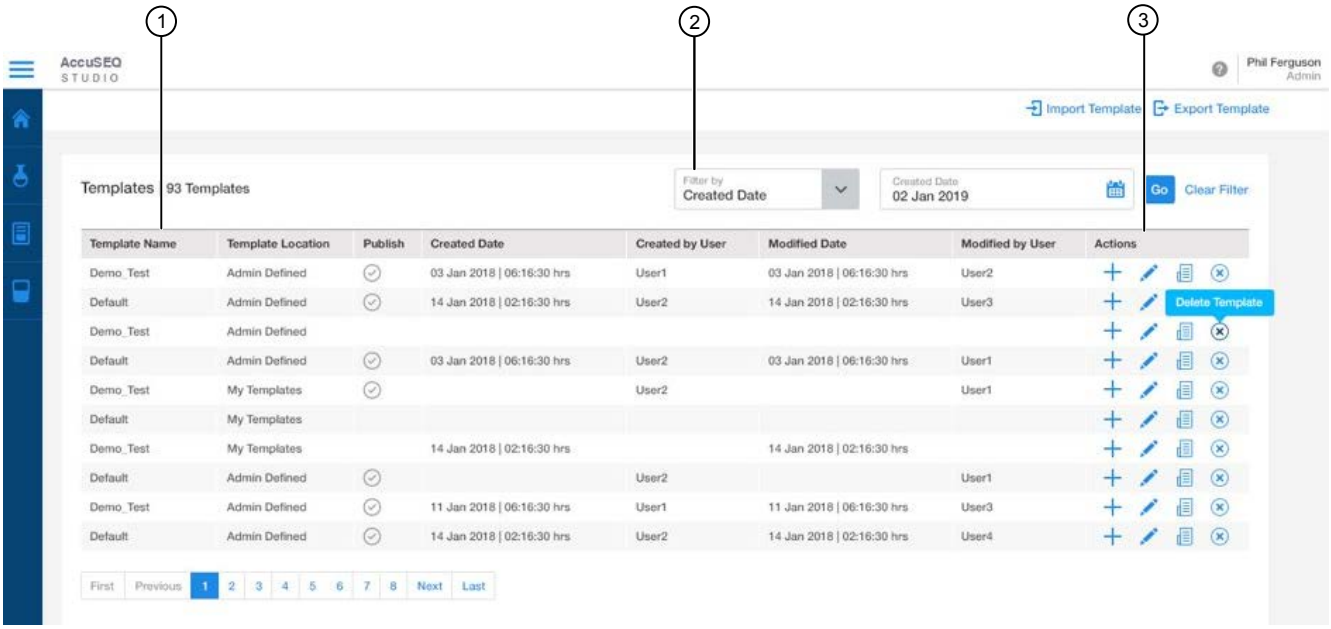
① Summary tab—Contains calibration information.
② Run History tab—Contains a log of instrument events.

Templates screen

Use the Templates screen to:

- View the full list of templates on the instrument (published and unpublished from the user's Default/Admin Defined and My Template lists).
- Create an experiment from a template
- Edit, delete, publish, or unpublish a template.

For information on templates, see Chapter 6, "Create a custom template".



① **Template Name**—View the published templates available for use. If your user account includes the View Unpublished template permission, unpublished templates are also listed. For information on publishing templates, see "Publish a template" on page 81.

② **Filter by**—Use to filter templates.

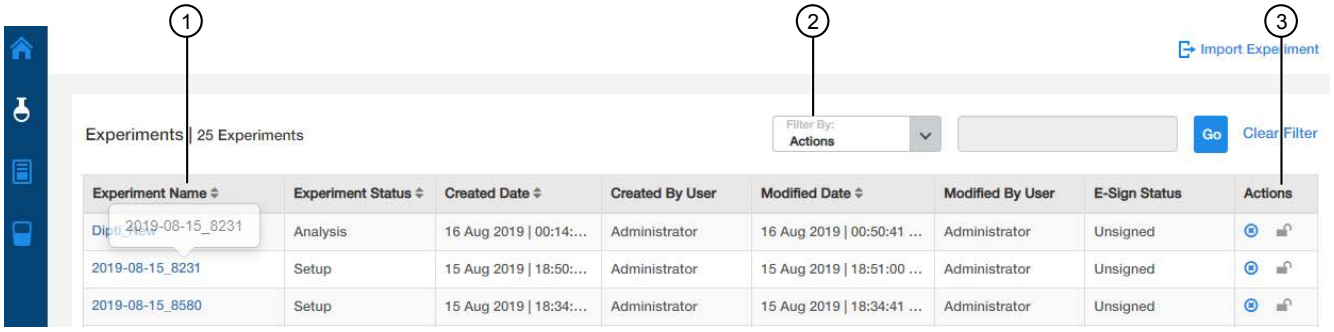
③ **Actions**—Depending on the role of the user that is signed in, the ability to + **Create**, **Edit**, **Publish**, or **Delete** templates.

Experiments screen

Use the Experiments screen to:

- View the full list of experiments
- Filter experiments (by parameters such as Created Date or User)
- Open an experiment
- Delete an experiment
- View if an experiment has an e-signature.

For more information on creating an experiment from an existing experiment, see "Create a copy of an existing experiment" on page 85.



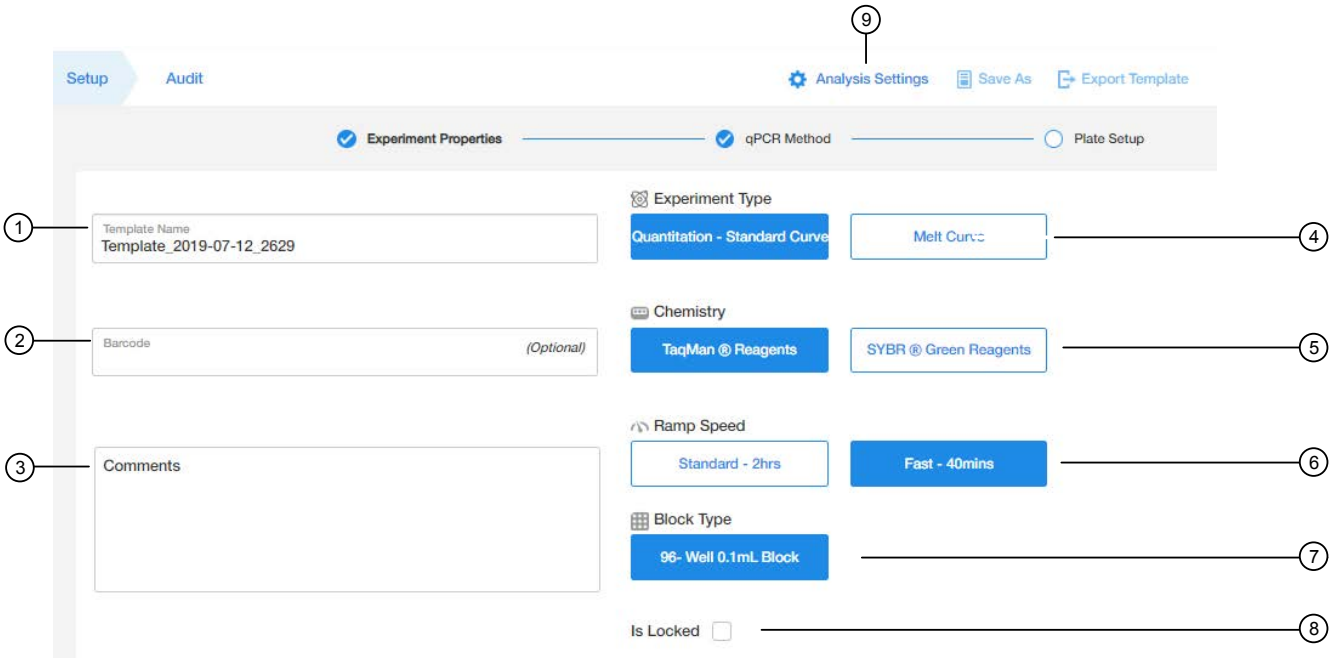
① **Experiment Name**—View a complete list of experiments run on the instrument (most recent first).
② **Filter by**—Use to filter experiments.
③ **Actions**

- ⊗—Delete the experiment; For permissions, see the *AccuSEQ Quantstudio5 Security, Audit, and E-signature (SAE) Administrator Console v1.0 User Guide* (Pub. No. 100084439)
- **(Locked gray icon)**—signed
- **(Unlocked gray icon)**—unsigned
- **(Locked blue icon)**—partially signed; click to go to the E-sign page

Setup screens

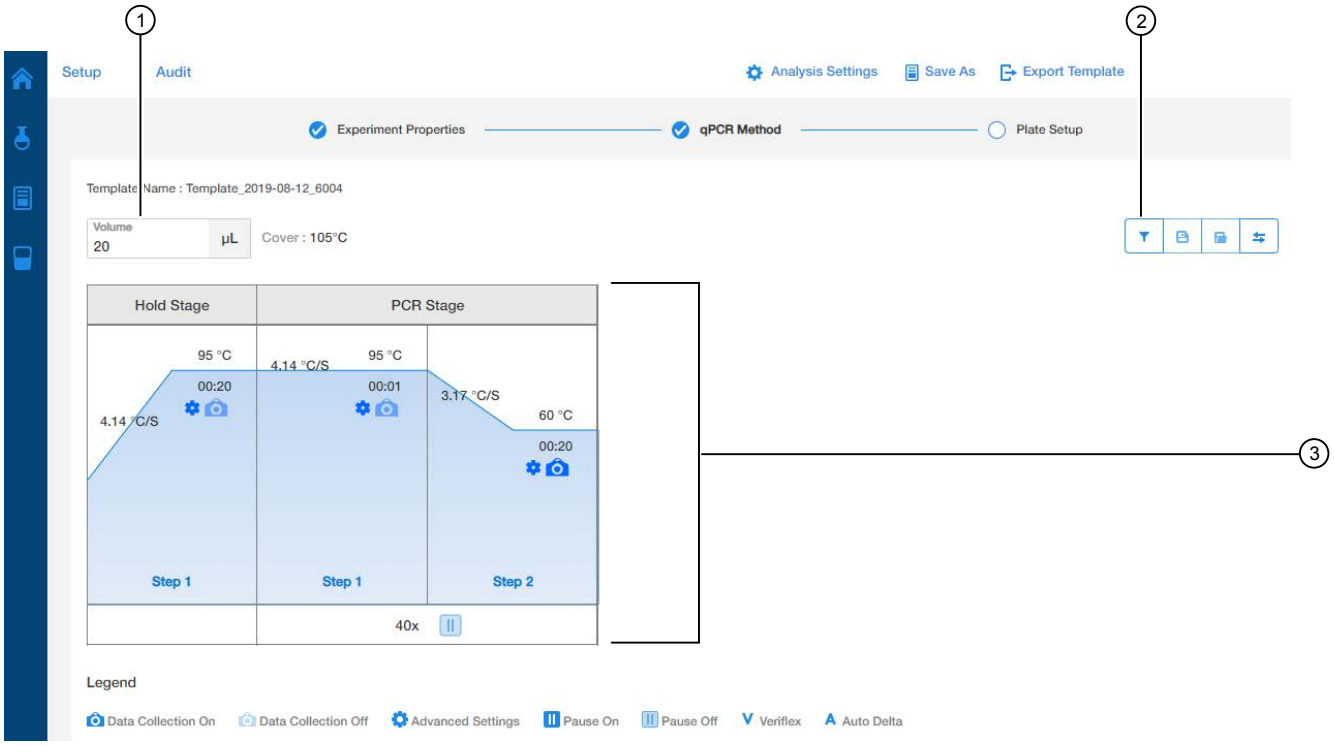
Use the **Setup** screen to confirm the properties and methods used in default templates or to create a custom template or experiment. For more information on creating a custom template, see Chapter 6, "Create a custom template".

1. **Experiment Properties** screen. For more information, see "Enter template properties" on page 73.



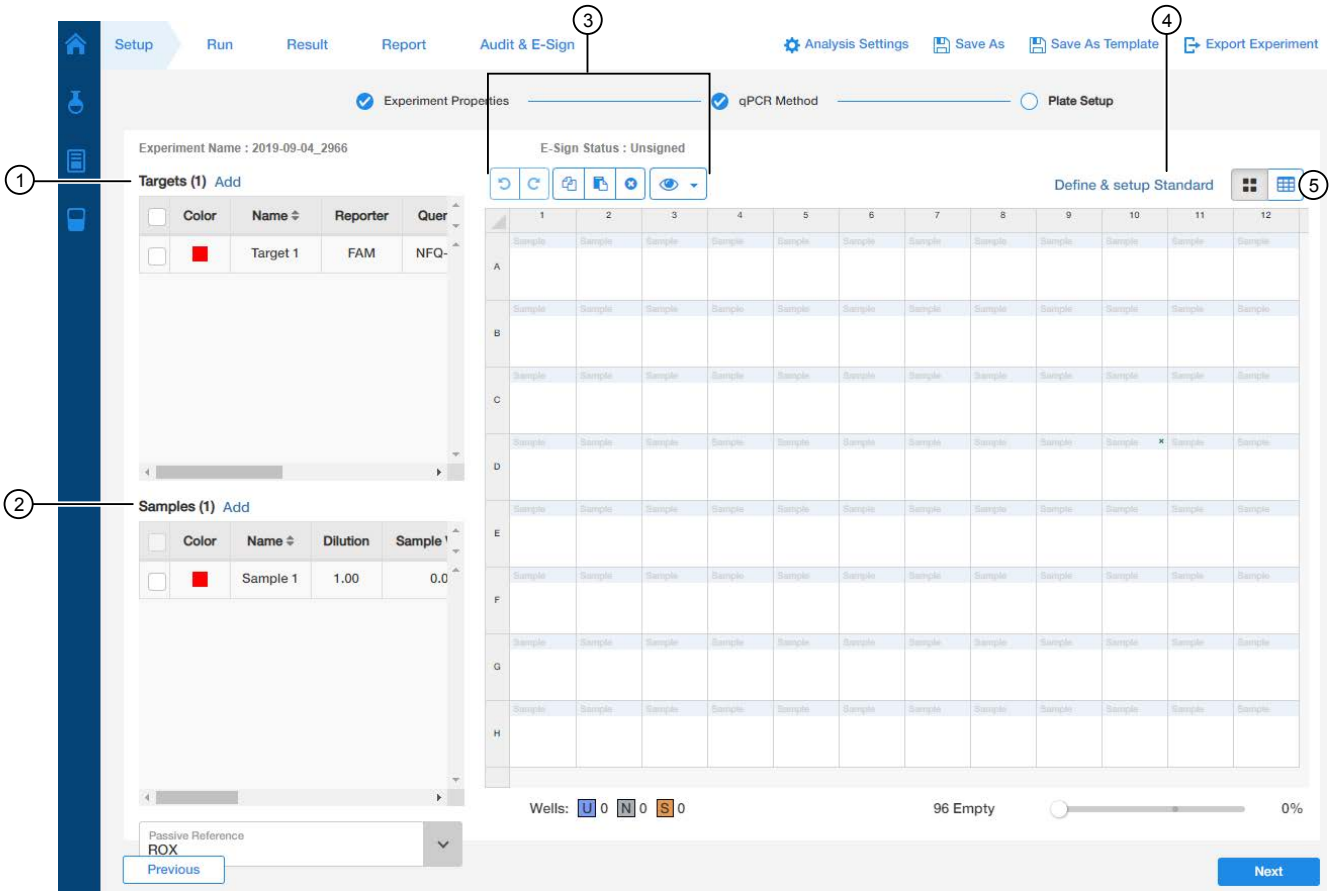
① **Template Name**—Use the software-generated name, or create a unique name.
② **Barcode**
③ **Comments**
④ **Experiment Type**
⑤ **Chemistry**
⑥ **Ramp Speed**
⑦ **Block Type**—fixed as a 96-Well 0.1mL block
⑧ **Is Locked** checkbox—check to lock the template
⑨ **Analysis Settings**

2. **qPCR method** screen. For more information, see "Confirm or edit the run method and optical filter selection" on page 74.



① Reaction volume. The volume is fixed in locked templates.
② Optical filters
③ PCR parameters

3. **Plate Setup** screen. For more information, see "Assign plate and well attributes" on page 76.

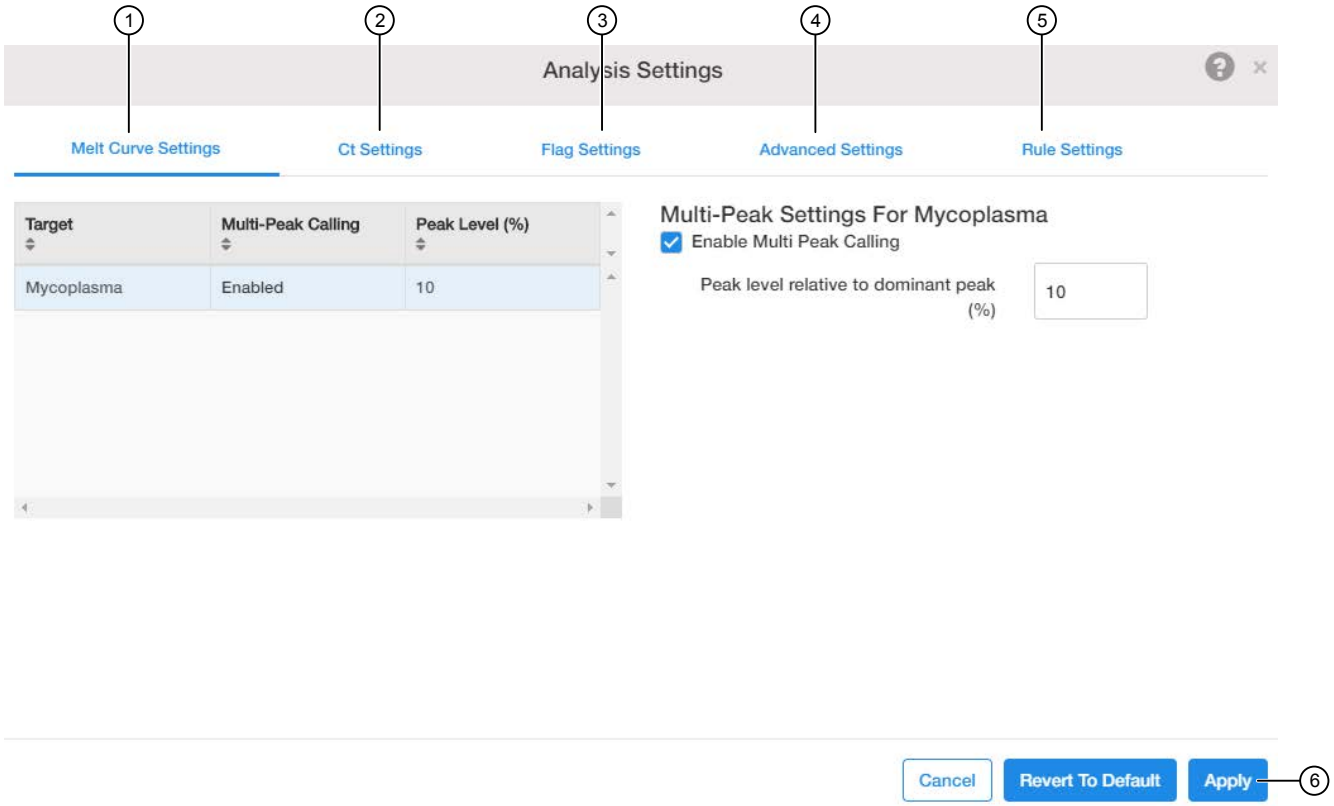


① Targets
② Samples
③ Plate setup toolbar
④ Define & setup Standard (standard curve experiments)
⑤ View (Grid View or Table View)

Analysis settings screen

Use the **Analysis settings** screen to:

- Adjust the **Melt Curve Settings** (MycoSEQ™ experiments only)
- Adjust the **Ct Settings**
- Adjust the **Flag Settings**
- Adjust baseline settings for individual wells (**Advanced Settings**)
- Adjust the **Rule Settings** (MycoSEQ™ experiments only)



① Melt Curve Settings
② Ct Settings
③ Flag Settings
④ Advanced Settings
⑤ Rule Settings
⑥ Actions (Click **Apply** to confirm changes before closing the window.)

Run screen

Use the **Run** screen to:

- View Amplification plot or Melt curve plot (MycoSEQ™ or custom melt curve experiments only)
- View the **Post Run Summary**
- Start a run

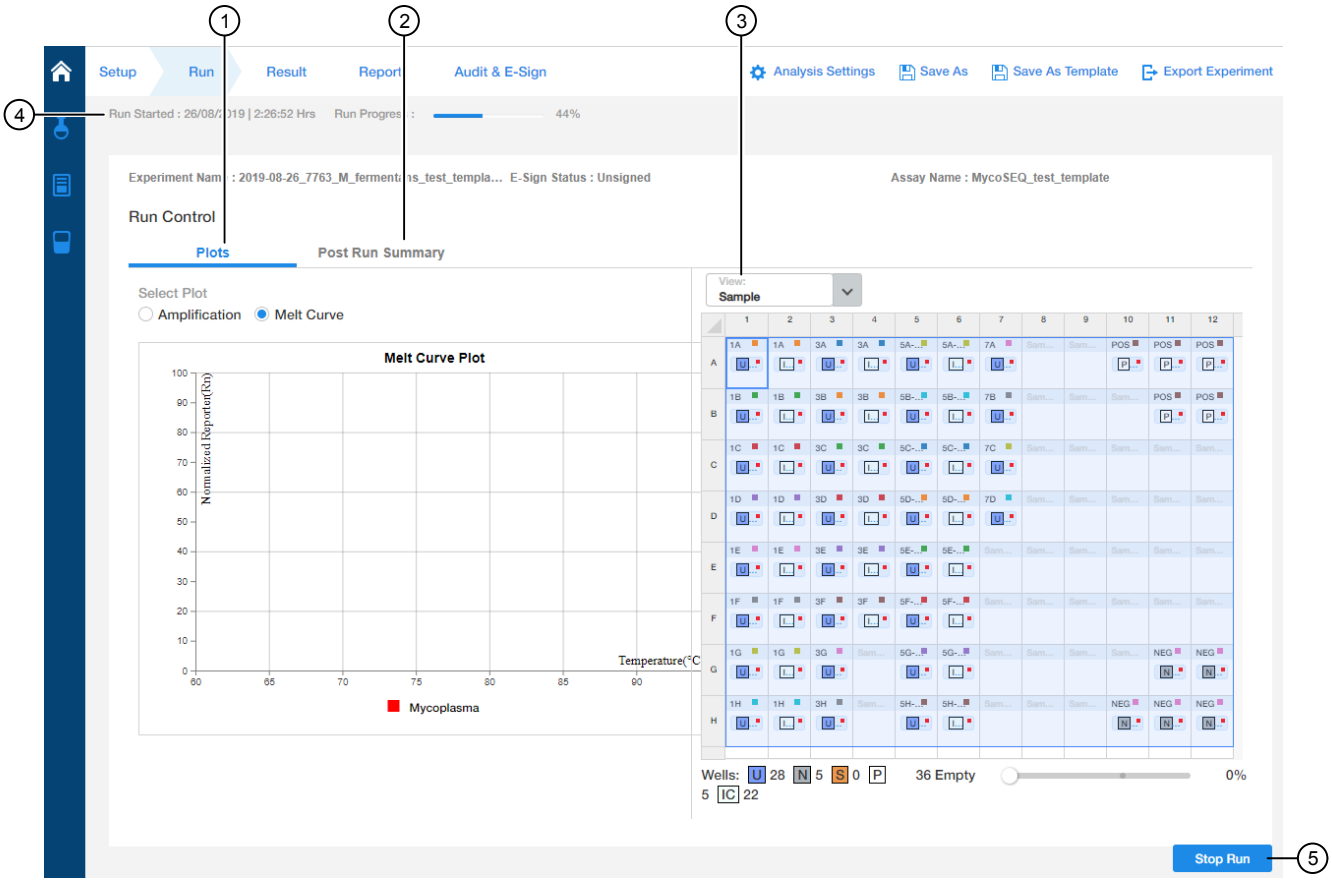


Figure 1 Example **Run Screen** (MycoSEQ™)

① **Plots**
② **Post Run Summary**
③ **View**—Select a parameter to view in **Plots**
④ Progress bar—Displays percentage of the run that is completed
⑤ **Stop Run**—Stops the run

Results screen

Use the **Results** screen to:

- View the plate call (MycoSEQ™ only)
- View the well calls (MycoSEQ™ only)
- Omit wells and reanalyze the experiment
- View plots
- E-Sign the experiment

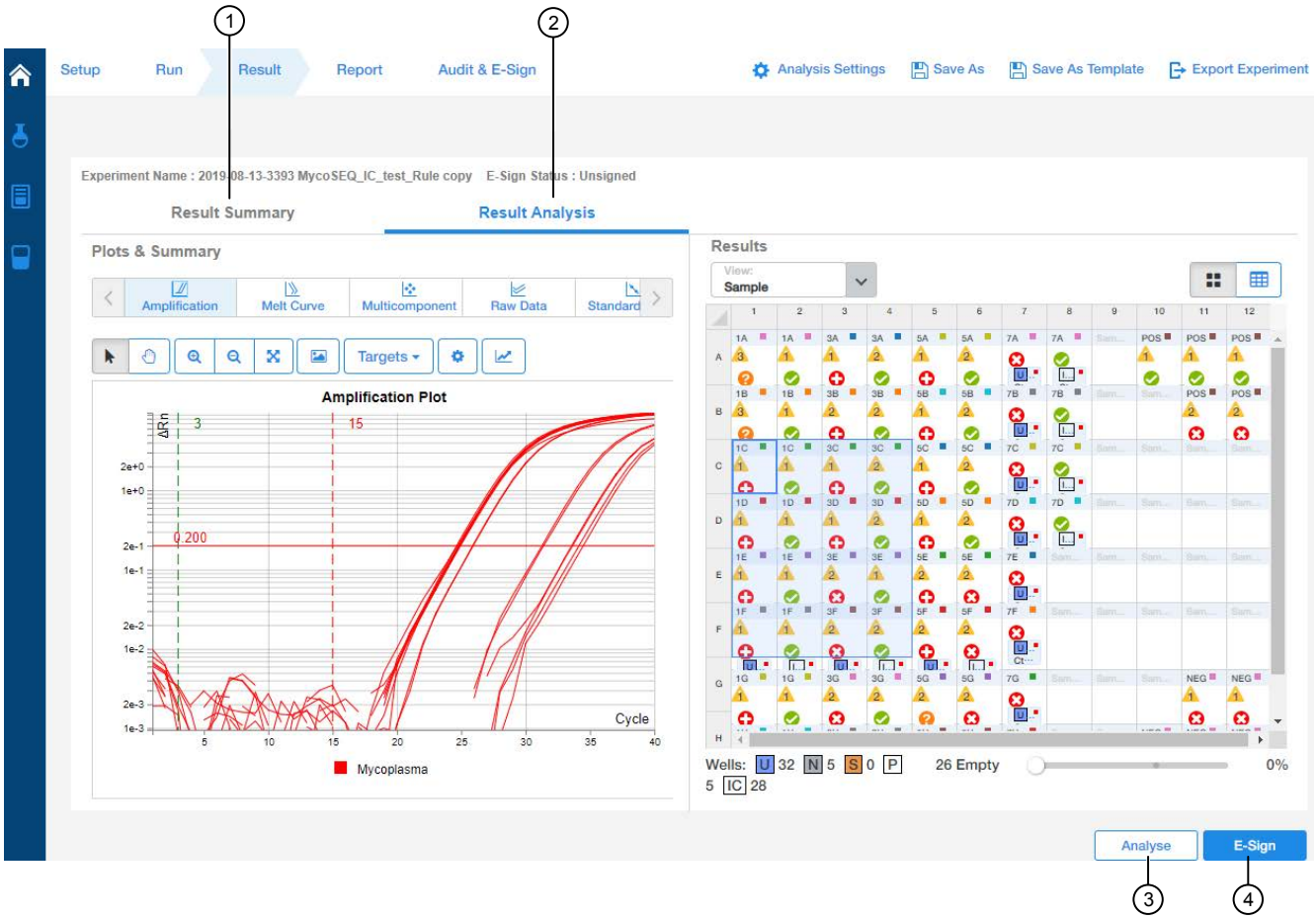


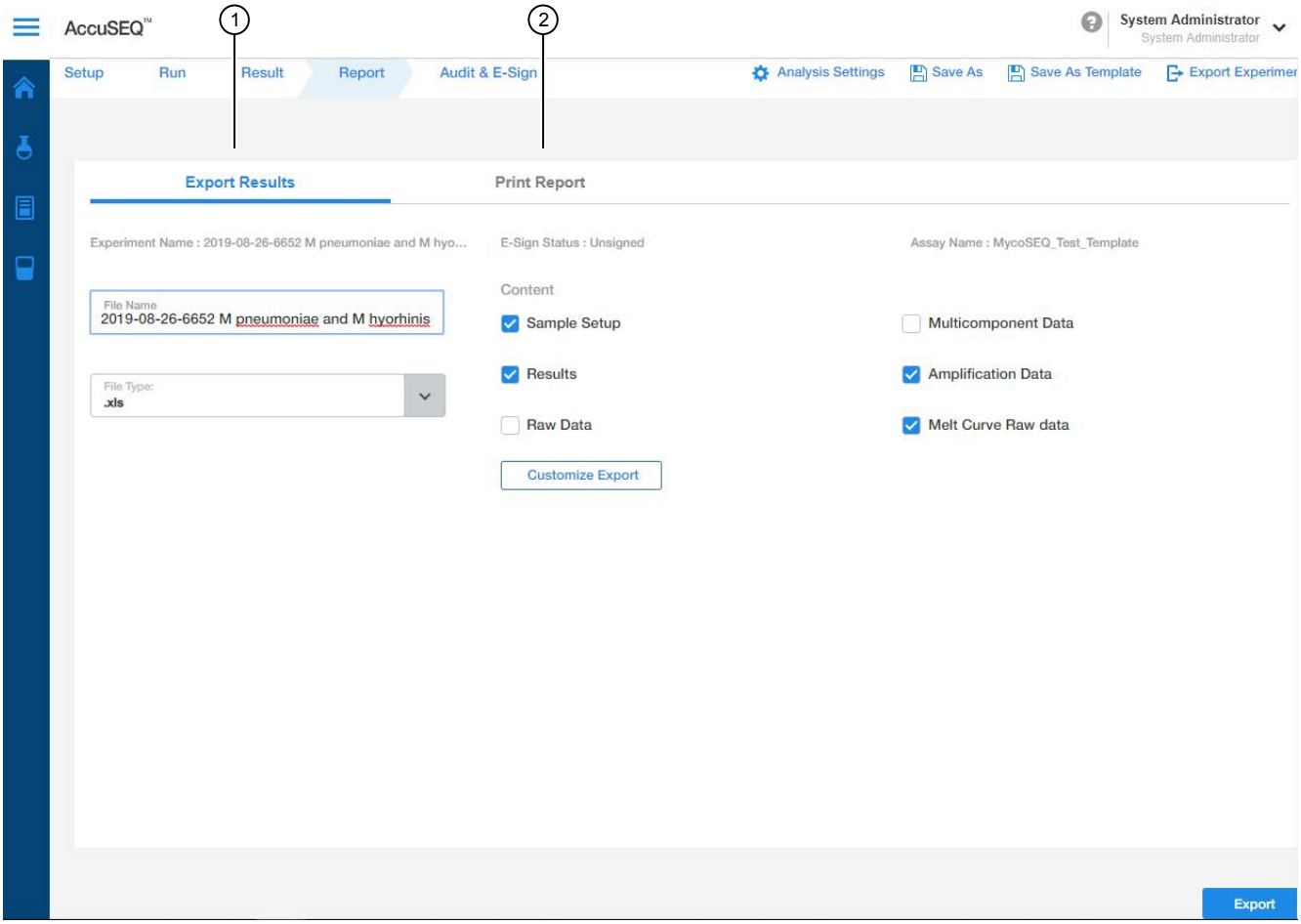
Figure 2 Example of Results screen (MycoSEQ™ assay)

① **Result Summary** pane—only available for MycoSEQ™ assays
② **Result Analysis** pane
③ **Analyze**—Use after omitting wells or changing the **Analysis Settings**.
④ **E-Sign**

Report screen

Use the **Report** screen to:

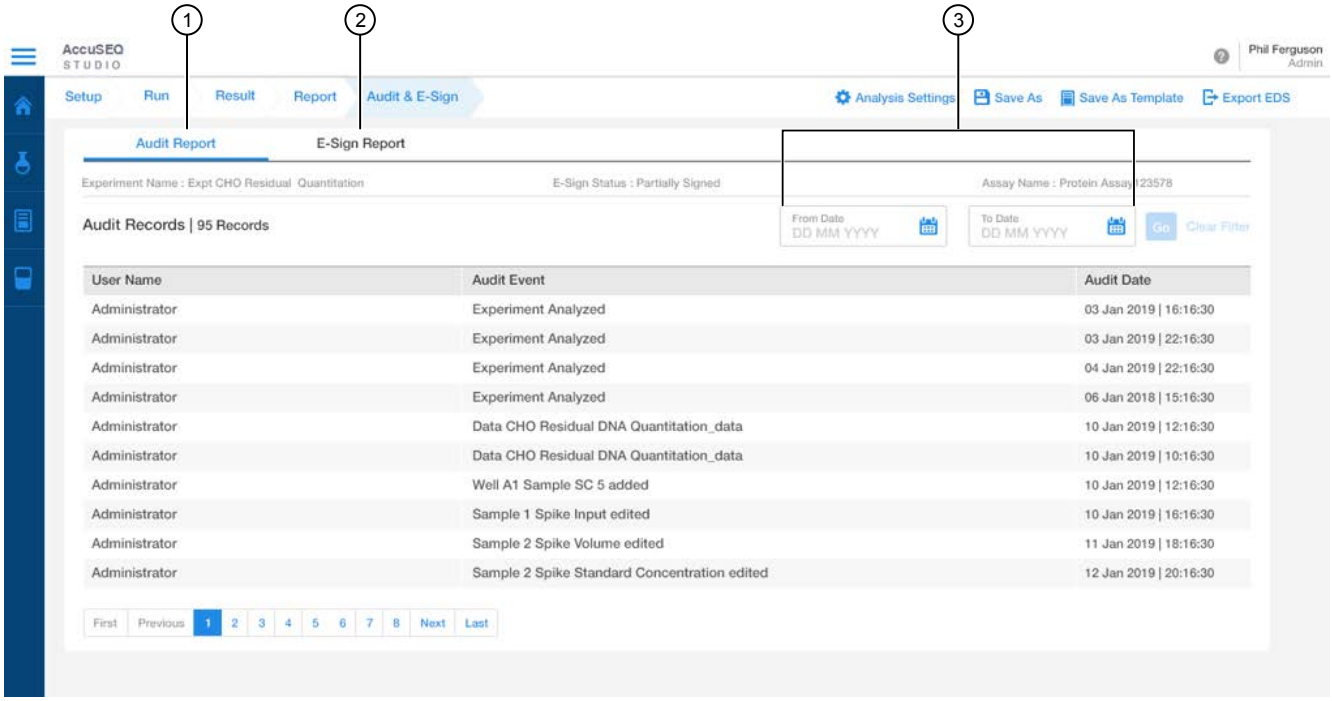
- Export results
- Customize export
- Print a report



① **Export Results**
② **Print Report**

Audit & E-sign screen

Use the **Audit & E-sign** screen to view the audit or e-signature report for an experiment.



① **Audit Report**
② **E-Sign Report**
③ Filter results by date

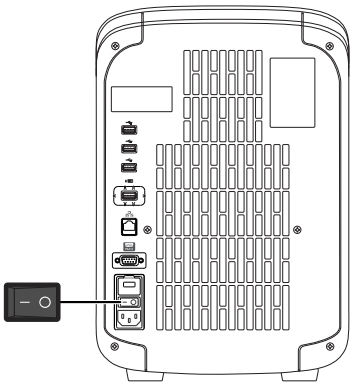
2 General procedures to operate the QuantStudio™ 5 Instrument

Before running experiments with the AccuSEQ™ Real-Time PCR Software, familiarize yourself with the QuantStudio™ 5 Instrument.

Before you begin

Power on the instrument

1. Touch anywhere on the touchscreen to determine if the instrument is in sleep mode. If the home screen is displayed, the instrument is already powered on.
2. If the home screen is not displayed, power on the instrument by pressing the switch on the rear panel.

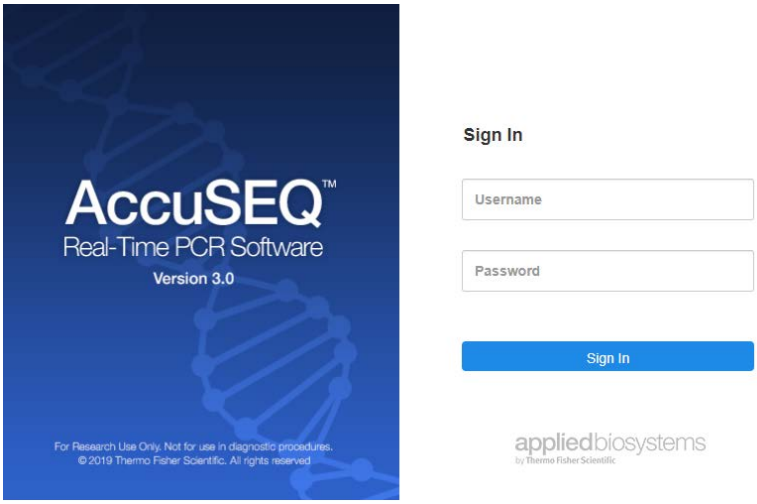


If left unattended (for about two hours), the instrument automatically enters sleep mode (enabled by default) to conserve power.

Note: To customize the sleep mode setting, touch ⚙ **(Settings) ▸ Instrument Settings ▸ Sleep Mode**.

Sign in to the AccuSEQ™ Real-Time PCR Software

1. Launch the AccuSEQ™ Real-Time PCR Software. In the Windows™ desktop, click ▸ **Applied Biosystems** ▸ **AccuSEQ** ▸ **AccuSEQ**.
2. Enter the **Username**, then **Password**.
3. Click **Sign in**.



Note the following restrictions you may see in the software:

- Your access to functions in the software is based on the permissions associated with your user account.
- If a user account does not have permission to perform a function, the function is grayed out in the software.
- If your system is configured for password expiration, you will be periodically prompted to change your password. If your system is configured to monitor failed log in attempts, you will be locked out of the software if you incorrectly enter your user name or password more than a specified number of times.

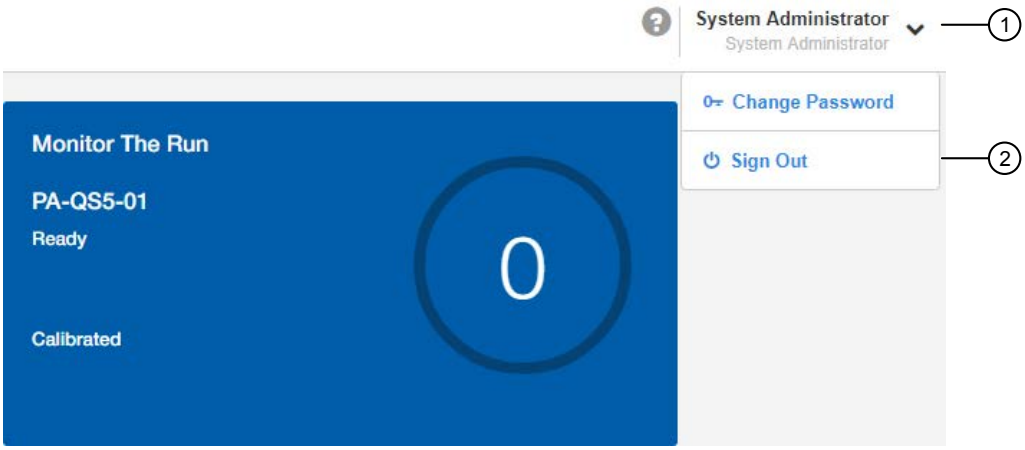
Change your password

When your password is about to expire, a message is displayed when you sign in.

1. Click your user name in the top right of the menu bar.
2. Click **Change Password**.
3. Enter the old password.
4. Enter a new password, confirm the new password, then click **OK**.

Sign out of the AccuSEQ™ Real-Time PCR Software

Click the username in the top right corner of the AccuSEQ™ Real-Time PCR Software home screen, then click **Sign out**.



① Username
② Sign out

Monitor Instrument Status

Basic instrument status is displayed in the **Monitor The Run** pane of the **(Home)** screen.

① Instrument name
② Instrument status (Ready, Running, Offline)
③ Calibration status

- Calibrated—All required calibrations are "Current". Required calibrations include: Background, ROI, Uniformity, and Dyes.
- Not calibrated—None of the required calibrations are complete.
- Requires calibration—One or more dyes are not calibrated.

④ Time lapsed (if a run is in progress)
⑤ Total run time
⑥ Experiment name

1. In the **(Home)** screen, click **(Instrument Status)**.
2. Click **Summary** to display a list of current instrument calibrations and their expiry dates and times. Ensure that the instrument is calibrated. For information on instrument calibration, see the *QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide* (Pub. No. A43322).
3. Click **Run History** to display a complete list of experiments that have been run on the instrument.

3 Set up, run, and review MycoSEQ™ experiments

Overview

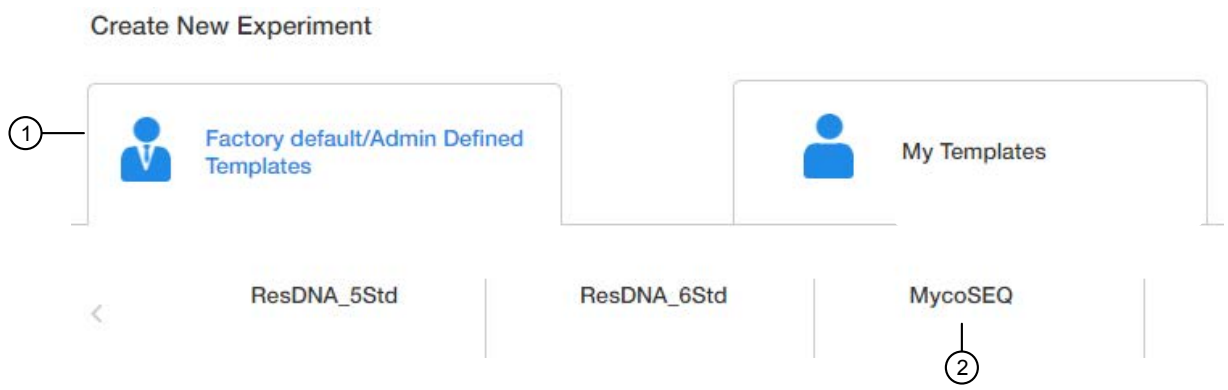
The MycoSEQ™ Mycoplasma Detection System integrates real-time PCR and *Power* SYBR™ Green technology, instruments, and software. It is used with the Applied Biosystems™ PrepSEQ™ nucleic acid extraction kits to detect more than 90 species of Mycoplasma.

MycoSEQ™ experiments are semi-quantitative experiments with a melt curve PCR stage. The MycoSEQ™ template in the AccuSEQ™ Software includes default cycle threshold (Ct), derivative value (DV), and melting temperature (Tm) values to make automated mycoplasma calls (Present, Absent, Review, or Fail).

Create a MycoSEQ™ experiment

1. In the **Home** screen, click the **Factory default/Admin Defined Template** tab, then select **MycoSEQ**.

   Note: To create a copy of an existing MycoSEQ™ experiment, see "Create a copy of an existing experiment" on page 85.



① **Factory default/Admin Defined Template** tab
② **MycoSEQ** template

2. In the **Experiment Properties** pane of the **Setup** tab:
   a. (*Optional*) Change the system-generated name of the experiment.
   b. (*Optional*) Enter the plate **Barcode**, then add **Comments**.
   Default MycoSEQ™ settings (cannot be changed).
      - **Experiment Type—Quantitation-Standard Curve**
      - **Chemistry—SYBR™ Green Reagents**
      - **Ramp Speed—Standard - 2hrs**
   c. Click **Next**.
3. In the **qPCR Method** pane of the **Setup** tab, view the default volume and cycling conditions (cannot be changed).

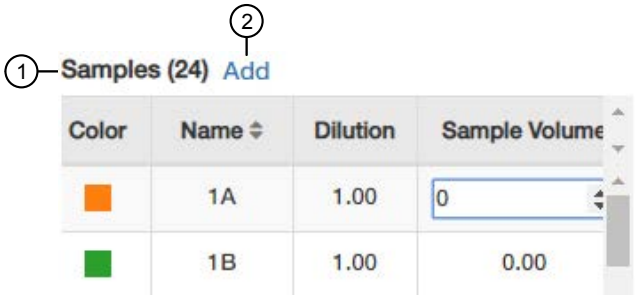
Figure 3 MycoSEQ™ template default cycling conditions

4. Click **Next**.

5. In the **Samples** pane of the **Setup** tab, enter the sample **Name**. Add additional **Samples** if needed.

   Note: Only the sample **Name** is necessary for experiments run from the factory default **MycoSEQ** template.

   IMPORTANT! Do not change the **Targets**.



① **Samples** pane
② **Add**—adds additional samples

For more information on plate setup, see "Assign plate and well attributes" on page 76.

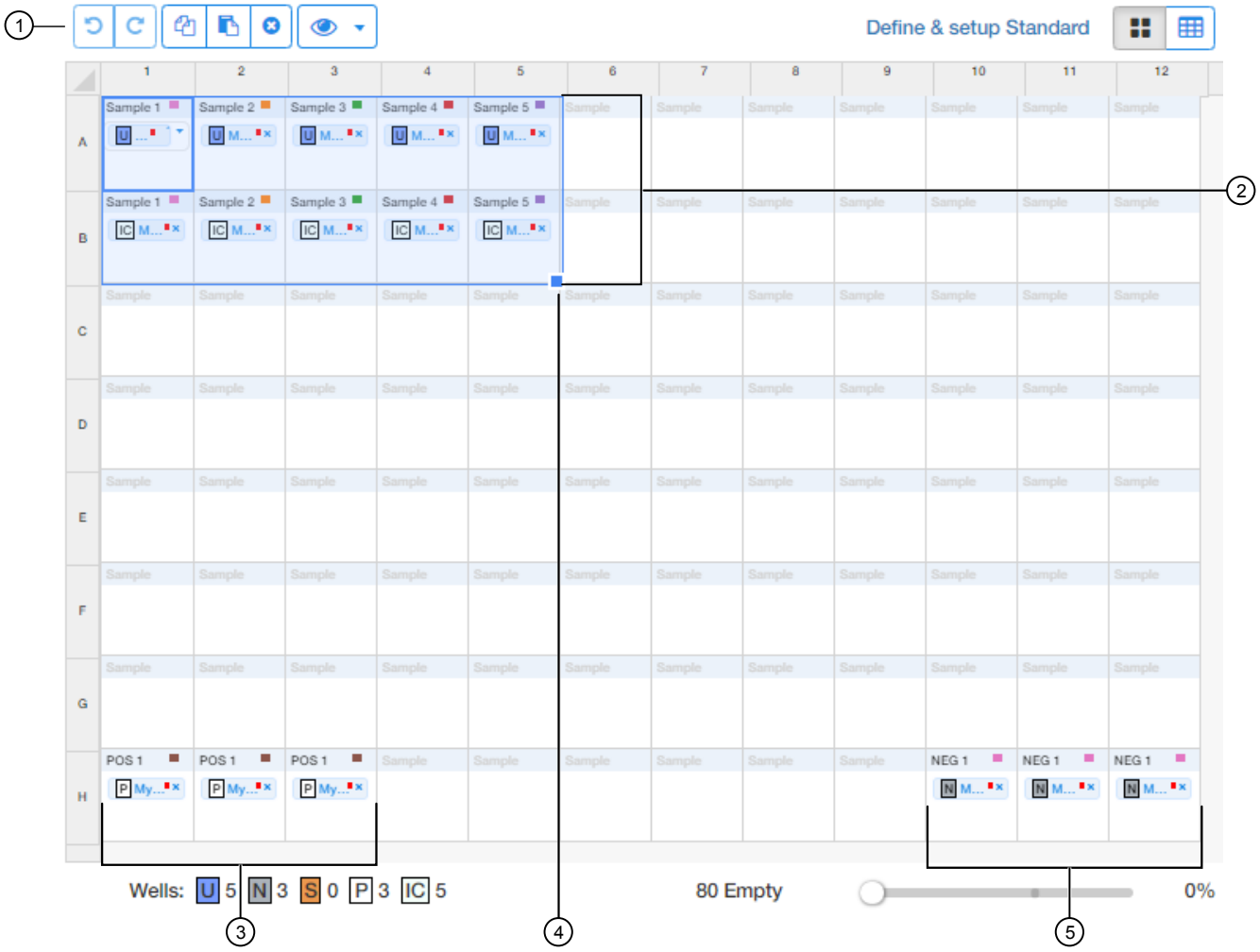


Figure 4 MycoSEQ™ template default sample plate layout

① Toolbar (in order: Undo, Redo, Copy, Paste, Delete, View)
② 5 default **Samples** with and without an inhibition control (IC)
③ Positive control (P) in triplicate (recommended)
④ Selected samples. Click-drag to add additional samples.
⑤ No template control (N) in triplicate (recommended)

6. Click **Next**.
   The **Run** tab is displayed.

7. Experiments are auto-saved in the software. To save, exit the experiment. The software prompts you to save changes. Click **Yes**.

   Note: Clicking **Save As** will create a copy of the experiment.

8. Assemble the PCR reactions following the manufacturer's instructions for the reagents and following the plate layout set up in the template. See *MycoSEQ™ Mycoplasma Real-Time PCR Detection Kit User Guide* (Pub. No. 4482248).

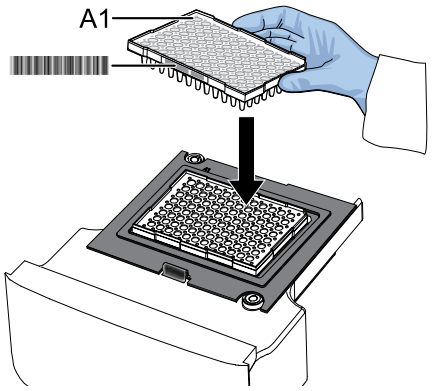
Immediately proceed to "Load the plate in the instrument" on page 33.

Load the plate in the instrument

CAUTION! Use optical flat caps for tubes. Rounded caps can damage the heated cover.

Load the plate.

1. Touch ⏏ to eject the instrument drawer.
2. Load the plate onto the plate adapter so that:
   - Well A1 of the plate is in the top-left corner of the plate adapter.
   - The barcode faces the front of the instrument.



IMPORTANT! The instrument should be used by trained operators who have been warned of the moving parts hazard.

Note: Do not remove the black plate adapter before loading a plate or tube strips. If used, tube strips can fit loosely in the adapter, but the heated cover applies the appropriate pressure to seat the tube strips securely in the adapter.

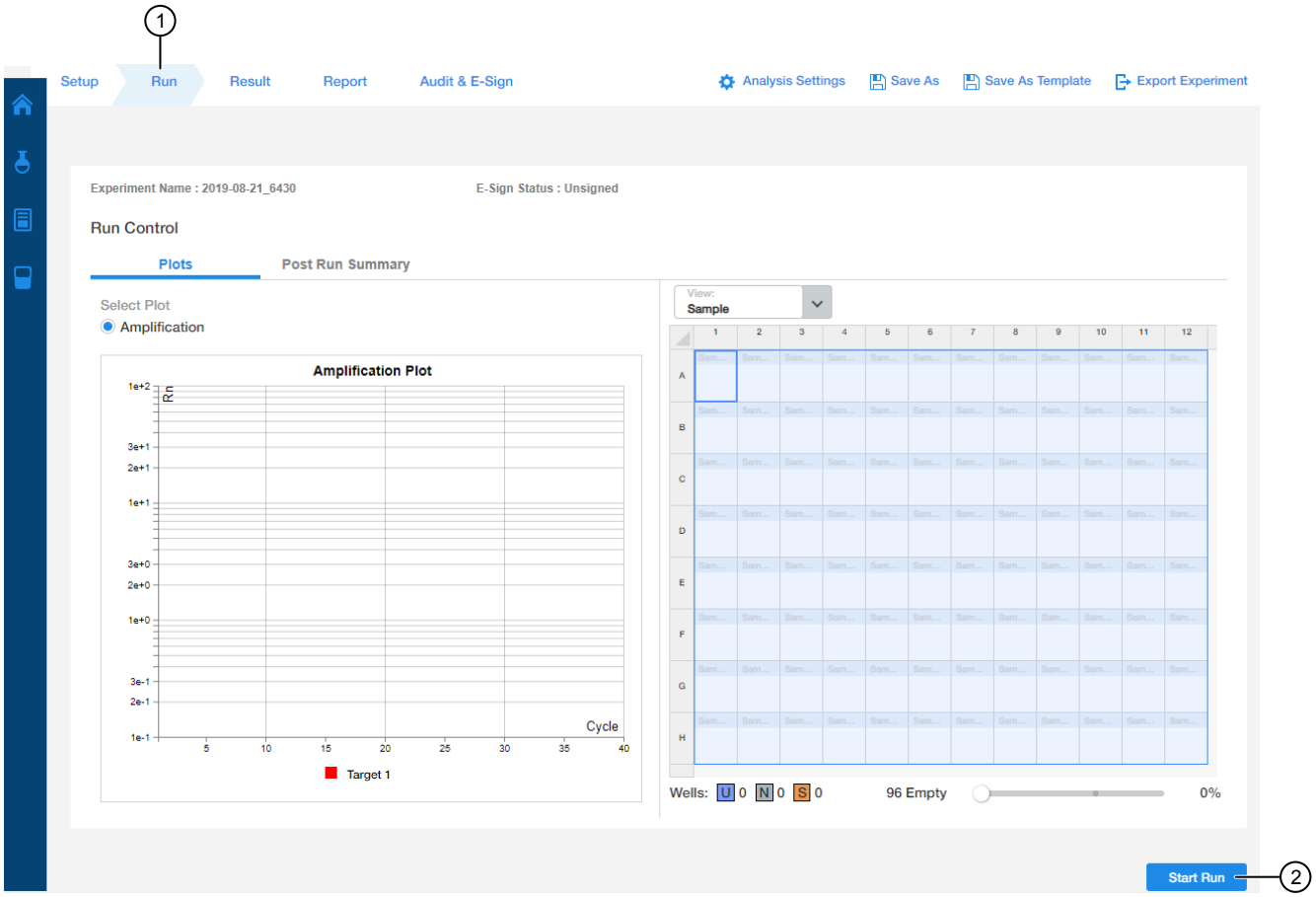
CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the plate temperature can reach 100°C. Allow it to cool to room temperature before handling.

3. Touch ⏏ to close the instrument drawer.

Start the run

Start the run in the AccuSEQ™ Software.

Option	Description
If the experiment is open	Click **Start Run**.
If the experiment is closed	1. Open the experiment. See "Open an experiment" on page 86. 2. Click the **Run** tab. 3. Click **Start Run**.



① **Run** tab
② **Start Run** button

A message stating **Run has been started successfully** is displayed when the run has started.

Monitor the run

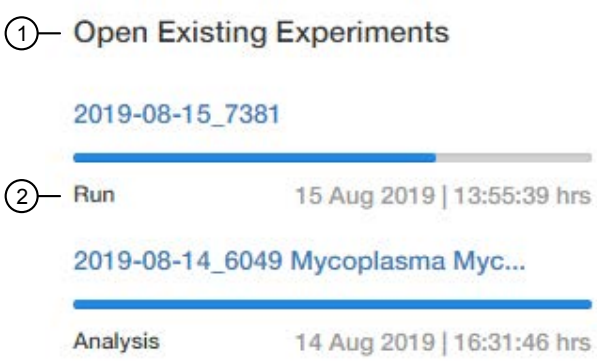
During the instrument run, you can monitor the run from the following places:

- On the instrument touchscreen.
- In the **Monitor the Run** pane of the AccuSEQ™ Software **(Home)** screen.

1. Instrument name
2. Instrument status (Ready, Running, Offline)
3. Calibration status
   - Calibrated—All required calibrations are "Current". Required calibrations include: Background, ROI/Uniformity, and Dyes.
   - Not calibrated—None of the required calibrations are complete.
   - Requires calibration—One or more dyes are not calibrated.
4. Time lapsed (if a run is in progress)
5. Total run time
6. Experiment name

- In the **Open Existing Experiments** pane of the AccuSEQ™ Software **(Home)** screen. The experiment being run is the first experiment listed. Status is **Run**.

  Note: When the run is complete, the status changes to **Analysis** and the bar changes to completely blue.



1. **Open Existing Experiments** pane
2. Experiment status

- In the **Run** tab of the AccuSEQ™ Real-Time PCR Software.
  You can perform the following actions.
  - Select wells in the plate layout to highlight respective curves in the plot.
  - Hover over curves in the plot for well information.
  - (*Optional*) Change what is displayed in the table wells, by selecting **Sample Name**, **Sample Color**, or **Target** in the **View** dropdown list.

  For more information, see "Run screen" on page 21.

On run completion, the **Post Run Summary** displays the run length, user and instrument information, and a list of any errors that occurred.

When the run is complete, unload the plate from the instrument.

Review results

Workflow: Review MycoSEQ™ experiments

"View the Result Summary" on page 37

▼

"Evaluate results in the Amplification Plot" on page 99

▼

"View and evaluate the Melt Curve Plot" on page 40

▼

Review data for outliers and *(optional)* "Omit outliers from analysis" on page 103

▼

(Optional) "Review the dye signal profile using the Multicomponent Plot" on page 105

▼

(Optional) "Review the signal profile using the Raw Data Plot" on page 107

▼

(Optional) "Review the flags in the QC Summary" on page 108)

▼

(Optional) "View and configure the analysis settings" on page 92

IMPORTANT! If you omit wells or configure the analysis settings, click **Analyze** to reanalyze the data. See "Results screen" on page 22.

View the Result Summary

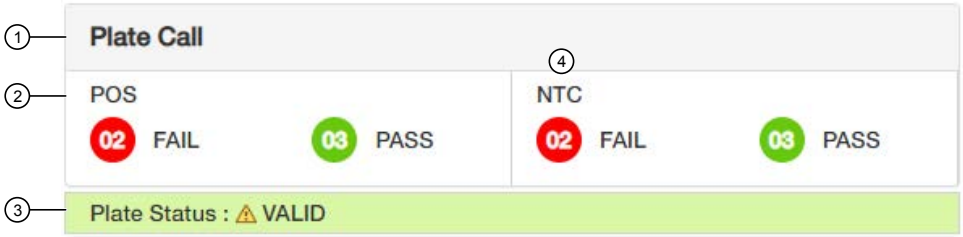
If the experiment is not open, see "Open an experiment" on page 86.

The **Result** tab is populated when the run is complete.

Calls are made based on the **Rule Settings** within the **Analysis Settings** for MycoSEQ™ experiments. See "Configure analysis settings" on page 92 and "Rule settings overview (MycoSEQ™ only)" on page 93 for more information.

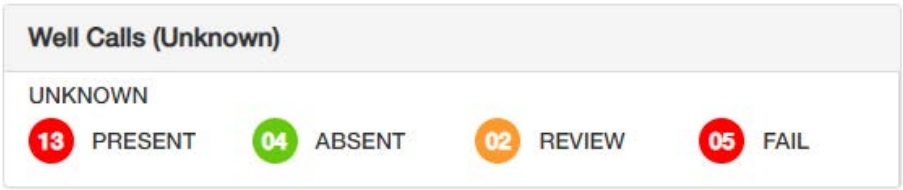
1. View the **Plate Call**.
   a. View the calls for the positive controls (POS).
   b. View the calls for the no template controls (NTC).

   The number inside the circle indicates the number of samples that passed or failed. The overall **Plate Status** (VALID or INVALID) is determined by the POS and NTC calls.



① Plate Call
② Positive controls (POS)
③ No Template Controls (NTC)
④ Plate Status

2. View the **Well Calls (Unknown)**.
   a. View the total number of wells for each call—Present, Absent, Review, or Fail.



   b. Use the **Legends** to view the calls in the plate

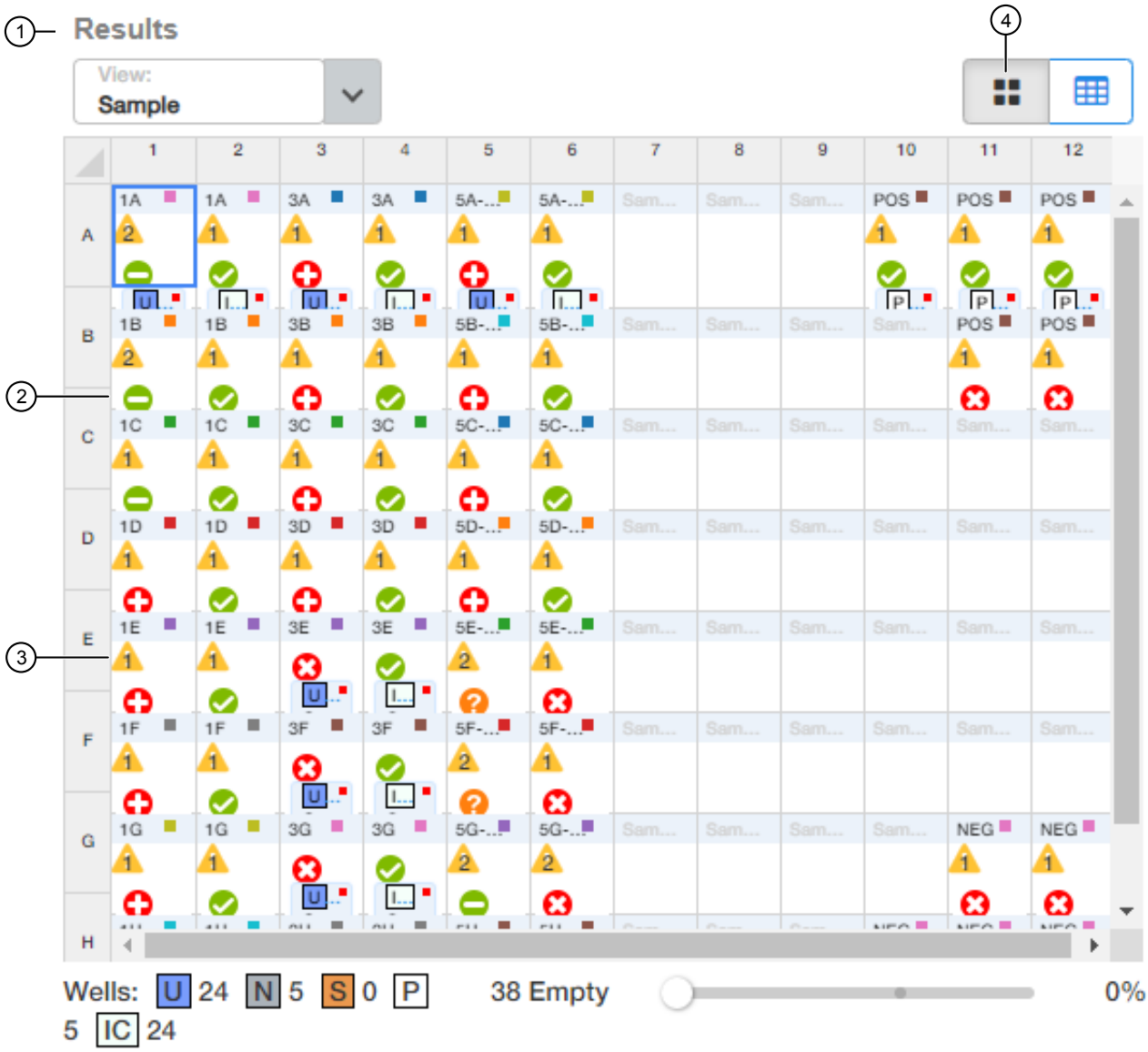


Figure 5 Example MycoSEQ™ Results (Grid View)

① **Results** pane
② Well call
③ Flags—the number within the triangle indicates the number of QC flag calls in the well; review in QC Summary. See "Flags" on page 112.
④ Grid View

Evaluate the overall shape of the Amplification Plot curves

You can evaluate the overall shape of the **Amplification** curves in the **Result** tab. Evaluating the shape in wells with a **Review** call can help determine if the sample should be rerun, or whether to edit the call to **Present** or **Absent**.

Open the experiment in the AccuSEQ™ Software. See "Open an experiment" on page 86.

1. In the **Results Analysis** pane of the **Result** tab, click **Amplification** in the horizontal scroll bar.

   Note: If no data are displayed in the **Result Analysis** pane, then click **Analyze**. See "Results screen" on page 22.

   ① Amplification Plot

   The **Amplification Plot** is displayed for the selected wells in the **(Grid View)** .

2. Ensure that the **Target** selected is **Mycoplasma**.

3. (*Optional*) Click **(Settings)** , then make the following selections:

   - **Plot Type**: **ΔRn, Rn, or C_T**
   - **Graph Type**: **Log or Linear**
   - **Show**: **Legend**, **Cq Mark**, or **Unselected**
   - **Plot Color**: **Target**, **Sample**, **Well**, or **Flag_Status**
   - **Threshold**: Select **Auto** or specify a threshold, then decide whether to **Show Threshold**.
   - **Baseline**: Decide whether to **Show Baseline**.

4. (*Optional*) Adjust the **(Plot Properties)**.

   a. (*Optional*) In the **General** tab, add a **Plot Title**, adjust the **Font** and **Color**, then click **Apply**.

   b. In the **X Axis** or **Y Axis** tabs, you can:

      - Add a **Label**
      - Select whether you want **Tick Marks**
      - Select **Auto-adjust range** or enter minimum and maximum values

        Note: The minimum value must be greater than 0.

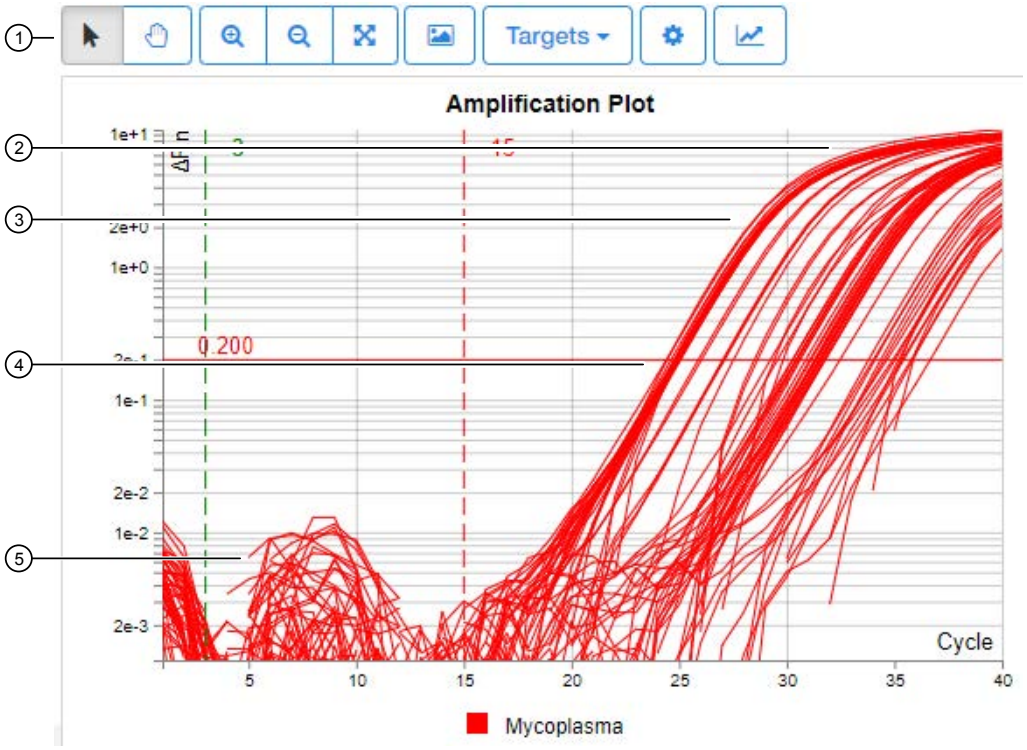


Figure 6 Typical Amplification Plot (4 phases)

① Amplification Plot tools
② Plateau phase
③ Linear phase
④ Exponential (geometric) phase
⑤ Baseline

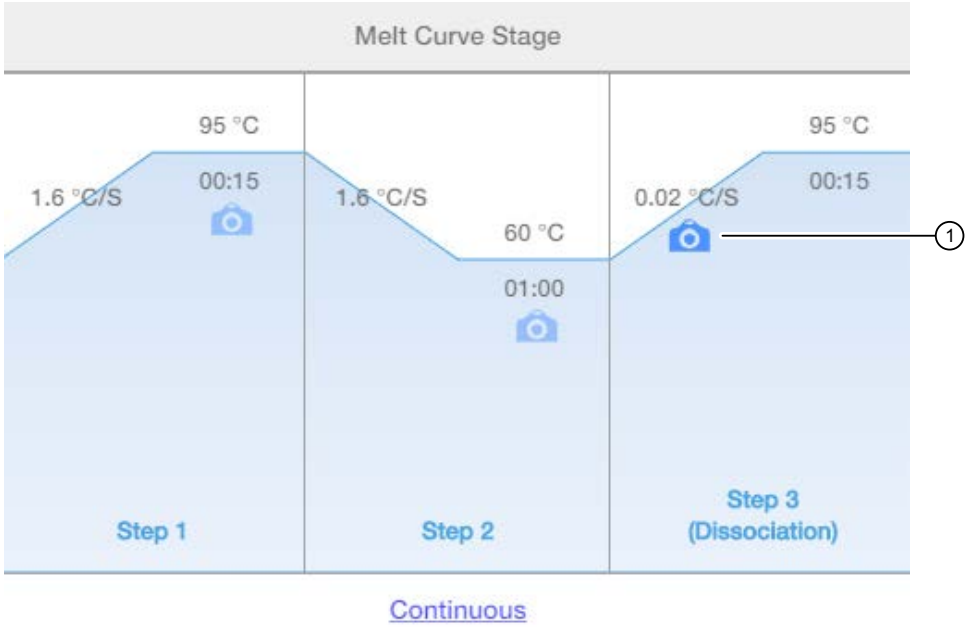
View and evaluate the Melt Curve Plot

You can view and evaluate the **Melt Curve** Plot in the **Result Analysis** window in the **Result** tab.

① **Melt Curve** plot
② **Melt Curve Stage** dropdown

1. In the **Result Analysis** window, select **Melt Curve** Plot from the horizontal scroll bar.

2. Keep the default **Melt Curve Stage**.
   Custom experiments with multiple data collections in the **Melt Curve Stage**, can select the stage that they want displayed.



① Stage 3 data collection point

3. Click ☰ to configure the plot, then make the following selections:
   - **Targets**: Select Mycoplasma.
   - **Plot Settings**: Select the **Plot Type** and **Plot Color**.
   - **Plot Properties**: Edit **Plot Title**, change fonts, colors, and labels.
   - **Save Image**: Save the image (PNG or SVG).
   - Use the select, pan, and zoom options to interact with the plot.

   The **Melt Curve Plot** is displayed for data points that are selected in the plot settings. The data points for selected wells in the **Grid View** or **Table View** are highlighted in the plot (see Figure 7).

4. (*Optional*) View the default **Melt Curve Stage**.

5. Confirm that amplification in the no template control and IC control wells is as expected. Use one of the following options:
   - Select control wells in the **Grid View** or **Table View**, then confirm the location of the data points in the **Melt Curve** Plot.
   - View the amplification plots for the no template controls.

6. In the **Melt Curve** Plot, view the signal intensity and calls for the unknown samples.

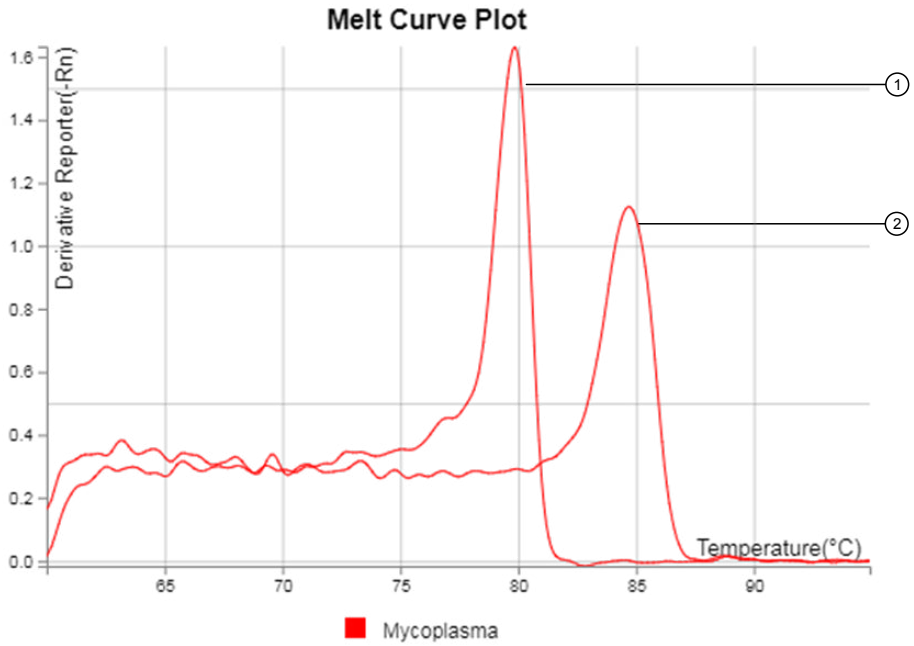


Figure 7 Example Melt Curve Plot

① Settings toolbar
② Melt Curve Stage dropdown list
③ Melting temperature (Mycoplasma)
④ Melting temperature (positive control)

Proceed to one of the following sections.

If	Then
The plate passed and there were no review calls in the wells	Proceed to "Export data and print reports" on page 110
The plate status was INVALID	Rerun the experiment
The plate had several FAIL or REVIEW wells	Proceed to Chapter 8, "Review the results and generate a report" and follow the more comprehensive instructions for reviewing the data. Data can be reanalyzed after omitting wells or changing the **Analysis Settings**.

4 Set up, run, and review ProteinSEQ™ experiments

Overview

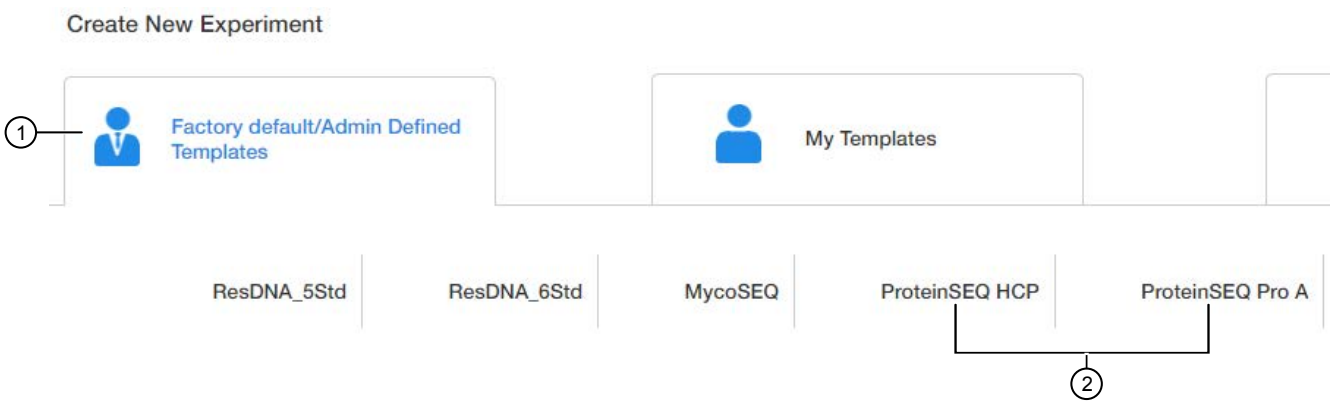
The ProteinSEQ™ Protein Detection System integrates real-time PCR assays, antibodies, instruments, and software to quantify host cell protein contaminants from cell lines.

ProteinSEQ™ experiments use a standard curve to determine absolute target quantity in your samples. The software measures amplification of the target in a standard dilution series and in test samples. The software then generates a non-linear standard curve using data from the standard dilution series. The standard curve is used to interpolate the absolute quantity of target in the test samples.

Create a ProteinSEQ™ experiment

1. In the **Home** screen, click the **Factory default/Admin Defined Template** tab, then select a **ProteinSEQ** template.

   Note: To create a copy of an existing ProteinSEQ™ experiment, see "Create a copy of an existing experiment" on page 85.



① **Factory default/Admin Defined Template** tab
② **ProteinSEQ** template (HCP or Protein A)

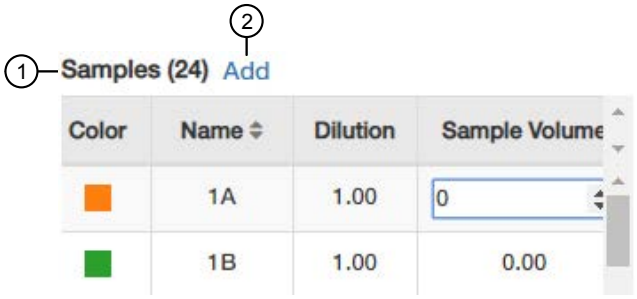
2. In the **Experiment Properties** pane of the **Setup** tab:
   a. (*Optional*) Change the system-generated name of the experiment.
   b. (*Optional*) Enter the plate **Barcode**, then add **Comments**.
   Default ProteinSEQ™ settings (cannot be changed)
   - **Experiment Type—Quantitation-Standard Curve**
   - **Chemistry—TaqMan™ Reagents**
   - **Ramp Speed—Fast - 40mins**
   c. Click **Next**.
3. In the **qPCR Method** pane of the **Setup** tab, view the default volume and cycling conditions (cannot be changed).

Figure 8 ProteinSEQ™ template default cycling conditions

4. Click **Next**.

5. In the **Samples** pane of the **Setup** tab, enter the sample **Name**, **Dilution**, and **Sample volume**. Add additional **Samples** if needed.

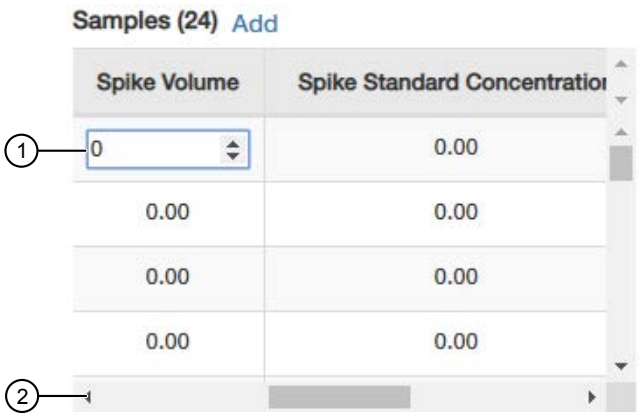
IMPORTANT! Do not change the **Targets**.



① **Samples** pane
② **Add**—adds additional samples

6. In the **Samples** pane of the **Setup** tab, scroll to the right, then enter the spike information.
   - **Spike Volume**—volume of sample to be spiked (for example, 180 µL).
   - **Spike Standard Concentration**—concentration of standard used in spiking (for example, 625 ng/mL). For Pro A, divide the concentration by a factor of 2 to account for the dilution by the sample preparation reagent.
   - **Reference**—the non-spiked sample; the mean quantity of reference is subtracted during % recovery calculation.
   - **Spike Input**—automatically calculated (double check that the amount is correct).

     Note: If incorrect, double check entries. Divide Spike Standard Concentration by a factor of 2 in Pro A assay.
   - (Optional) **Comments**



① Textbox—type in the value, or use the up and down arrows
② Scroll bar—scroll to find the spike parameter

For more information on plate setup, see "Assign plate and well attributes" on page 76.

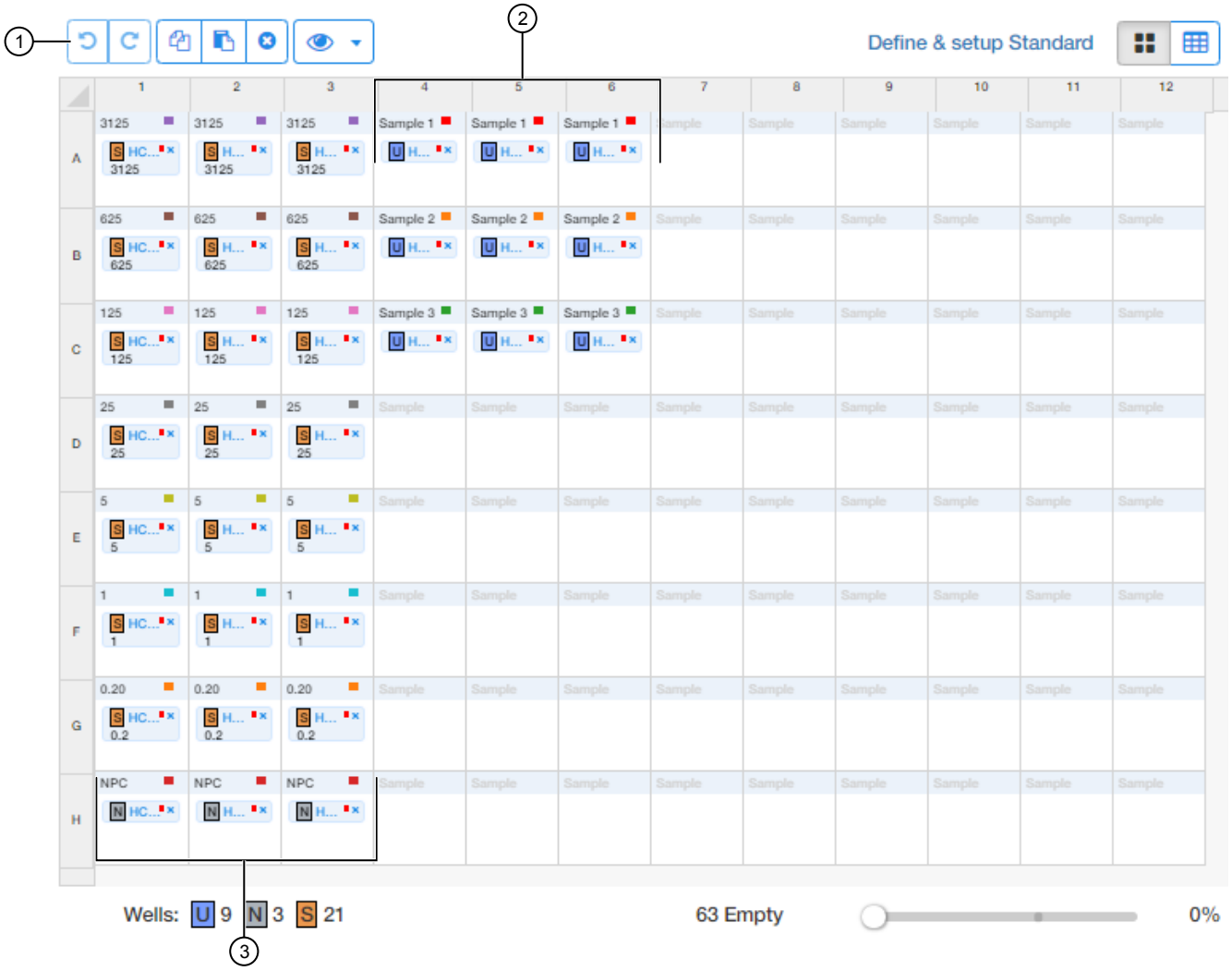


Figure 9 ProteinSEQ™ HCP template default sample plate layout

① Toolbar (in order: Undo, Redo, Copy, Paste, Delete, View)
② 7 standards in triplicate (rows A–G); No Protein Controls (NPCs) in triplicate (row H)
③ 3 default **Samples**

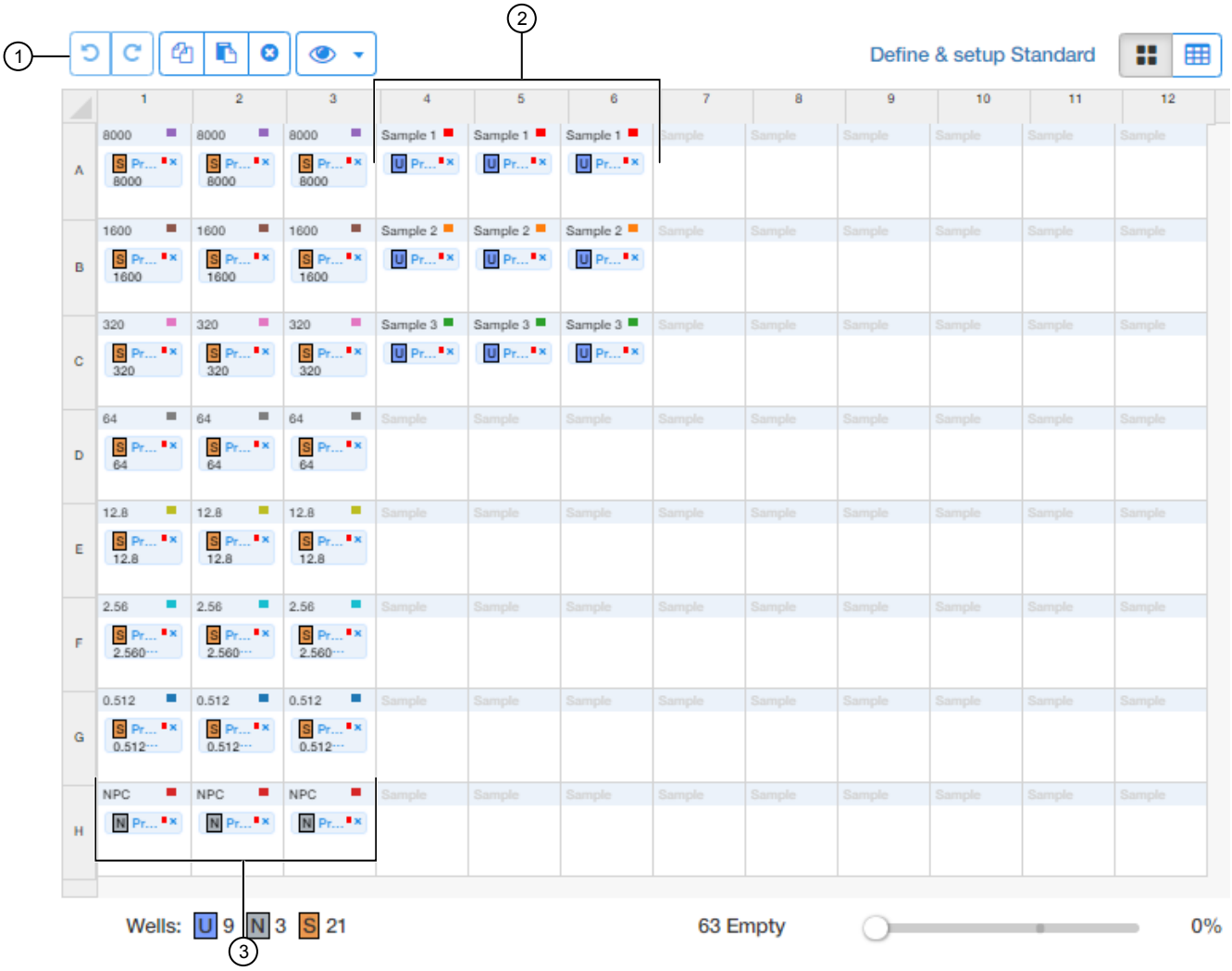


Figure 10 ProteinSEQ™ Pro A template default sample plate layout

① Toolbar (in order: Undo, Redo, Copy, Paste, Delete, View)
② 7 standards in triplicate (rows A–G); No Protein Controls (NPCs) in triplicate (row H)
③ 3 default **Samples**

7. Click **Next**.
   The **Run** tab is displayed.

8. Experiments are auto-saved in the software. To save, exit the experiment. The software prompts you to save changes. Click **Yes**.

   Note: Clicking **Save As** will create a copy of the experiment.

9. Assemble the PCR reactions following the manufacturer's instructions for the reagents and following the plate layout set up in the template. See *ProteinSEQ™ CHO Host Cell Protein Quantification Kit User Guide* (Pub. No. MAN0010806) or *ProteinSEQ™ Protein A Quantification Kit User Guide* (Pub. No. MAN0013524).

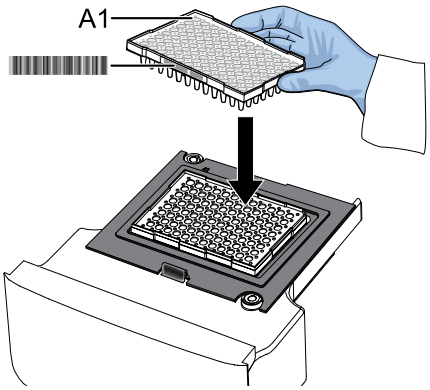
Immediately proceed to "Load the plate in the instrument" on page 33.

Load the plate in the instrument

CAUTION! Use optical flat caps for tubes. Rounded caps can damage the heated cover.

Load the plate.

1. Touch ⏏ to eject the instrument drawer.
2. Load the plate onto the plate adapter so that:
   - Well A1 of the plate is in the top-left corner of the plate adapter.
   - The barcode faces the front of the instrument.



IMPORTANT! The instrument should be used by trained operators who have been warned of the moving parts hazard.

Note: Do not remove the black plate adapter before loading a plate or tube strips. If used, tube strips can fit loosely in the adapter, but the heated cover applies the appropriate pressure to seat the tube strips securely in the adapter.

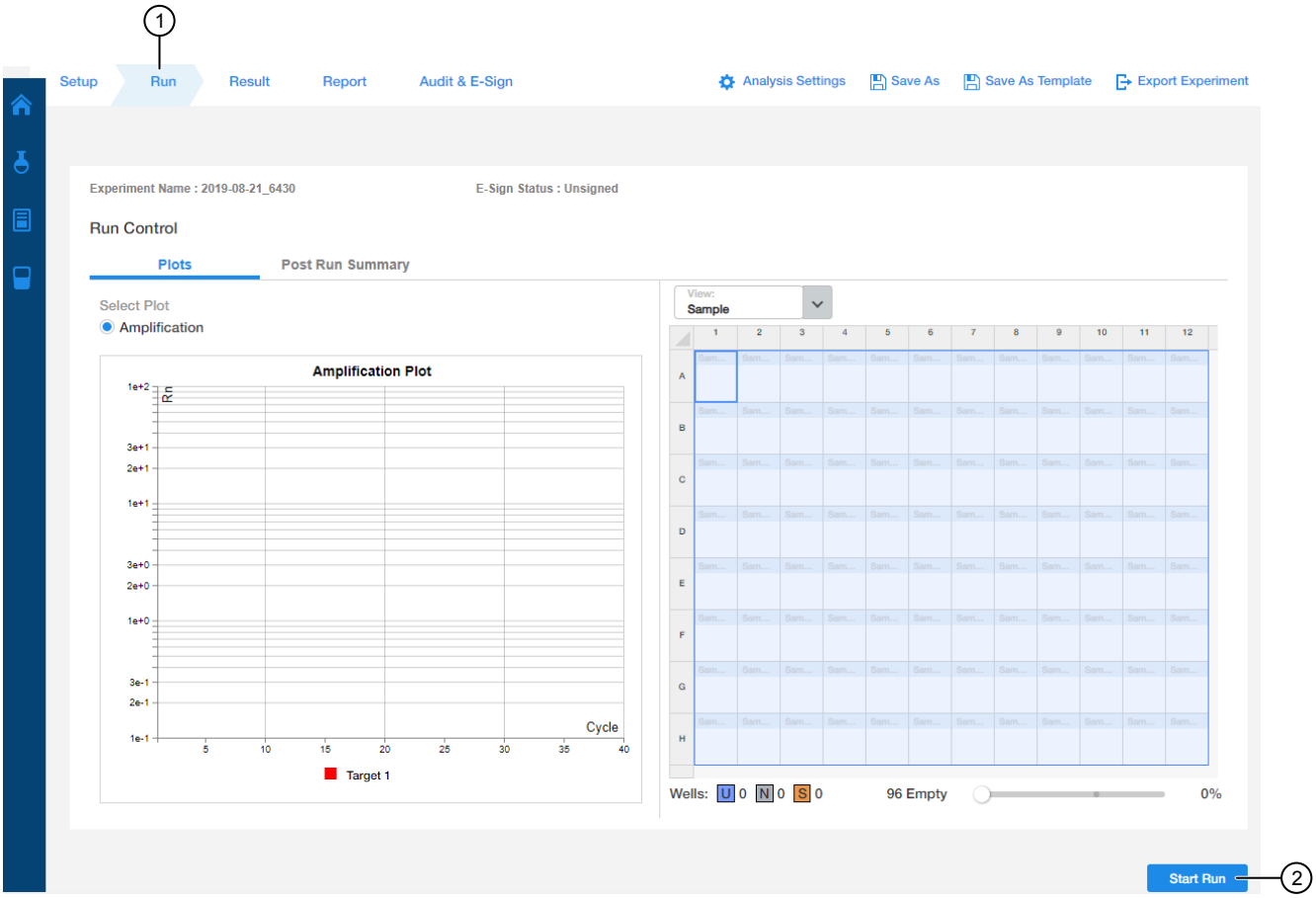
CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the plate temperature can reach 100°C. Allow it to cool to room temperature before handling.

3. Touch ⏏ to close the instrument drawer.

Start the run

Start the run in the AccuSEQ™ Software.

Option	Description
If the experiment is open	Click **Start Run**.
If the experiment is closed	1. Open the experiment. See "Open an experiment" on page 86. 2. Click the **Run** tab. 3. Click **Start Run**.



① **Run** tab
② **Start Run** button

A message stating **Run has been started successfully** is displayed when the run has started.

Monitor the run

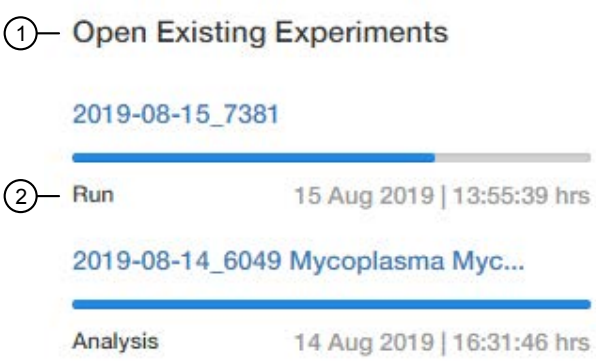
During the instrument run, you can monitor the run from the following places:

- On the instrument touchscreen.
- In the **Monitor the Run** pane of the AccuSEQ™ Software **(Home)** screen.

1. Instrument name
2. Instrument status (Ready, Running, Offline)
3. Calibration status
   - Calibrated—All required calibrations are "Current". Required calibrations include: Background, ROI/Uniformity, and Dyes.
   - Not calibrated—None of the required calibrations are complete.
   - Requires calibration—One or more dyes are not calibrated.
4. Time lapsed (if a run is in progress)
5. Total run time
6. Experiment name

- In the **Open Existing Experiments** pane of the AccuSEQ™ Software **(Home)** screen. The experiment being run is the first experiment listed. Status is **Run**.

  Note: When the run is complete, the status changes to **Analysis** and the bar changes to completely blue.



1. **Open Existing Experiments** pane
2. Experiment status

- In the **Run** tab of the AccuSEQ™ Real-Time PCR Software.
  You can perform the following actions.
  - Select wells in the plate layout to highlight respective curves in the plot.
  - Hover over curves in the plot for well information.
  - (*Optional*) Change what is displayed in the table wells, by selecting **Sample Name**, **Sample Color**, or **Target** in the **View** dropdown list.

  For more information, see "Run screen" on page 21.

On run completion, the **Post Run Summary** displays the run length, user and instrument information, and a list of any errors that occurred.

When the run is complete, unload the plate from the instrument.

Review results

Workflow: ProteinSEQ™ experiments

"Evaluate the overall shape of the Amplification Plot curves" on page 51

▼

"View and evaluate the Standard Curve Plot" on page 54

▼

Review data for outliers and *(optional)* Omit outliers from analysis

▼

(Optional) Review the dye signal profile using the Multicomponent Plot

▼

(Optional) View and evaluate the Raw Data Plot

▼

(Optional) Review the flags in the QC Summary

▼

(Optional) Configure the analysis settings (View and configure the analysis settings)

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

Evaluate the overall shape of the Amplification Plot curves

You can evaluate the overall shape of the **Amplification** curves in the **Result** tab.

Open the experiment in the AccuSEQ™ Software. See "Open an experiment" on page 86.

1. In the **Results Analysis** pane of the **Result** tab, select **Amplification** from the horizontal scroll bar.

   Note: If no data are displayed in the **Result Analysis** pane, then click **Analyze**.

① Amplification Plot

The **Amplification Plot** is displayed for the selected wells in the **(Grid View)** .

2. Ensure that the **Target** is correct.

3. (*Optional*) Click **(Plot Settings)** , then make the following selections:

   - **Plot Type**: **ΔRn, Rn, or C_T**
   - **Graph Type**: **Log or Linear**
   - **Show**: **Legend**, **Cq Mark**, or **Unselected**
   - **Plot Color**: **Target**, **Sample**, **Well**, or **Flag_Status**
   - **Threshold**: Select **Auto** or specify a threshold, then decide whether to **Show Threshold**.
   - **Baseline**: Decide whether to **Show Baseline**.

4. (*Optional*) Adjust the **(Plot Properties)**.

   a. In the **General** tab, add a **Plot Title**, adjust the **Font** and **Color**, then click **Apply**.

   b. In the **X Axis** or **Y Axis** tabs, you can:

      - Add a **Label**
      - Select whether you want **Tick Marks**
      - Select **Auto-adjust range** or enter minimum and maximum values

      Note: The minimum value must be greater than 0.

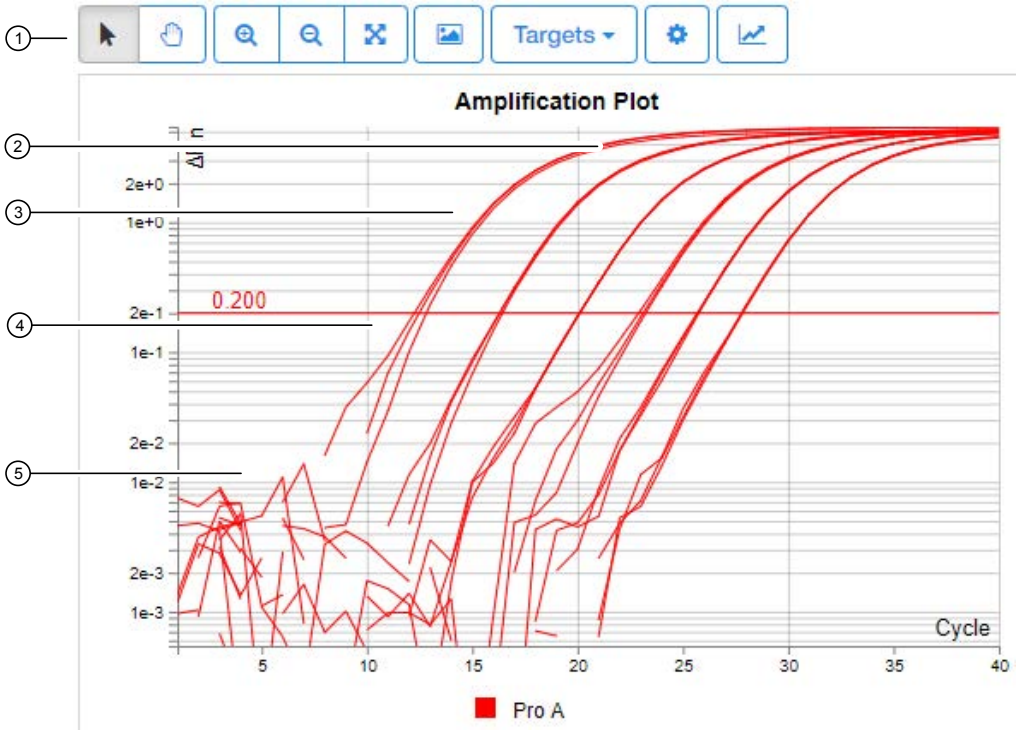


Figure 11 Typical Amplification Plot

① Amplification Plot tools
② Plateau phase
③ Linear phase
④ Exponential (geometric) phase
⑤ Baseline

Standard Curve Plot overview

The Standard Curve Plot displays the standard curve for samples that are designated as standards. The software calculates the quantity of an unknown target from the standard curve.

AccuSEQ™ Software v3.0 fits ProteinSEQ™ experiment standards to a standard curve using non-linear method to obtain interpolated values for unknown samples.

- 4PL is commonly used for symmetric curves with asymptotes for both the lower and upper Protein A concentrations.
- 5PL is commonly used if the curve is asymmetric or if either the lower or upper asymptote is not present.
- Apply 1/Y or 1/Y^2 weighting according to your criteria

Evaluate dynamic range using the %CV and the quality of the curve fit.

- R^2 is appropriate for judging linear fits but it is not an appropriate metric for evaluating the quality of a non-linear fit.
- Common acceptance criteria for non-linear curve fits are back-calculation values of 80-120% throughout the curve and 75-125% at the Lower Limit of Quantification (LLOQ).
- Common acceptance criteria for precision are %CV ≤20% throughout the curve and ≤25% at the LLOQ.

View and evaluate the Standard Curve Plot

1. In the **Result** tab, select **Standard Curve** in the horizontal scroll bar.

① Standard Curve plot

2. (*Optional*) Click **Table View** to configure the plot, then make the following selections:
   - **Targets**: Select the target of interest
   - **Plot Settings**: Select the **Plot Color Sample**, **Target**, or **Task**
   - **Plot Properties**: Edit **Plot Title**, change fonts, colors, labels, and select whether to auto-adjust or manually enter the range.

     Note: The minimum value must be greater than 0.
   - **Save Image**: Save the image.
   - Use the select, pan, and zoom options to interact with the plot.
3. Select all wells in the **Grid View**
   The Standard Curve Plot is displayed.
4. Select **4PL** and **None** for plot settings.
   The equation and **Target** are displayed below the plot.
5. Confirm that the back-calculation values meet the experimental criteria.

6. Visually check that all unknown sample values fall within the standard curve range.

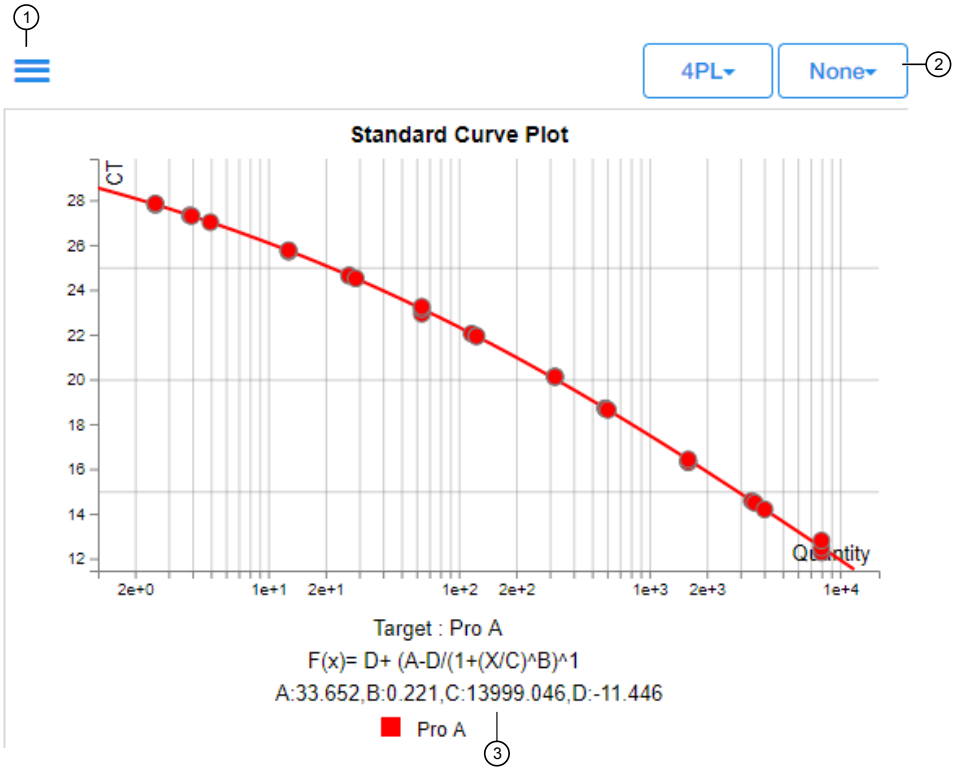


Figure 12 Example Standard Curve Plot

① Settings toolbar
② Default plot settings
③ Target, plot, and slope information:

- F(x) is the final protein concentration.
- A—Minimum asymptote. In a standard curve assay, this is the response value at 0 standard concentration.
- B—Hill's slope; refers to the steepness of the curve; should be positive or negative.
- C—Concentration at the inflection point; where the curvature changes direction or signs.
- D—Maximum asymptote. In a standard curve assay, this is the response value for infinite standard concentration.

Proceed to one of the following sections.

If	Then
The amplification and standard curve plots were normal and there were no flags in the wells	Proceed to "Export data and print reports" on page 110
The plate had several wells with abnormal amplification or which contained flags	Repeat the experiment, adjusting the template setup and analysis settings to improve results. or Proceed to Chapter 8, "Review the results and generate a report" and follow the more comprehensive instructions for reviewing the data. Data can be reanalyzed after omitting wells or changing the **Analysis Settings**.

5 Set up, run, and review resDNASEQ™ experiments

Overview

The resDNASEQ™ Quantitative DNA Kits are used to quantify residual host-cell DNA from CHO, *E. coli*, HEK293, Human, Vero, *Pichia*, NS0, and MDCK cell lines, which are used for production of biopharmaceutical products.

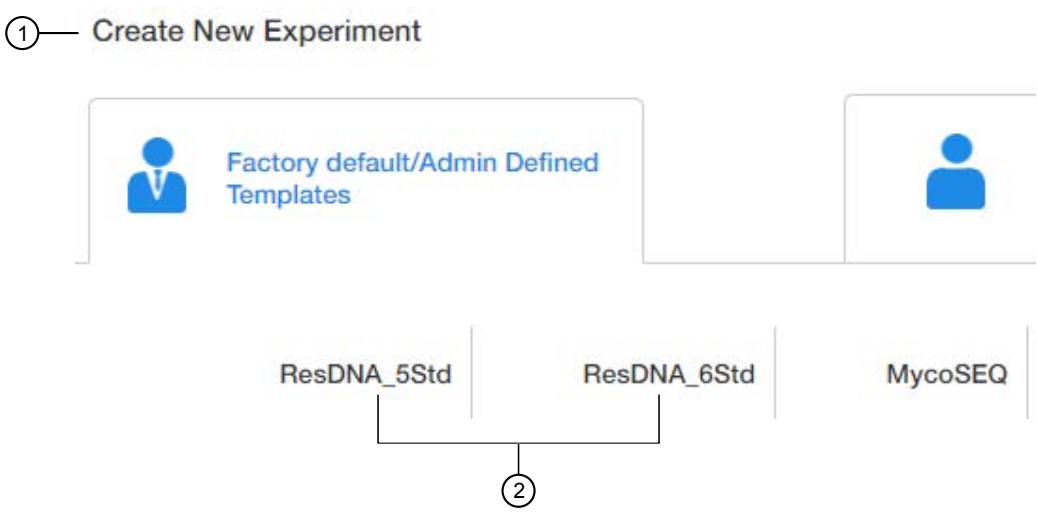
resDNASEQ™ experiments use a standard curve to determine absolute target quantity in your samples. The software performs the following tasks.

1. The software measures amplification of the target in a standard dilution series and in test samples.
2. The software generates a standard curve using data from the standard dilution series.
3. The software uses the standard curve to interpolate the absolute quantity of target in the test samples.

Create a resDNASEQ™ experiment

1. In the **Home** screen, click the **Factory default/Admin Defined Template** tab, then select a **resDNASEQ** template.

   Note: To create an experiment from an existing resDNASEQ™ experiment, see "Create a copy of an existing experiment" on page 85.



① **Factory default/Admin Defined Template** tab
② **resDNASEQ** template (ResDNA_5Std or ResDNA_6Std)

Template	Assays
5 standards (_5Std)	*E. coli*, HEK293, Human, and *Pichia*
6 standards (_6Std)	CHO, Vero, MDCK, and NS0

2. In the **Experiment Properties** pane of the **Setup** tab:
   a. (*Optional*) Change the system-generated name of the experiment.
   b. (*Optional*) Enter the plate **Barcode**, then add **Comments**.
   Default resDNASEQ™ settings (cannot be changed)
   - **Experiment Type** is **Quantitation-Standard Curve**
   - **Chemistry** is **TaqMan™ Reagents**
   - **Ramp Speed** is **Standard - 2hrs**
   c. Click **Next**.

3. In the **qPCR Method** pane of the **Setup** tab, view the default volume and cycling conditions (cannot be changed).

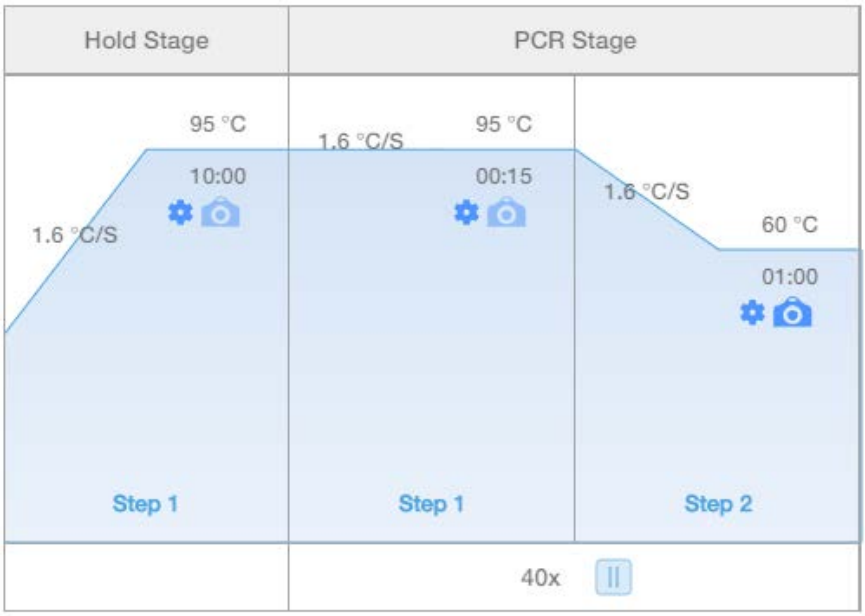
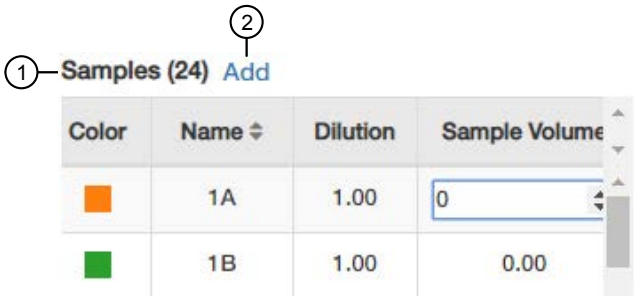


Figure 13 resDNASEQ™ template default cycling conditions

4. Click **Next**.

5. In the **Samples** pane of the **Setup** tab, enter the sample **Name** and **Dilution**. **Sample Volume** is not applicable. Add additional **Samples** if needed.

IMPORTANT! Do not change the **Targets**.



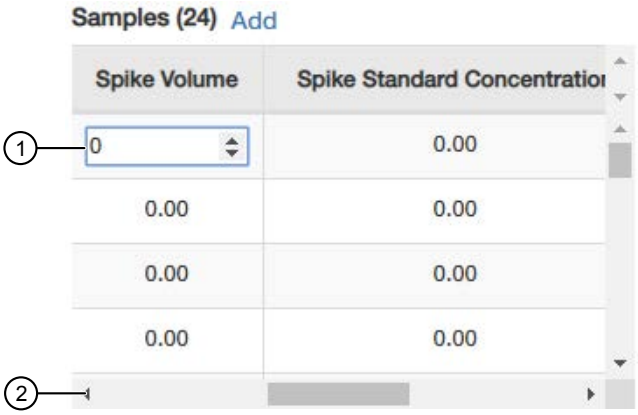
① **Samples** pane
② **Add**—adds additional samples

6. In the **Samples** pane of the **Setup** tab, scroll to the right, then enter the spike information.

   For more information on plate setup, see "Assign plate and well attributes" on page 76.

   - **Sample Volume**—not applicable; leave as default (0).
   - **Spike Volume**—volume of DNA added to the PCR (set to 10).
   - **Spike Standard Concentration**—expected spike amount per reaction (for example, 10pg).
   - **Reference**—the non-spiked sample; the mean quantity of reference is subtracted during % recovery calculation.
   - **Spike Input**—automatically calculated (double check if correct).

     Note: If incorrect, be sure **Spike Volume** is set to 10 and **Spike Standard Concentration** is the expected pg spike per PCR reaction.
   - (Optional) **Comments**
   - **Protein Concentration**—Drug substance protein concentration (if Total DNA in pg DNA/mg Protein is required).



① Textbox—type in the value, or use the up and down arrows
② Scroll bar—scroll to find the spike parameter

For more information on plate setup, see "Assign plate and well attributes" on page 76.

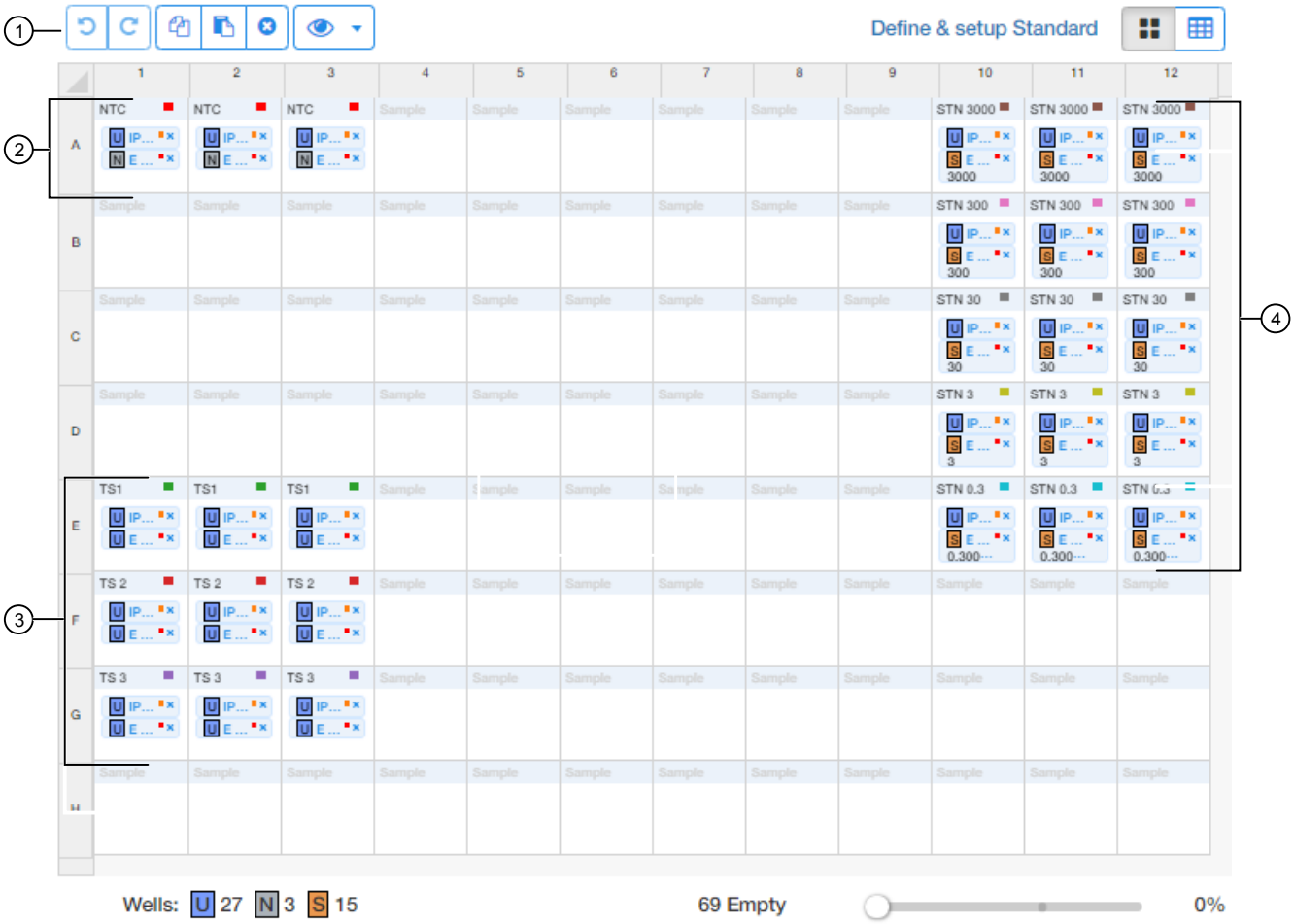


Figure 14 resDNASEQ™_5Std template default sample plate layout

① Toolbar (in order: Undo, Redo, Copy, Paste, Delete, View)
② 3 No Template Control (NTC) samples
③ 3 default **Samples**
④ Standard curve dilutions (S) in triplicate

7. Click **Next**.
   The **Run** tab is displayed.

8. Experiments are auto-saved in the software. To save, exit the experiment. The software prompts you to save changes. Click **Yes**.

   Note: Clicking **Save As** will create a copy of the experiment.

9. Assemble the PCR reactions following the manufacturer's instructions for the reagents and following the plate layout set up in the template. See *resDNASEQ™ Quantitative DNA Kits User Guide* (Pub. No. 4469836).

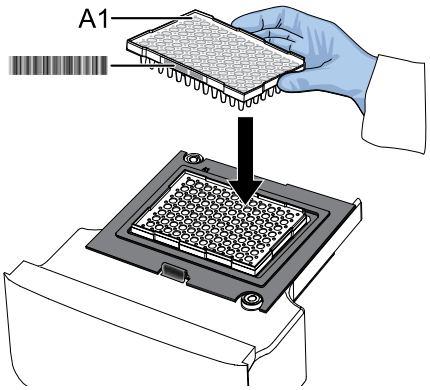
Immediately proceed to "Load the plate in the instrument" on page 33.

Load the plate in the instrument

CAUTION! Use optical flat caps for tubes. Rounded caps can damage the heated cover.

Load the plate.

1. Touch ⏏ to eject the instrument drawer.
2. Load the plate onto the plate adapter so that:
   - Well A1 of the plate is in the top-left corner of the plate adapter.
   - The barcode faces the front of the instrument.



IMPORTANT! The instrument should be used by trained operators who have been warned of the moving parts hazard.

Note: Do not remove the black plate adapter before loading a plate or tube strips. If used, tube strips can fit loosely in the adapter, but the heated cover applies the appropriate pressure to seat the tube strips securely in the adapter.

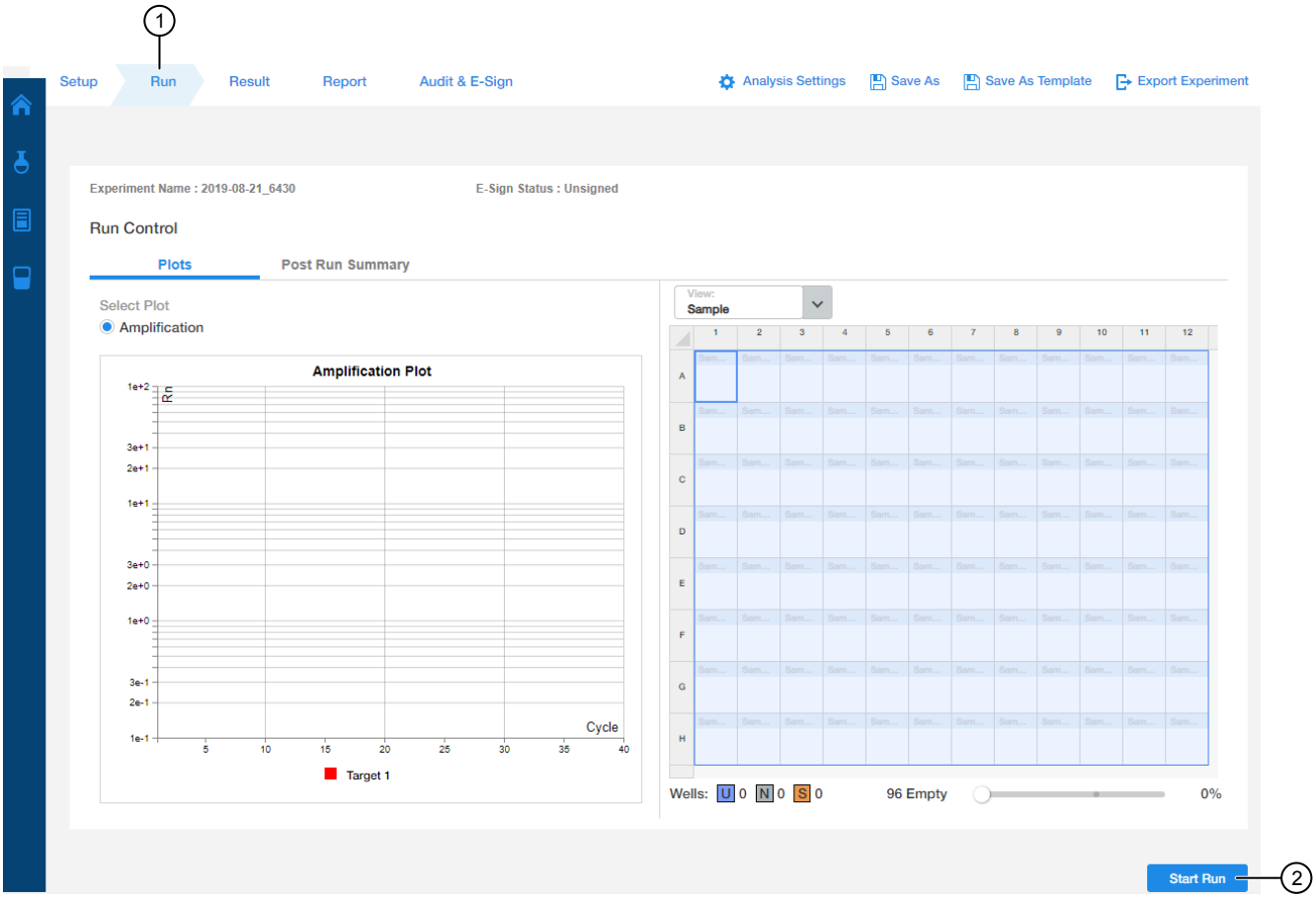
CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the plate temperature can reach 100°C. Allow it to cool to room temperature before handling.

3. Touch ⏏ to close the instrument drawer.

Start the run

Start the run in the AccuSEQ™ Software.

Option	Description
If the experiment is open	Click **Start Run**.
If the experiment is closed	1. Open the experiment. See "Open an experiment" on page 86. 2. Click the **Run** tab. 3. Click **Start Run**.



1 **Run** tab
2 **Start Run** button

A message stating **Run has been started successfully** is displayed when the run has started.

Monitor the run

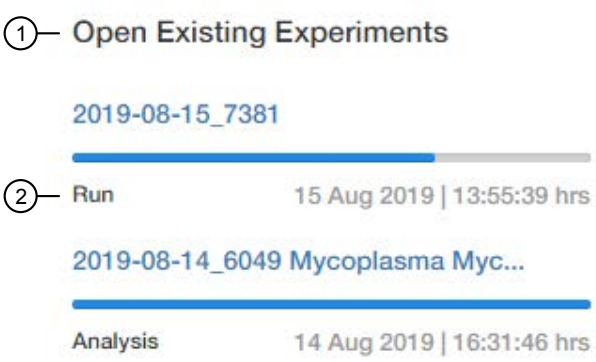
During the instrument run, you can monitor the run from the following places:

- On the instrument touchscreen.
- In the **Monitor the Run** pane of the AccuSEQ™ Software **(Home)** screen.

1. Instrument name
2. Instrument status (Ready, Running, Offline)
3. Calibration status
   - Calibrated—All required calibrations are "Current". Required calibrations include: Background, ROI/Uniformity, and Dyes.
   - Not calibrated—None of the required calibrations are complete.
   - Requires calibration—One or more dyes are not calibrated.
4. Time lapsed (if a run is in progress)
5. Total run time
6. Experiment name

- In the **Open Existing Experiments** pane of the AccuSEQ™ Software **(Home)** screen. The experiment being run is the first experiment listed. Status is **Run**.

  Note: When the run is complete, the status changes to **Analysis** and the bar changes to completely blue.



1. **Open Existing Experiments** pane
2. Experiment status

- In the **Run** tab of the AccuSEQ™ Real-Time PCR Software.
  You can perform the following actions.
  - Select wells in the plate layout to highlight respective curves in the plot.
  - Hover over curves in the plot for well information.
  - (*Optional*) Change what is displayed in the table wells, by selecting **Sample Name**, **Sample Color**, or **Target** in the **View** dropdown list.

  For more information, see "Run screen" on page 21.

On run completion, the **Post Run Summary** displays the run length, user and instrument information, and a list of any errors that occurred.

When the run is complete, unload the plate from the instrument.

Review results

Workflow: resDNASEQ™ experiments

Evaluate results in the Amplification Plot

▼

View and evaluate the Standard Curve Plot

▼

Review data for outliers and *(optional)* Omit outliers from analysis

▼

(Optional) Review the dye signal profile using the Multicomponent Plot

▼

(Optional) View and evaluate the Raw Data Plot

▼

(Optional) Review the flags in the QC Summary

▼

(Optional) Configure the analysis settings (View and configure the analysis settings)

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

Evaluate the overall shape of the Amplification Plot curves

You can evaluate the overall shape of the **Amplification** curves in the **Result** tab.

Open the experiment in the AccuSEQ™ Software. See "Open an experiment" on page 86.

1. In the **Results Analysis** pane of the **Result** tab, select **Amplification** from the horizontal scroll bar.

   Note: If no data are displayed in the **Result** tab, click **Result Analysis**, then click **Analyze**.

① Amplification Plot

The **Amplification Plot** is displayed for the selected wells in the **(Grid View)** .

2. Ensure that the **Target** is correct.

3. (*Optional*) Click **(Settings)** , then make the following selections:

   - **Plot Type**: **ΔRn, Rn, or C_T**
   - **Graph Type**: **Log or Linear**
   - **Show**: **Legend**, **Cq Mark**, or **Unselected**
   - **Plot Color**: **Target**, **Sample**, **Well**, or **Flag_Status**
   - **Threshold**: Select **Auto** or specify a threshold, then decide whether to **Show Threshold**.
   - **Baseline**: Decide whether to **Show Baseline**.

4. (*Optional*) Adjust the **(Plot Properties)**.

   a. In the **General** tab, add a **Plot Title**, adjust the **Font** and **Color**, then click **Apply**.

   b. In the **X Axis** or **Y Axis** tabs, you can:

      - Add a **Label**
      - Select whether you want **Tick Marks**
      - Select **Auto-adjust range** or enter minimum and maximum values

      Note: The minimum value must be greater than 0.

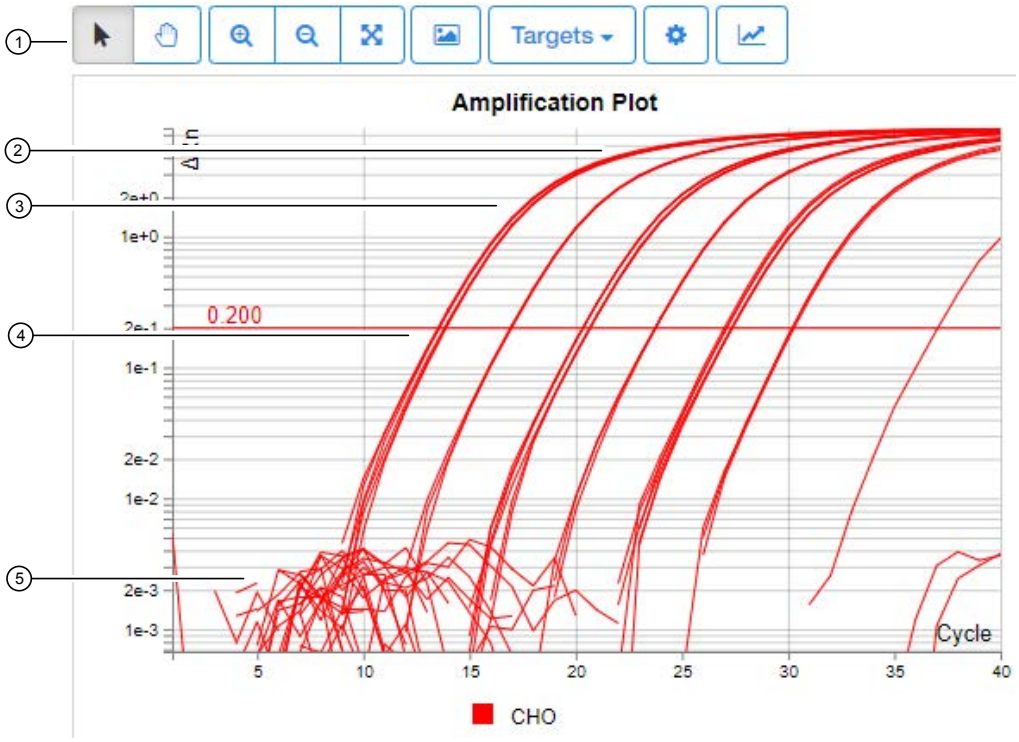


Figure 15 Typical Amplification Plot

① Amplification Plot tools
② Plateau phase
③ Linear phase
④ Exponential (geometric) phase
⑤ Baseline

Standard Curve Plot overview

The Standard Curve Plot displays the standard curve for samples that are designated as standards. The software calculates the quantity of an unknown target from the standard curve.

Table 1 Results or metrics to review in the Standard Curve Plot

Results or metrics	Description	Criteria for evaluation
Slope and amplification efficiency	The amplification efficiency is calculated using the slope of the regression line in the standard curve.	A slope close to –3.3 indicates optimal, 100% PCR amplification efficiency. Factors that affect amplification efficiency: • Range of standard quantities—use a broad range of standard quantities, 5 to 6 logs. • Number of standard replicates—include replicates to decrease the effects of pipetting inaccuracies. • PCR inhibitors—PCR inhibitors and contamination in the reaction can reduce amplification efficiency. • Other possible factors: – Inaccurate sample or reagent pipetting – Incorrect plate setup

Results or metrics	Description	Criteria for evaluation
R^2 value (correlation coefficient)	The R^2 value is a measure of the closeness of fit between the regression line and the individual C_q data points of the standard reactions.	• A value of 1.00 indicates a perfect fit between the regression line and the data points. • An R^2 value > 0.9900 is desirable.
C_t values	The threshold cycle (C_t) is the PCR cycle number at which the fluorescence level meets the threshold.	(Custom assays only) A C_t value > 8 and < 35 is desirable. • C_t value < 8—there may be too much template in the reaction. • C_t value > 35—there may be a low amount of target in the reaction; for C_t values > 35, expect a higher standard deviation.

View and evaluate the Standard Curve Plot

1. In the **Result** tab, select **Standard Curve** in the horizontal scroll bar.

① Standard Curve plot

2. (*Optional*) Click **Table View** to configure the plot, then make the following selections:
   - **Targets**: Select the target of interest
   - **Plot Settings**: Select the **Plot Color Sample**, **Target**, or **Task**
   - **Plot Properties**: Edit **Plot Title**, change fonts, colors, labels, and select whether to auto-adjust or manually enter the range.

     Note: The minimum value must be greater than 0.
   - **Save Image**: Save the image.
   - Use the select, pan, and zoom options to interact with the plot.

3. Select all wells in the **Grid View**
   The Standard Curve Plot is displayed. The slope, R^2 value, amplification efficiency, and error are displayed below the plot.

4. Confirm that the slope, R^2 value, and standard error meet the experimental criteria.
   (Recommended) $R^2 \geq 0.99$.

5. Visually check that all unknown sample values fall within the standard curve range.

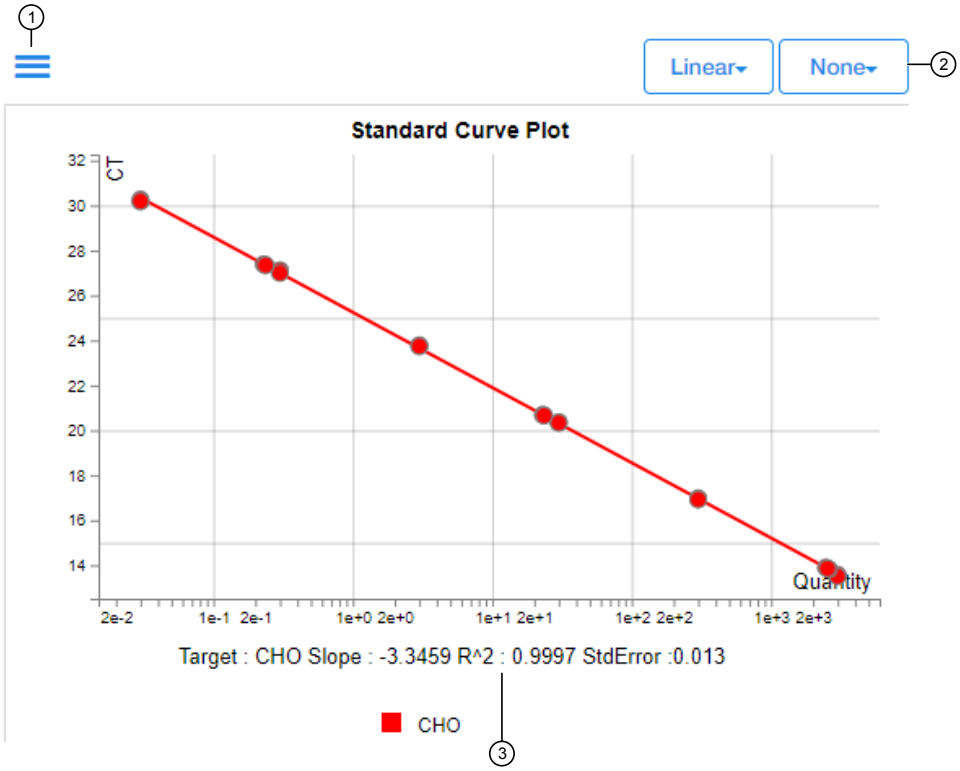


Figure 16 Example Standard Curve Plot

① Settings toolbar
② Default plot settings
③ R^2 value

Proceed to one of the following sections.

If	Then
The amplification and standard curve plots were normal and there were no flags in the wells	Proceed to "Export data and print reports" on page 110
The plate had several wells with abnormal amplification or which contained flags	Repeat the experiment, adjusting the template setup and analysis settings to improve results. or Proceed to Chapter 8, "Review the results and generate a report" and follow the more comprehensive instructions for reviewing the data. Data can be reanalyzed after omitting wells or changing the **Analysis Settings**.

6 Create a custom template

This section describes the general procedures to create a template in the software. To use the default or admin-defined templates in the software, see the following sections:

- Chapter 3, "Set up, run, and review MycoSEQ™ experiments"
- Chapter 4, "Set up, run, and review ProteinSEQ™ experiments"
- Chapter 5, "Set up, run, and review resDNASEQ™ experiments"

Template types

There are three types of templates in the AccuSEQ™ Real-Time PCR Software v3.0.

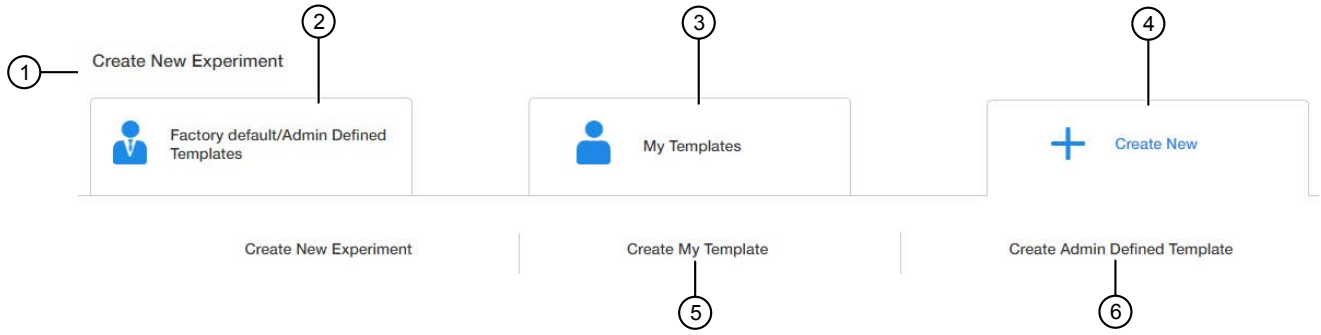
- Factory default Templates—templates supplied with the AccuSEQ™ Real-Time PCR Software v3.0; locked to editing by any user.
- Admin Defined Templates—templates that are created by the Administrator.
- My Templates—templates that are imported or created by the user that is signed in; no other users have permission to view these templates.

Templates can be locked or unlocked. See "Lock a template" on page 81.

Create or open a template

Create a new template or open an existing template in the **(Home)** screen.

- In the **Create New Experiment** pane, perform one of the following tasks to create a new template.

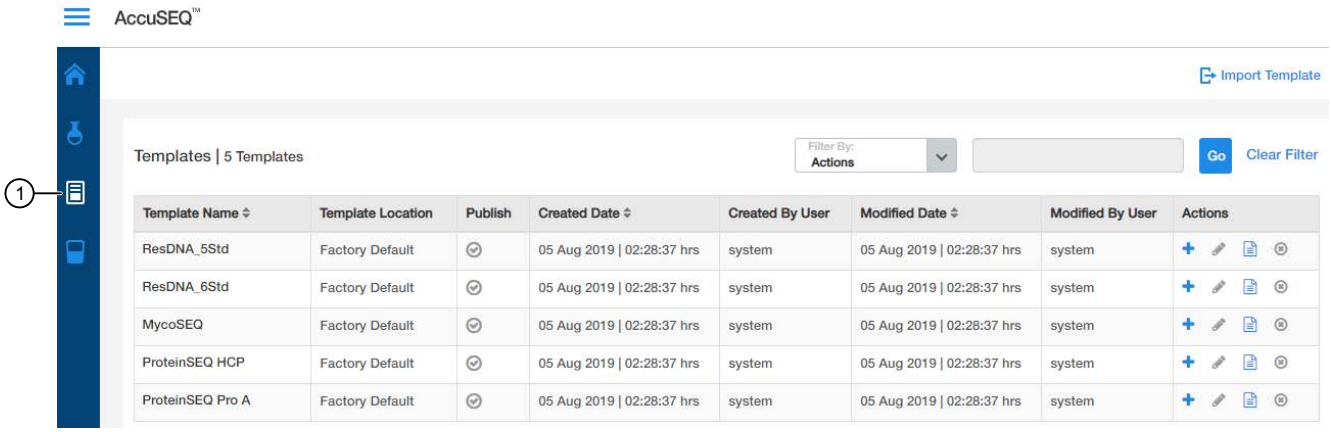


① **Create New Experiment** pane
② **Factory default/Admin Defined Templates**—List of existing default or Admin Defined templates. These templates can be used as templates for new experiments.
③ **My Templates**—List of templates available to the user that is signed in. These templates can be used as templates for new experiments.
④ **Create New**—Used to create an experiment or template with no pre-existing settings.
⑤ **Create My Template**—Used to create a new template (stored locally in **My Templates**).
⑥ **Create Admin Defined Template**—Used to create a new template (Administrator only).

Note: Available to other users when **Published**. See "Publish a template" on page 81.

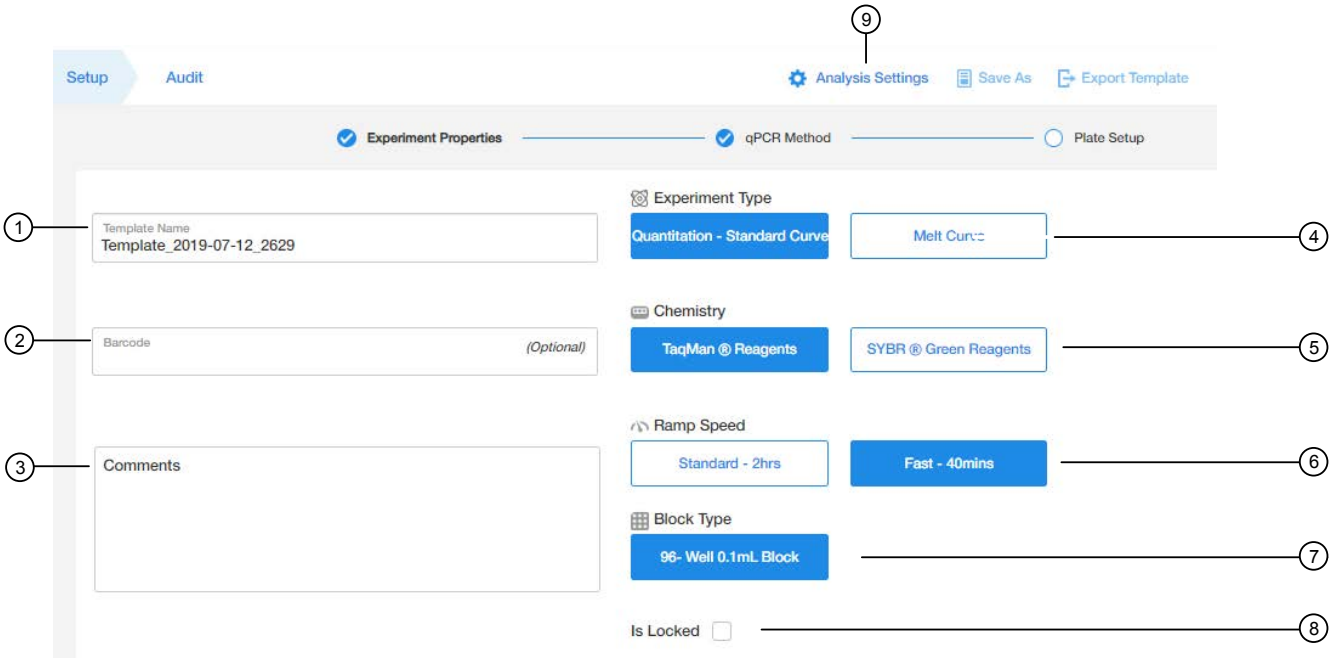
To	Action
Create a template without pre-existing settings	1. Click + **Create New**. 2. Select **Create My Template** or **Create Admin Defined Template**. 3. Edit the **Experiment Properties**, **qPCR Method**, and **Plate Setup** as required. 4. Click **Save**.
Create a template from an existing **Factory default/Admin Defined Template** template	1. Open a **Factory default/Admin Defined Template**. 2. Edit the **Experiment Properties**, **qPCR Method**, and **Plate Setup** as required. A new experiment is created. 3. Save the template (do one of the following). a. Click **Home**. The template is auto-saved. b. Click **Save As**, enter the **Template Name** and properties, select if the template **Is Locked** or **Is Admin Defined**, then click **Save**.
Create a template from an **Admin Defined** template (alternate)	1. Navigate to **Templates**. 2. Select the desired file, then click **Edit**. 3. Edit the **Experiment Properties**, **qPCR Method**, and **Plate Setup** as required. A new experiment is created. 4. Save the template (do one of the following). a. Click **Home**. The template is auto-saved. b. Click **Save As**, enter the **Template Name** and properties, select if the template **Is Locked** or **Is Admin Defined**, then click **Save**.
Create a template from an existing user-created template **Note:** Only templates that are created by the signed-in user are listed under **My Templates**.	1. Select **My Templates**. 2. Open the desired template. 3. Edit the **Experiment Properties**, **qPCR Method**, and **Plate Setup** as required. A new experiment is created. 4. Save the template (do one of the following). a. Click **Home**. The template is auto-saved. b. Click **Save As**, enter the **Template Name** and properties, select if the template **Is Locked** or **Is Admin Defined**, then click **Save**.

- Click **Templates** in the left navigation pane for a full list of templates.



① **Templates** icon

Enter template properties



① Software-generated **Template Name**
② **Barcode** field
③ **Comments** field
④ **Experiment Type**
⑤ **Chemistry**
⑥ **Ramp Speed**
⑦ **Block Type** (fixed as a 96-Well 0.1mL block).
⑧ **Is Locked** checkbox (Only Administrators can create locked **Admin Defined Templates**).
⑨ **Analysis Settings**

1. (*Optional*) In the **Template Name** field, modify the file name.
2. (*Optional*) Click the **Barcode** field, then enter a plate barcode.
3. (*Optional*) Enter information in the **Comments** field.
4. In the **Setup** tab, select, or edit the **Experiment Type**, **Chemistry**, **Ramp Speed**, and **Block Type**.

   Note: The experiment type defines the available options for the template setup.
5. (*Optional*) Select **Is Locked** to lock the template. If locked, users are unable to edit the template.
6. (*Optional*) Click **Analysis settings** to change the default C_t Settings, Flag Settings, and Advanced Settings. See "Configure analysis settings" on page 92.

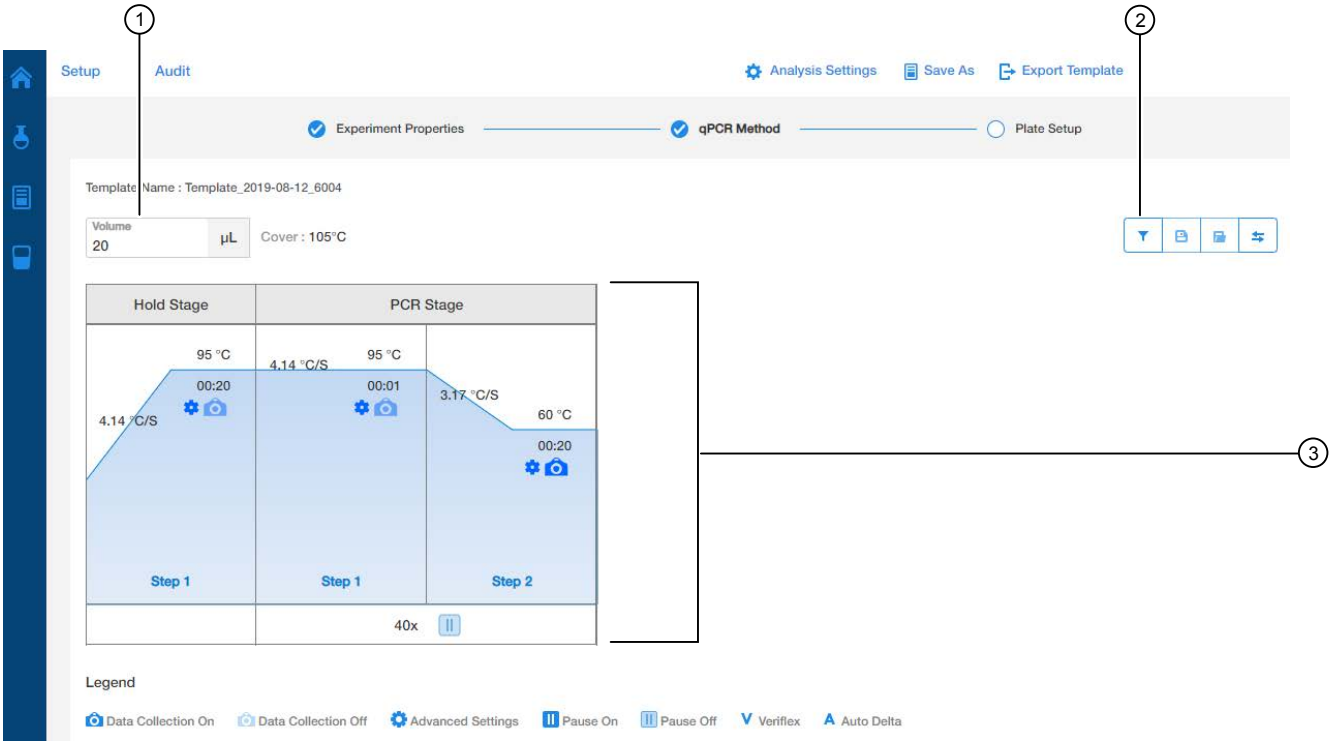
   Note: Click **Apply** to save any changes before closing the window.
7. Click **Next**.

The **qPCR Method** screen is displayed.

Confirm or edit the run method and optical filter selection

Note: This section provides general procedures to edit the run method and optical filter selection in the qPCR Method.

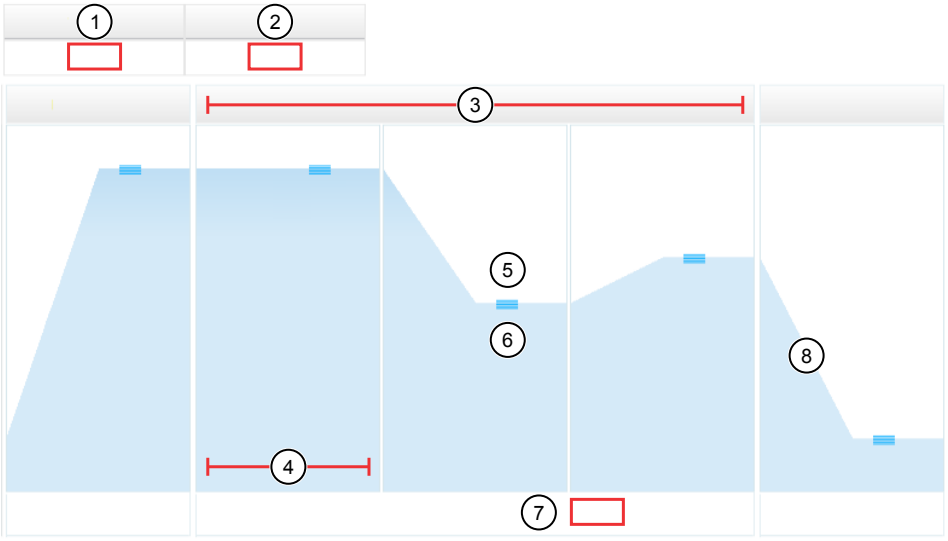
For specific instructions for each assay type, see the corresponding chapter in this guide.



① Reaction volume. The volume is fixed in locked templates.
② Optical filters.
③ PCR parameters.

1. (*Optional*) In the **qPCR Method** tab, adjust the reaction volume.
2. (*Optional*) Edit the default run method (thermal protocol).
   - The default run method is optimized for TaqMan™ assays and a broad range of other reagents.
   - To edit the default run method, see "Adjust method parameters" on page 87.
3. (*Optional*) Click **(Optical Filter Settings)** to view or edit the default filter settings.
   - The default optical filter selection is for factory-calibrated (system) dyes.
   - For more information about system dyes and their calibration and optical filter selection, see *QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide* (Pub. No. MAN0010407).
4. Click **Next**.

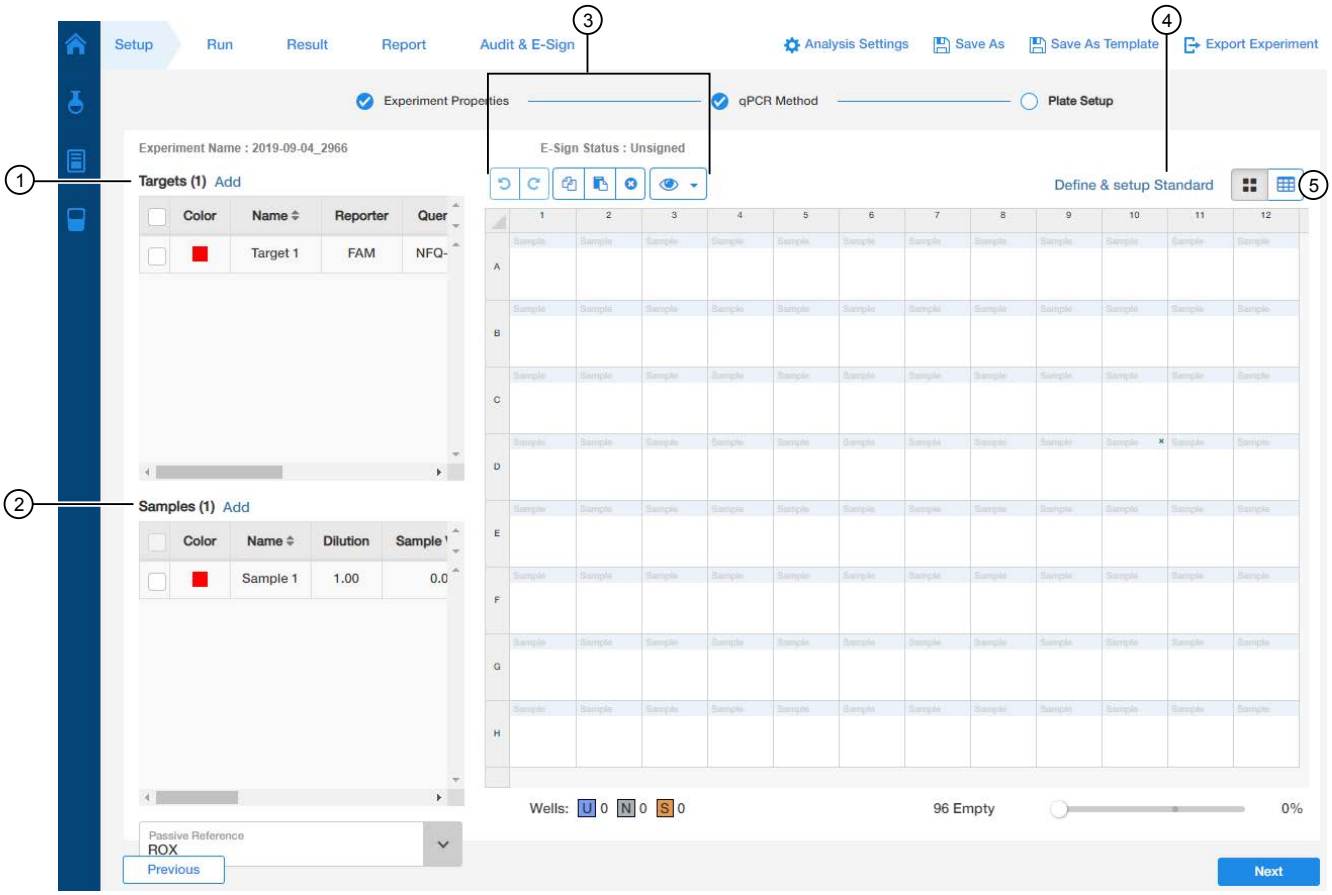
Method elements



① Reaction volume
② Heated cover temperature
③ Stage of thermal protocol
④ Step within a stage
⑤ Step temperature
⑥ Step hold time
⑦ Number of cycles
⑧ Temperature ramp rate

Assign plate and well attributes

Note: This section provides general procedures to set up the plate. For specific instructions for each assay type, see the corresponding chapter in this guide. Do not change **Targets** for default assay templates.



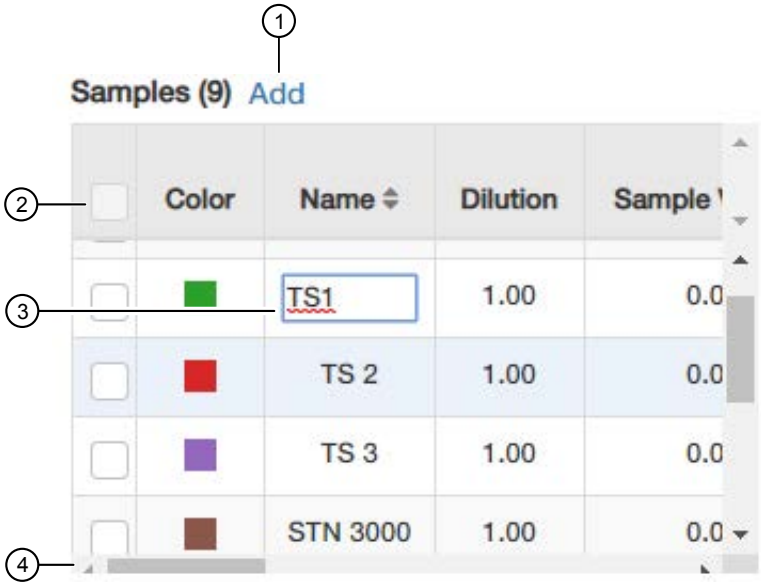
① **Targets**
② **Samples**
③ Plate setup toolbar
④ **Define & setup Standard** (standard curve experiments only)
⑤ View (Grid View or Table View)

1. In **Plate Setup** screen, click or click-drag to select plate wells in the **(Grid View)** of the plate.

2. Assign the well attributes for the selected wells.

   - To add new **Samples** or **Targets**, click **Add** in the appropriate column on the left of the screen, then edit the new Name and other properties as required. The new sample or target is then selectable within the wells of the plate.

     Note: To change sample names, click the name in the Name column, then type the new name. To change Reporters and Quenchers, click the dye, then select from the dropdown list.



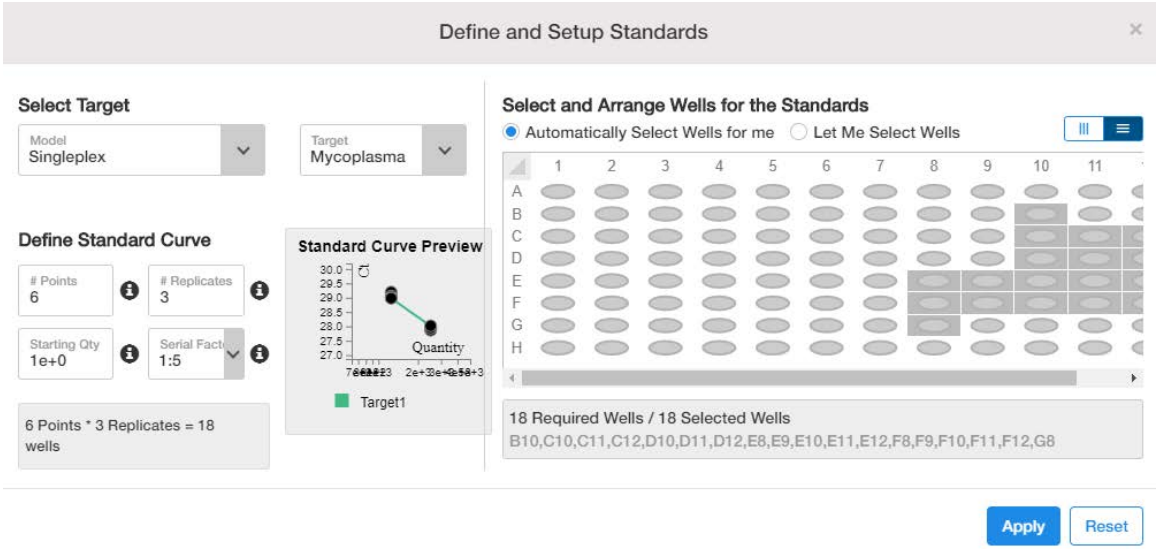
① **Add** button
② Checkbox—Select **Targets** and **Samples** to go in the selected well.
③ Textbox—Click the name to edit.
④ Scrollbar—Use to scroll to additional properties.

- Use the plate setup toolbar (above the plate) to make edits to the plate.
  - Click **View** to show/hide the Sample Name, Sample Color, or Target from the view.
- To add consecutive samples (with the same **Target**), select a well, then click-drag the dark blue box to the right. See "Select plate wells" on page 79 for additional actions.

- (*Optional*) Click **Define & setup Standard** to set up a standard curve.

  Note: This option is not available for MycoSEQ™ assays.

  a. Select Singleplex or Multiplex.
  b. Select or type in the Target.
  c. Define the number of points and replicates, the starting quantity, and the serial dilution factor.
  d. (*Optional*) Select **Automatically Select Wells for me** or **Let Me Select Wells** used for the standard curve.
  e. Click **Apply** to save before closing the window.



3. (*Optional*) Double-click a well to enter comments for the selected well.

4. Select a **Passive Reference** from the dropdown list (bottom left of screen).

Select plate wells

- Select plate wells in the **(Grid View)** .

To	Action
Select a single well	Click a well in the plate
Select multiple wells	Click-drag in the plate
Select contiguous wells	Shift+click wells in the plate
Select a column of wells	Click a column header
Select all wells	Click the top-left corner of the plate grid
Select a block of wells	Click a well to define a corner, then shift+click another well on the opposite corner

Note: It is not currently possible to Ctrl+click to add wells in different areas of the plate.

- Select plate wells in the **(Table View)**.

To	Action
Select a single well	Click a row in the table
Select non-contiguous wells	Ctrl+click rows in the table
Deselect a single well	Ctrl+click the selected row

Save the custom template

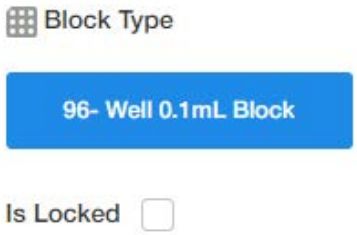
To save the template, do one of the following.

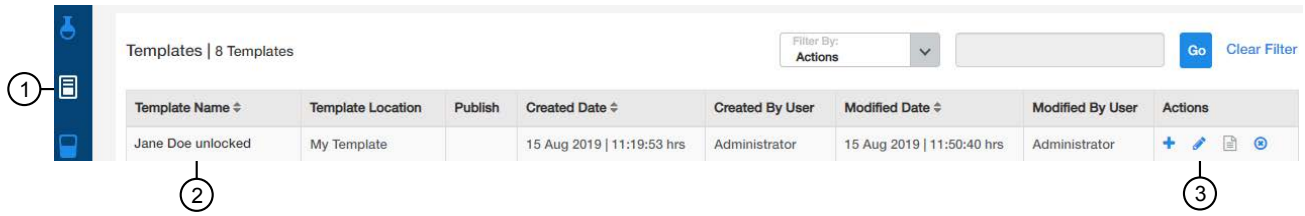
Option	Description
To save a custom template that you created new	Enter the **Experiment Properties**, **qPCR Method** and **Plate Setup** information, then click **Save**. **Template saved successfully** appears.
To save a template from an open Experiment	1. Click **Save As Template** in any tab. 2. Enter a **Template Name**. 3. (*Optional*) Enter a description of the template. 4. Select whether the template **Is Admin Defined** or **Is Locked**. 5. Click **Save**.
To save a template (from an experiment) created from an existing template	1. Open the template that you want to copy. 2. Click **Save As Template** in any tab. 3. Enter a new **Template Name**. 4. (*Optional*) Enter a description of the template. 5. Select whether the template **Is Admin Defined** or **Is Locked**. 6. Click **Save**. Once saved, the new template can be opened and edited if needed.
To save a template from an existing template (excluding factory default templates)	1. Navigate to **Templates**. 2. Click to open the template that you want to copy. 3. Click **Save As** in any tab. 4. Enter a new **Template Name**. 5. (*Optional*) Enter a description of the template. 6. Select whether the template **Is Admin Defined** or **Is Locked**. 7. Click **Save**. Once saved, the new template can be opened and edited if needed.

Lock a template

New templates or unlocked templates can be locked. **Experiment Properties** cannot be edited in locked templates.

Lock the template.

Template type	Action
New	1. Check the **Is Locked** checkbox in the **Experiment Properties** screen of the **Setup** tab.  2. Click **Next** to proceed with locked template creation.
Unlocked template	1. Navigate to the Templates screen. 2. Select the template, then click **Edit**. 3. Check the **Is Locked** checkbox in the **Experiment Properties** screen of the **Setup** tab. 4. Click **Next** to proceed with locked template creation.

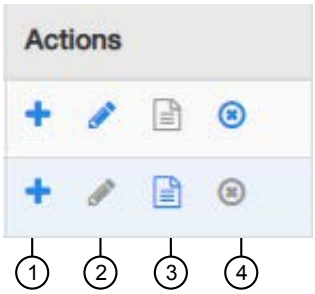


1. **Templates** list
2. **Template Name**
3. **Edit**

Publish a template

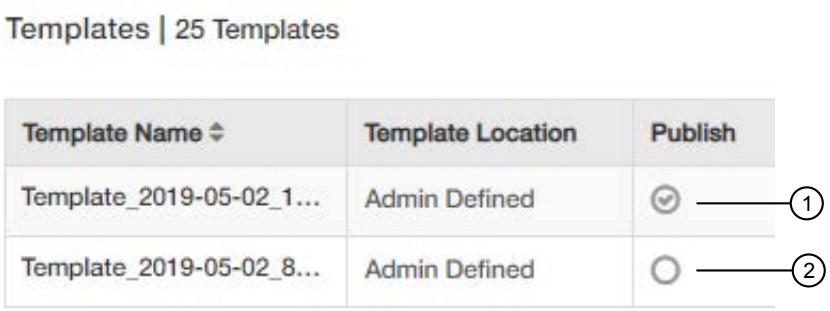
To make a template available for other users, publish the template.

1. Click the **Templates** tab, then select the template of interest.
2. Click **Publish Template** in the **Actions** column.
   Blue icons are active. Gray icons are inactive.



① Create Experiment
② Edit Template
③ Publish Template
④ Delete Template

3. Click **Yes** to verify that you want to publish the template.
   A message is displayed if the template was successfully published, click **OK**.
   Publish is then selected in the templates list.



① Published
② Unpublished

Import template

Import Template is used to import a template created in a different AccuSEQ™ Software v3.0 location.

IMPORTANT! Only EDT files from QuantStudio™ 5 Real-Time PCR Instruments with 0.1-mL blocks are supported for import in the AccuSEQ™ Software v3.0. EDT version 1.3.2 is recommended.

---

1. In the navigation pane of the **(Home)** screen, click **(Templates)**.
2. Click **Import Template**.
3. Browse to the file of interest, then click **Open**.

The template is displayed in the list of templates in the **Templates** pane.

Export a template

Export Template is used to export an experiment to a different AccuSEQ™ Software v3.0 location.

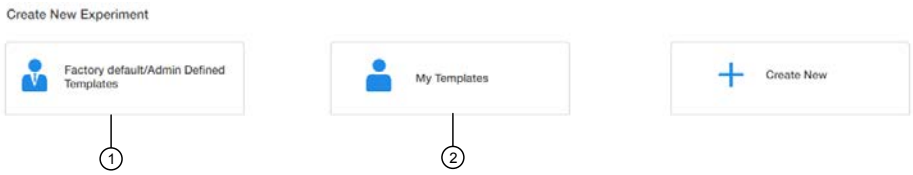
1. In the navigation pane of the **(Home)** screen, click **(Templates)**.
   The **Templates** pane is displayed.
2. Browse to the file of interest, then click **Edit**.
3. In the **Setup** or **Run** tab, click **Export Template**.
4. An EDT file is created, then downloaded.

   Note: You can change the Chrome™ browser settings to ask where to save each file before downloading.

7 General Experiment procedures

Create an experiment from a template

1. Open a template by doing one of the following:
   - In the **Create New Experiment** pane of the **(Home)** screen, select an existing template from the **Factory default/Admin Defined Templates** or **My Templates** tabs.



   ① **Factory default/Admin Defined Templates** tab
   ② **My Templates** tab

   - From the **(Home)** screen, click **(Templates)** for a full list of templates. Select a template, then click **(Create Experiment)**.

2. In the **Experiment Properties** pane of the **Setup** tab:
   a. (*Optional*) Change the system-generated name of the experiment.
   b. (*Optional*) Enter the plate **Barcode**.
   c. (*Optional*) Enter **Comments**.
   d. Ensure that the **Experiment Type**, **Chemistry**, and **Ramp Speed** are correct for the assay that you are running.
   e. Click **Next**.

3. In the **qPCR Method** pane of the **Setup** tab:
   a. Ensure that the reaction volume and cycling conditions are correct for the assay that you are running.
   b. Click **Next**.
4. In the **Plate Setup** pane of the **Setup** tab:
   a. Enter **Samples**, **Targets**, and standards.
   b. Click **Next**.

   The **Run** tab opens.

Create a copy of an existing experiment

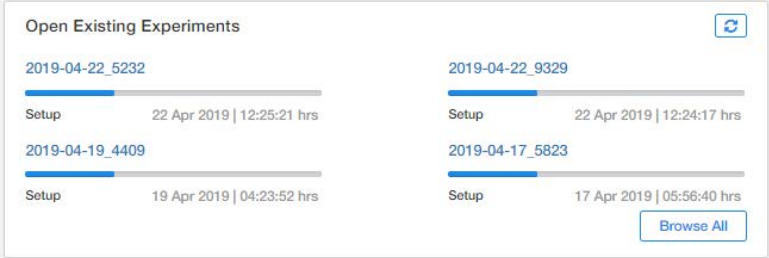
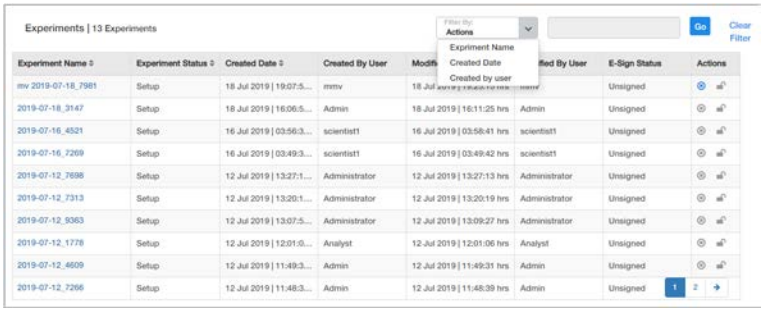
Usually, experiments are run from existing template files. To create a new template or experiment file, see Chapter 6, "Create a custom template".

1. Open an existing experiment. See "Open an experiment" on page 86.
   The **Setup** tab opens.
2. In any tab, click **Save As**.
3. Enter the new **Experiment Name**, then click **Save**.
4. Navigate to **Experiments**, open the copied experiment, then edit as needed.
5. In the **Experiment Properties** pane of the **Setup** tab:
   a. (*Optional*) Enter the plate barcode.
   b. Ensure that the **Experiment Type**, **Chemistry**, and **Ramp Speed** are correct.
   c. Click **Next**.
6. In the **qPCR Method** pane of the **Setup** tab:
   a. Ensure that the reaction volume and cycling conditions are correct.
   b. Click **Next**.
7. In the **Plate Setup** pane of the **Setup** tab:
   a. Enter or edit **Samples**, **Targets**, and standards.
   b. Click **Next**.

   The **Run** tab opens.
8. Click **Start Run**.

Open an experiment

You can open an experiment from 2 locations:

Location	Description
Home screen	To open the most recently run experiments: 1. Click (Home). 2. Click an experiment name, or click **Browse All**. 
Experiments screen	To open previously run experiments: 1. Click (Experiment). 2. If needed, filter, or display the next pages of the list. 3. Click an experiment name. 

Export an experiment

Export Experiment is used to export an experiment that is run on a QuantStudio™ 5 Real-Time PCR Instrument with a 0.1-mL block to a different AccuSEQ™ Software v3.0 location.

1. In the navigation pane of the **(Home)** screen, click **(Experiments)**.
   The **Experiments** pane is displayed.
2. Browse to the file of interest, then click the **Experiment Name** to open.
3. Click **Export Experiment** (top right corner).

4. An EDS file is created, then downloaded.

   Note: You can change the Chrome™ browser settings to ask where to save each file before downloading.

Import an experiment

Import Experiment is used to import an experiment created in a different AccuSEQ™ Software location.

IMPORTANT! Only EDS files from QuantStudio™ 5 Real-Time PCR Instruments with 0.1-mL blocks are supported for import in the AccuSEQ™ Software v3.0. EDS version 1.3.2 is recommended.

---

1. In the navigation pane of the **(Home)** screen, click **(Experiments)**.
   The **Experiments** pane is displayed.
2. Click **Import Experiment**.
3. Browse to the file of interest, then click **Open**.

The experiment is displayed in the list of experiments in the **Experiments** pane.

Note: The e-signature status of imported experiments is set to **Unsigned**.

Modify a qPCR method

Adjust method parameters

For an overview of the method as it is graphically represented, see "Method elements" on page 88.

- In the **qPCR Method** screen of the **Setup** tab, click a method parameter field to edit the following information:
  - Reaction volume
  - Temperature ramp rate
  - Step temperature
  - Step hold time
  - Number of cycles
- Hover over the temperature, then type in a new temperature to change a step temperature.
- Click to switch data collection on or off at each step.
  Data Collection On enables analysis of data that is collected throughout the PCR, for real-time analysis and troubleshooting.

- Click ⚙ to configure settings for **Auto Delta** or VeriFlex™ Zones for individual steps (see "Set up advanced temperature zones (Auto Delta Settings and VeriFlex™ Zones)" on page 89).

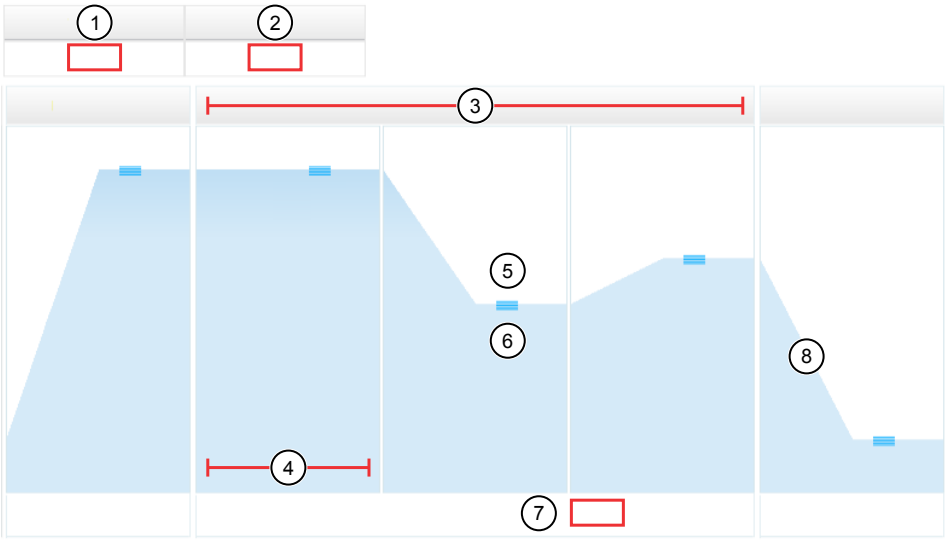
  Note: In melt curve stages, **Advanced Settings** are not applicable.

  An A or V is displayed in the PCR stage when **Auto Delta** or VeriFlex™, respectively, is enabled.

- Click ⏸ to configure pause settings.

- See the Help to learn more about adjusting the following parameters:
  - Adding or subtracting a stage
  - Adding or subtracting a step from a stage
  - Configuring optical filter settings

Method elements



① Reaction volume
② Heated cover temperature
③ Stage of thermal protocol
④ Step within a stage
⑤ Step temperature
⑥ Step hold time
⑦ Number of cycles
⑧ Temperature ramp rate

Add or remove a stage

1. In the **qPCR Method** tab, hover the cursor over the header or footer of a stage to display − and + on the stage borders.

   Note: Hovering within a stage on a step displays the same icons, but they apply to the step only.

2. Add or remove a stage.

Option	Action
Add a stage	1. Click + on the left or right border. 2. Select the type of stage to add.
Remove a stage	1. Click −.

To edit stages, see "Add or remove a step" on page 89.

Add or remove a step

1. In the **qPCR Method** tab, hover the cursor over a step to display − and +.
2. Add or remove a step.
   - Click + on the left or right border of a step to add a step before or after, respectively.
   - Click − to remove the step.

Set up advanced temperature zones (Auto Delta Settings and VeriFlex™ Zones)

Configure settings for **Auto Delta Settings** and **VeriFlex™ Zones**.

- **Auto Delta Settings**—Incremental increase or decrease of a cycle's temperature or hold time for a step in a cycling stage (not applicable for **Hold** or **Infinite Hold** stages).
- **VeriFlex™ Zones**—Independent temperature zones within 5°C of adjacent zones. The instrument block has 6 zones.

1. In the **qPCR Method** tab, click **(Advanced Setting)** in a step.

   Note: Any changes apply only to the step in which you clicked.

2. Configure either the **VeriFlex™ Zones** or **Auto Delta** for the selected step.
   - Select **Enable VeriFlex™**, then enter a temperature for each zone.

     Note: In the **Plate Setup** tab, the **VeriFlex™ Zones** display on the plate layout.
   - Select **Enable Auto Delta**, then enter a starting cycle, temperature, and time.
3. Click **Save**.

Add or adjust a pause step

1. In the **qPCR Method** tab, click ⏸ in the step.
2. Select **Pause Cycle**.
3. Enter a pause temperature between 4°C and 99°C.

   CAUTION! PHYSICAL INJURY HAZARD. During instrument operation, the plate temperature can reach 100°C. If you want to access the plate during a run pause, enter room temperature as the pause temperature and allow the plate to cool to room temperature before handling.

4. Enter the cycle after which the pause should occur.
5. Click outside of the pause dialog box to return to the method.
6. (*Optional*) To remove a pause, click ⏸, then deselect **Pause Cycle**.

Select optical filters

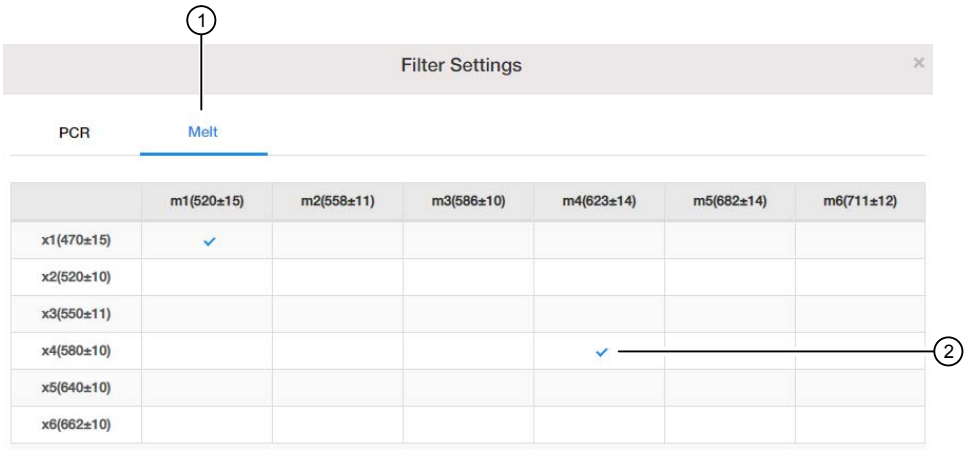
The need to edit optical filter settings is rare, and it is for advanced or custom uses only.

Use the optical filters settings to select a filter set to match the profile of a custom dye.

1. In the **qPCR Method** tab, select **(Optical Filter Settings)**.
   The excitation (x) and emission (m) wavelengths that correspond to each filter are shown on the screen.

2. Select the checkboxes to enable or disable filters.
   A **Melt** table is accessible if the method contains a melt curve stage. Otherwise, use the **PCR** table to select optical filters.

   IMPORTANT! Select filter 4 for custom melt curve experiments that use ROX™.



   ① **Melt** tab
   ② Filter 4

3. Click **Save** to save changes, or **Cancel** to cancel any changes.

4. (*Optional*) Click **Revert to Default** to reset filters.

For information on the dyes read by each filter, see your instrument user guide.

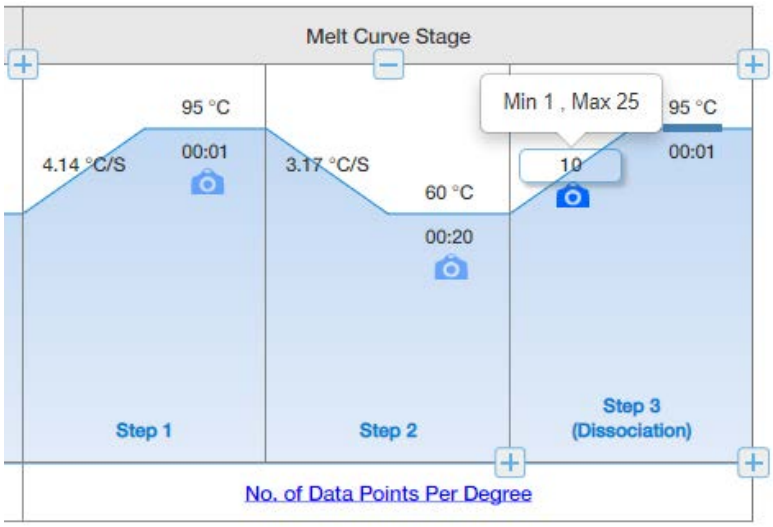
Edit the ramp increment for the melt curve dissociation step

In the **qPCR Method** section of the **Setup** tab, you can perform the following tasks to edit the ramp increment for the melt cure dissociation step.

- Select the ramp increment method for the dissociation step (Click **Continuous** found under the graphical representation of the thermal protocol to change the default).

Option	Description
Continuous (default)	Continuously increases the temperature by the ramp increment (°C/sec).
Step & Hold	Increases the temperature by the ramp increment (°C), then holds at that temperature for the specified time.
No. of Data Points per Degree	Increases the temperature by the ramp increment (°C) and collects the specified number of data points per degree increased.

- (*For all options*) Edit the temperature ramp increment.
  a. Click the ramp increment element in the **Dissociation** step.
  b. Enter a value or use the up/down arrows (default is 0.15°C/s).
- (*Step and Hold only*) Edit the hold time after each temperature increase.
  a. Click the time field under **Step & Hold**.
  b. Enter a value (default is 5 seconds).
- (*No. of Data Points per Degree only*) Edit the number of data points to be collected with each degree increase.
  a. Click the number of data points element in the **Dissociation** step.
  b. Enter a value or use the up/down arrows (default is 10 data points).



Configure analysis settings

This section describes the analysis settings that apply to all experiment types, unless otherwise noted.

Guidelines for the analysis settings

- We recommend analyzing the experiment with the default analysis settings.
- If the default analysis settings are not appropriate for the experiment, modify the settings in the **Analysis Settings** dialog box, then reanalyze the experiment.

The default analysis settings are different for each experiment type. The analysis settings determine the following parameters.

- How the baseline, threshold, and threshold cycle (C_t) are calculated
- Which flags are enabled
- Other analysis options that are specific to an experiment type

View and configure the analysis settings

1. In any open **Experiment**, click **Analysis Settings**. To open an experiment see "Open an experiment" on page 86.
2. View and (*optional*) configure the analysis settings.
3. (*Optional*) To return to the default settings, click **Revert to Default**.
4. Click **Apply**.
   Click **Cancel** to discard your changes.
   Data are reanalyzed automatically.

Melt Curve Settings overview

Use the **Melt Curve Settings** to configure melt curve parameters.

- Enable or disable **Multi Peak Calling**.
- Adjust the peak level relative to the dominant peak (%).

C_t settings overview

The default C_t settings are appropriate for most applications. Configuration of the settings is an option for analysis of not typical or unexpected run data.

Note: The **C_t Settings** feature is not available for experiments without a PCR stage, such as melt curve experiments.

Table 2 C_t Settings

Setting	Description
Data Step Selection	Determines the stage/step combination for C_t analysis (when there is more than one data collection point in the run method).
Default C_t Setting	Determines how the **Baseline Threshold** Algorithm is set. The **Default C_t Setting** are used for targets unless they have custom settings. **Note:** Use **Edit Default Settings** to edit the default settings for the assay.

Setting	Description
C_t Settings for Target	• **Default Settings** selected—The **Default C_t Settings** are used to calculate the C_t values for the target. • **Default Settings** deselected—The software allows manual setting of the baseline or the threshold.

Table 3 Recommendations for manual threshold and baseline settings

Setting	Recommendation
Threshold	Enter a value for the threshold so that the threshold is: • Above the background. • Below the plateau and linear phases of the amplification curve. • Within the exponential phase of the amplification curve.
Baseline	Select the **Start Cycle** and **End Cycle** values so that the baseline ends before significant fluorescence signal is detected.

Flag settings overview

Use the **Flag Settings** to configure the flag parameters.

- Adjust the sensitivity so that more wells or fewer wells are flagged.
- Change the flags applied by the software for each experiment type.

Advanced settings overview

Use the **Advanced Settings** tab to change baseline settings for individual wells.

Note: The **Advanced Settings** feature is not available for experiments without a PCR stage, such as melt curve experiments.

Rule settings overview (MycoSEQ™ only)

Note: The **Rule Settings** feature is currently only available for MycoSEQ™ experiments.

The default **Rule Settings** are appropriate for most MycoSEQ™ applications. Configuration of the settings is an option for analysis of not typical or unexpected run data. See "View and configure the analysis settings" on page 92.

Table 4 Rule Setting Calls

Setting	Description
Plate Call	Determines whether the plate has passed or failed (by the pass of both the positive and no template controls).
Inhibition Control	Monitors for inhibition in the unknown sample (inhibition in the presence of a positive control).
Well Calls (Unknown)	Determines whether individual wells have passed (called as present or absent), failed, or require review.

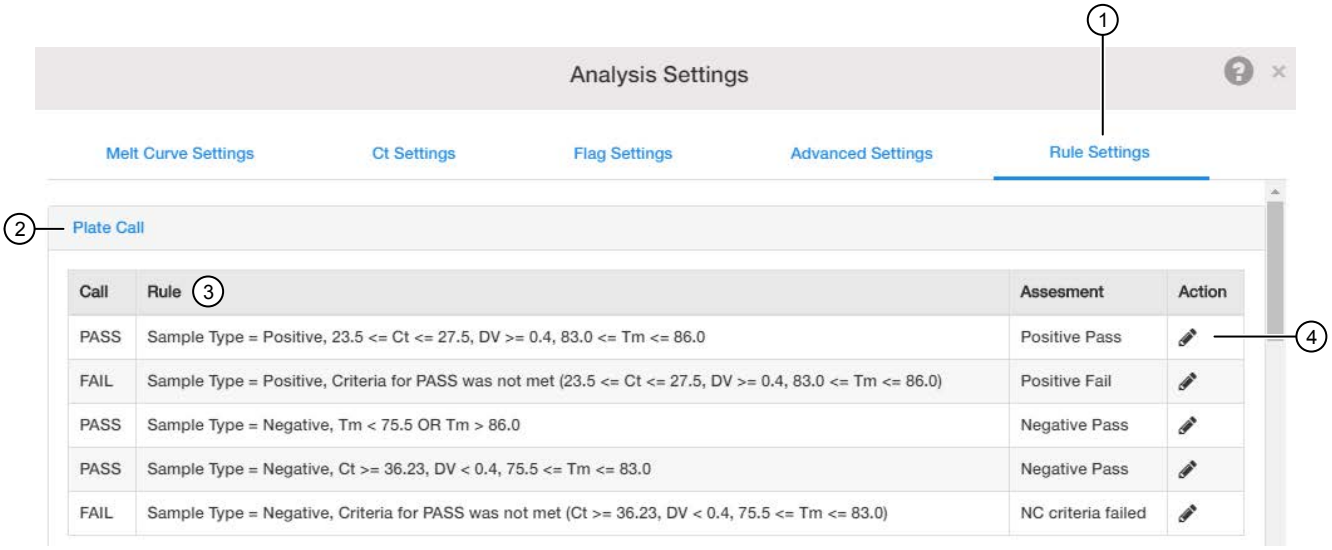


Figure 17 Example **Rule Settings** (MycoSEQ™ Plate Call)

① **Rule Settings** tab
② **Plate Call** pane
③ **Rule**—Ct, DV, and T_m values for each Sample type call.
④ **Edit**—The Ct, Δ CT (inhibition controls only), DV, and T_m parameters can be adjusted as required after completion of the run.

Note: **Rule Settings** are editable only after completion of a run.

Set up and run the PCR reactions

1. Assemble the PCR reactions. Follow the manufacturer's instructions for the reagents, then follow the plate layout set up in the software. See the appropriate kit user guide for more information.

2. Load the reaction plate into the instrument (see "Load the plate in the instrument" on page 33), then start the run (see "Monitor the run" on page 35).

8 Review the results and generate a report

This section includes general information to help in the review and export of the results. For more detailed information about the type of review required for a specific experiment type, see the corresponding chapter in this guide.

Overview of the Result tab

Review and analyze run data in the **Result** tab.

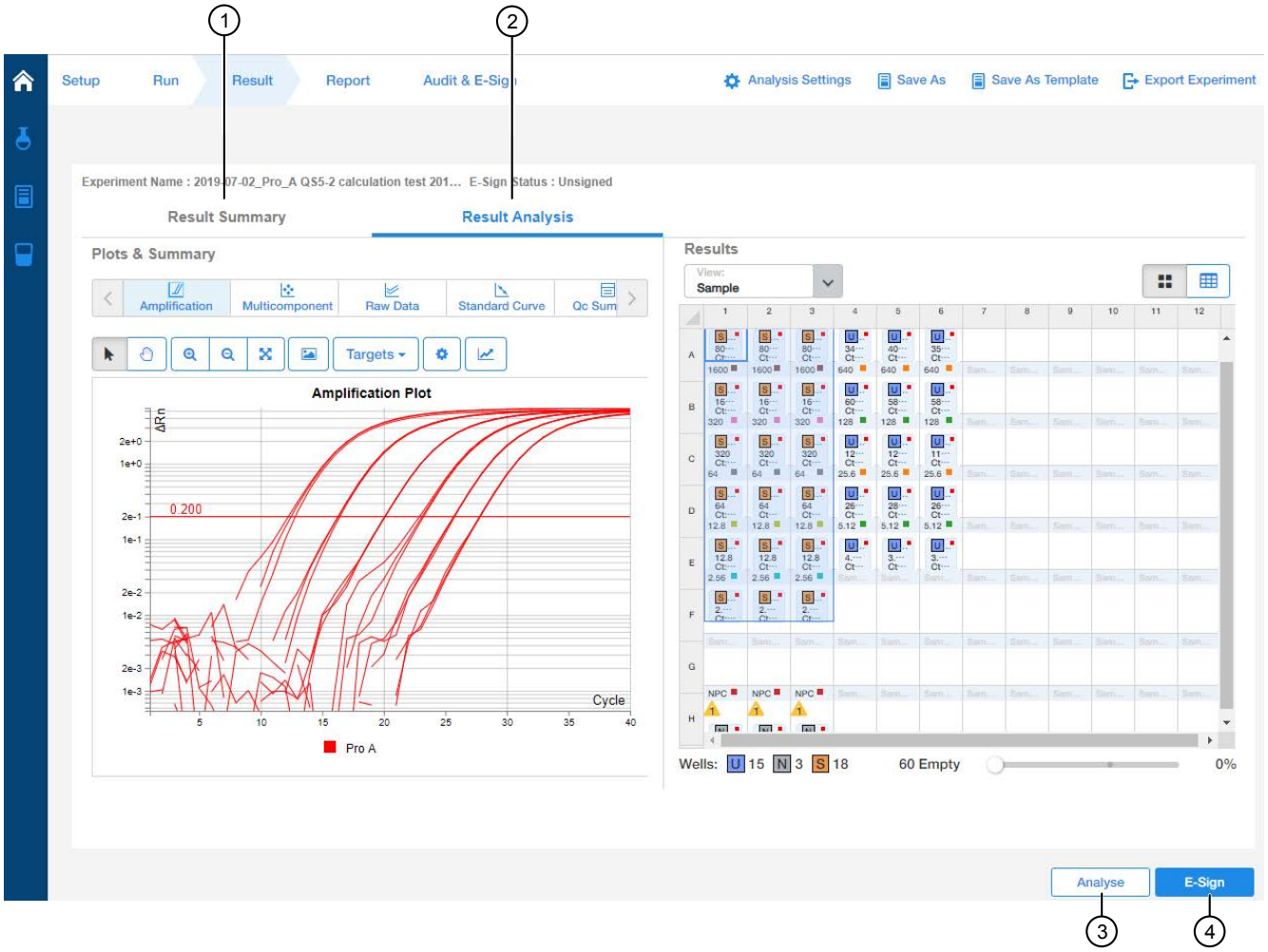


Figure 18 **Result** tab

① **Result Summary**—Populated for MycoSEQ™ experiments only; displays the overall **Plate Call** and the **Well Call** for individual wells. See Chapter 3, "Set up, run, and review MycoSEQ™ experiments".

② **Result Analysis**— Displays plots and well information such as sample name and flags.

③ **Analysis Settings**— Click **Analysis Settings** to access analysis settings.

④ **Analyze** button—Click **Analyze** after omitting wells or changing the analysis settings.

⑤ **E-sign** button—Click **E-sign to** to sign the experiment. For information, see "E-sign an experiment" on page 108.

Note: The analysis settings and plots that are available vary by experiment type.

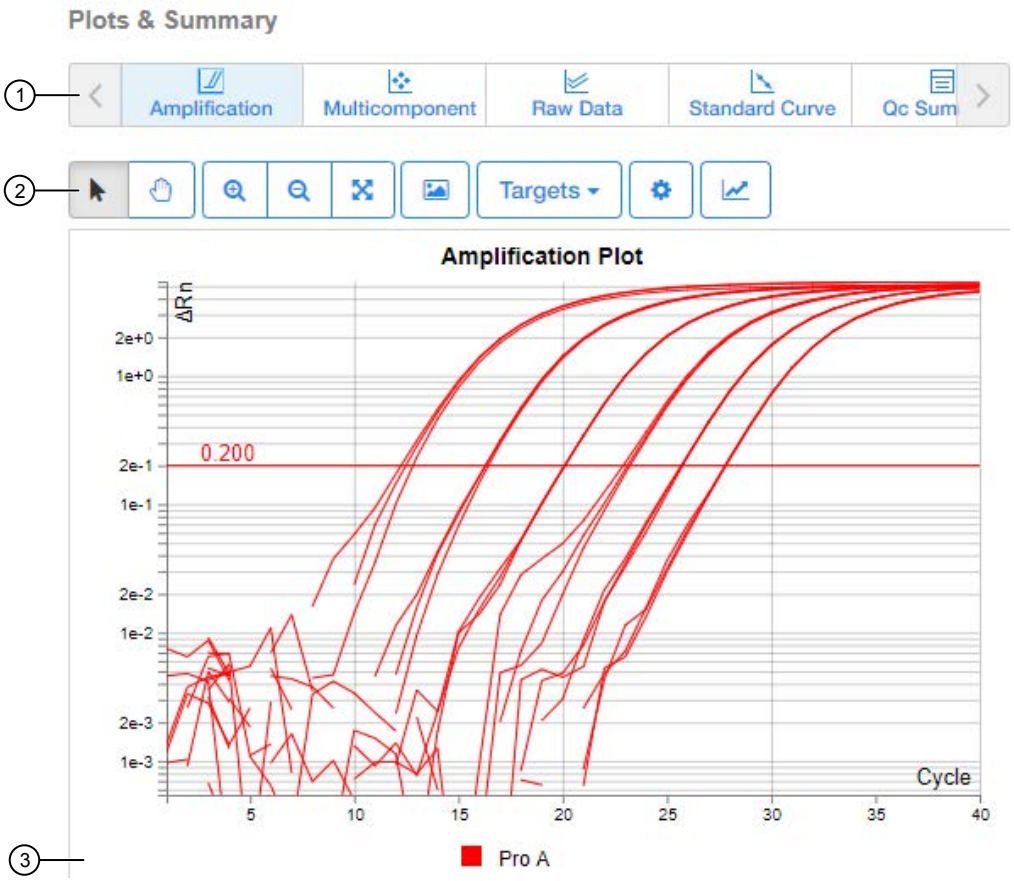


Figure 19 Plots & Summary pane

1. Plot horizontal scrollbar
2. Plot toolbar
3. Plot legend

The Plot toolbar includes the following options:

- Select and move
- Zoom in and out
- Return to original plot display
- Save plot as image file
- Select targets or dyes
- Configure plot properties
- Show/hide plot legend

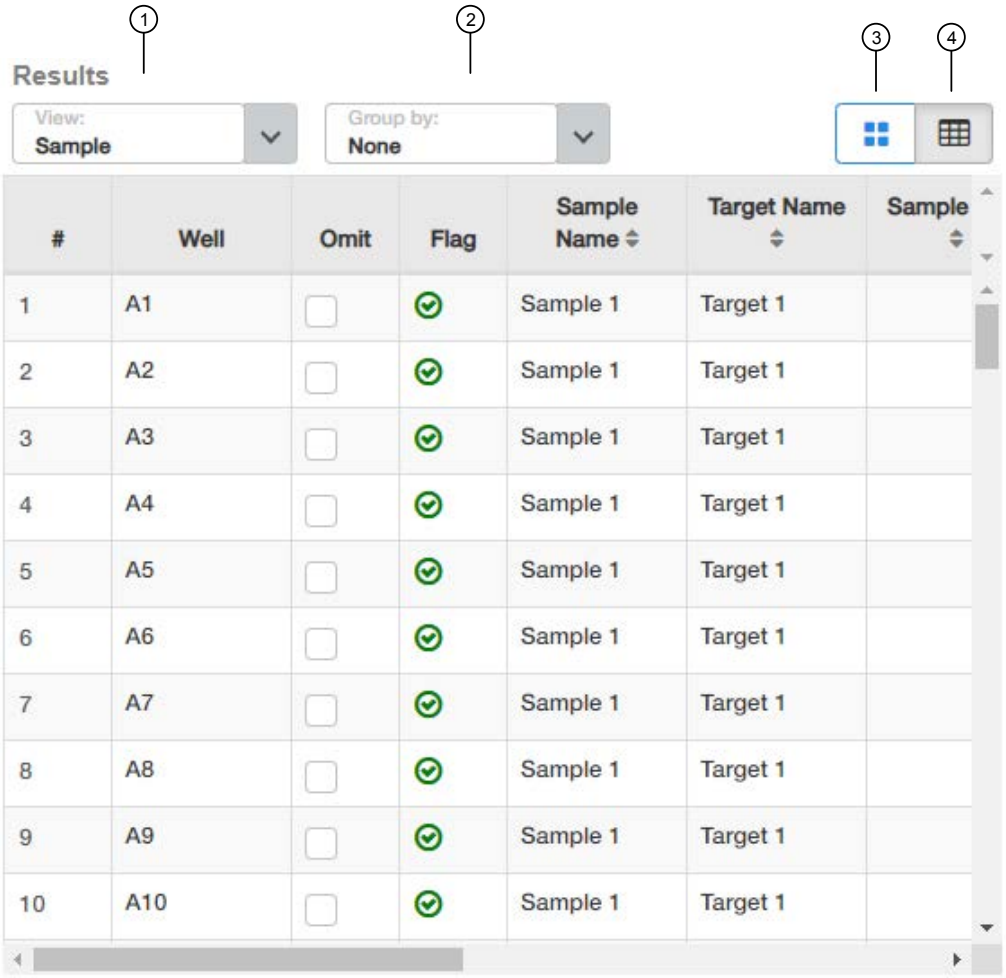


Figure 20 Results pane

① **View**—Select properties to display
② **Group by**—Select a parameter by which to group well rows
③ **Grid View**
④ **Table View** (shown)

Workflow: General procedures to review run results

When a run is complete, the AccuSEQ™ Real-Time PCR Software automatically analyzes the run data using the analysis settings that are specified in the experiment. The software then displays the run results in the **Results** tab.

Evaluate results in the Amplification Plot

▼

Assess the relevant experiment plot for the experiment
(for example, view the **Standard Curve Plot** for resDNASEQ™ experiments)

(see the corresponding chapter in this guide)

▼

Review data for outliers and *(optional)* Omit outliers from analysis

▼

(Optional) Review the dye signal profile using the Multicomponent Plot

▼

(Optional) Review the signal profile using the Raw Data Plot

▼

(Optional) Review the flags in the QC Summary

▼

(Optional) Configure analysis settings

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

Evaluate results in the Amplification Plot

Amplification Plot overview

The **Amplification Plot** displays sample amplification as a function of cycle number or well. You can use the amplification plot to perform the following tasks:

- Confirm or correct baseline and threshold values.
- Identify outliers.
- Identify and examine abnormal amplification. Abnormal amplification can exhibit one of the following characteristics:
  - Increased fluorescence in negative control wells
  - Absence of detectable fluorescence at an expected cycle

    Note: If you notice abnormal amplification or a complete absence of fluorescence, see the instrument user guide for troubleshooting information.

Three plots are available. Some plots can be viewed as a linear or $\log_{10}$ graph.

Table 5 Amplification Plot types

Plot type	Description	Use to
ΔRn	ΔRn is the magnitude of normalized fluorescence signal, relative to the baseline fluorescence, generated by the reporter at each cycle during the PCR amplification.	• Identify and examine irregular amplification. • View threshold values for the run.
Rn	Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference.	• Identify and examine irregular amplification. • View baseline values for the run.
C_t	C_t is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot.	• Locate outlying amplification (outliers).

Evaluate the overall shape of the Amplification Plot curves

You can evaluate the overall shape of the **Amplification** curves in the **Result** tab.

Open the experiment in the AccuSEQ™ Software. See "Open an experiment" on page 86.

1. In the **Results Analysis** pane of the **Result** tab, select **Amplification** from the horizontal scroll bar.

   Note: If no data are displayed in the **Result** tab, click **Result Analysis**, then click **Analyze**.

   ① Amplification Plot

   The **Amplification Plot** is displayed for the selected wells in the **(Grid View)** .

2. Ensure that the **Target** is correct.

3. (*Optional*) Click **(Settings)** , then make the following selections:

   - **Plot Type**: **ΔRn, Rn, or C_T**
   - **Graph Type**: **Log or Linear**
   - **Show**: **Legend**, **Cq Mark**, or **Unselected**
   - **Plot Color**: **Target**, **Sample**, **Well**, or **Flag_Status**
   - **Threshold**: Select **Auto** or specify a threshold, then decide whether to **Show Threshold**.
   - **Baseline**: Decide whether to **Show Baseline**.

4. (*Optional*) Adjust the **(Plot Properties)**.
   a. In the **General** tab, add a **Plot Title**, adjust the **Font** and **Color**, then click **Apply**.

b. In the **X Axis** or **Y Axis** tabs, you can:
   - Add a **Label**
   - Select whether you want **Tick Marks**
   - Select **Auto-adjust range** or enter minimum and maximum values

   Note: The minimum value must be greater than 0.

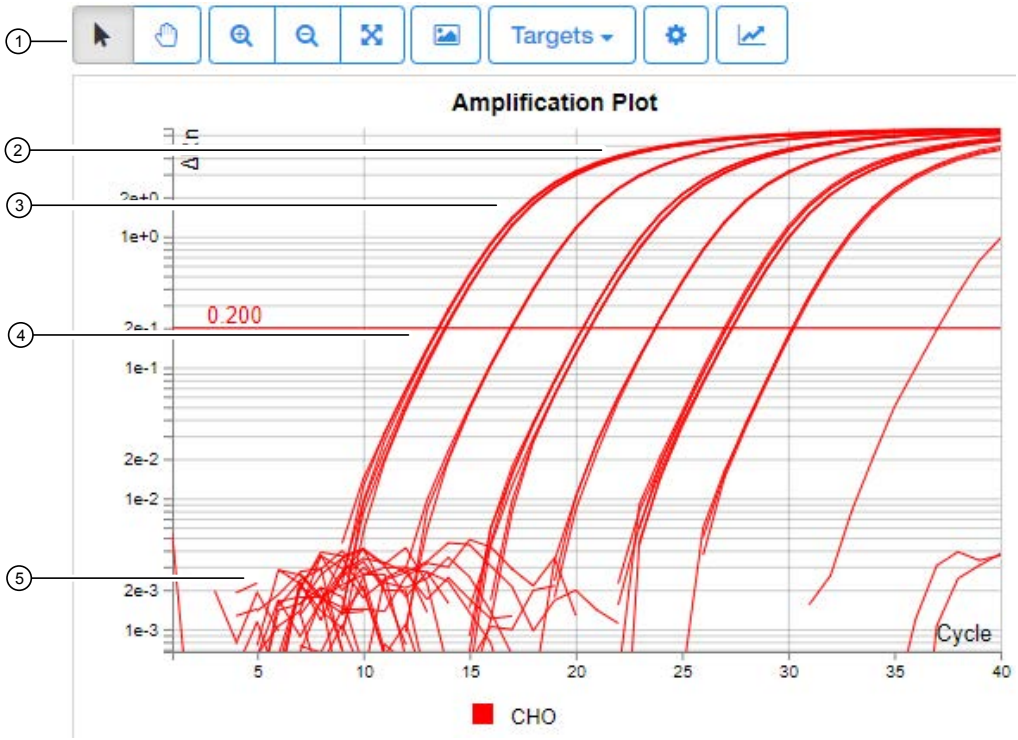


Figure 21 Typical Amplification Plot

① Amplification Plot tools
② Plateau phase
③ Linear phase
④ Exponential (geometric) phase
⑤ Baseline

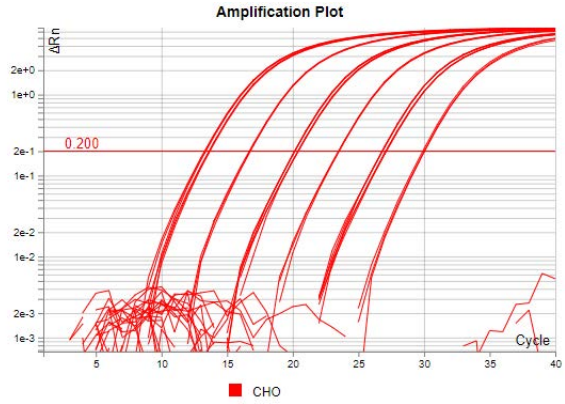
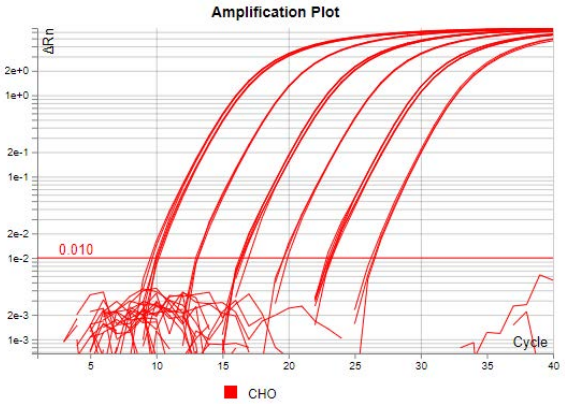
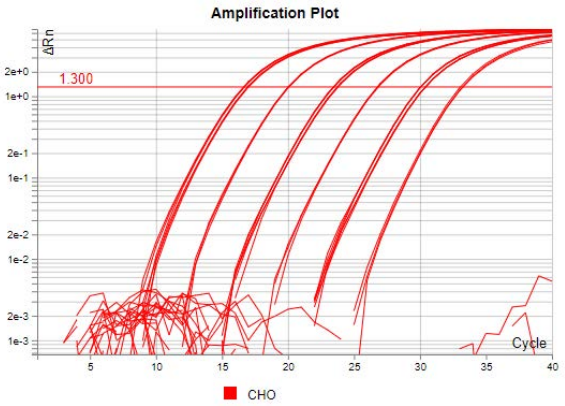
Confirm or correct threshold settings

1. In the **Result** tab, select **Amplification** from the horizontal scroll bar. The **Amplification Plot** is displayed for the selected wells in the **(Grid View)** .
2. Click **Targets** to configure what is displayed in the plot.
3. Click **(Settings)** , then make the following selections:
   - **Plot Type**: **ΔRn, Rn, or C_T**
   - **Graph Type**: **Log or Linear**
   - Select what to **Show**: **Legend**, **Cq Mark**, or **Unselected**
   - **Plot Color**: **Target**, **Sample**, **Well**, or **Flag_Status**
   - **Threshold**: Select **Auto** or specify a threshold, then select whether to **Show Threshold**.
   - **Baseline**: Select **Auto** or specify a threshold, then select whether to **Show Baseline**.

4. (*Optional*) Adjust the threshold.
   - Click-drag the threshold bar into the exponential phase of the curve.
   - Configure the C_t analysis settings (see "Ct settings overview" on page 92).

Table 6 Examples of threshold settings

Set the threshold in the exponential phase of the amplification curve. A threshold set above or below the optimum can increase the standard deviation of the replicate groups.

Threshold setting evaluation	Example
Threshold set correctly.	
Threshold set too low.	
Threshold set too high.	

Omit outliers from analysis

1. In the **Result** tab, select **Amplification** from the horizontal scroll bar.
   The **Amplification Plot** is displayed for the selected wells in the **(Grid View)** .
2. Click **Targets** to configure what is displayed in the plot.
3. Click **(Settings)** , then make the following selections:
   - **Plot Type**: **ΔRn, Rn, or C_T**
   - **Graph Type**: **Log or Linear**
   - Select what to **Show**: **Legend**, **Cq Mark**, or **Unselected**
   - **Plot Color**: **Target**, **Sample**, **Well**, or **Flag_Status**
   - **Threshold**: Select **Auto** or specify a threshold, then select whether to **Show Threshold**.

     Note: Threshold must be set at 0.2 for all **Factory default** assays (MycoSEQ™, resDNASEQ™, and ProteinSEQ™. Auto or any other threshold settings are applicable only to custom assays.
   - **Baseline**: Select **Auto** or specify a threshold, then select whether to **Show Baseline**.
4. Click **Table View** to look for outliers.
   a. Select **Group by ▸ Replicates**.
   b. Identify outliers in each replicate group.
      Outlier wells typically have one or more QC flags.
5. Omit outliers in the **(Table View)** by selecting **Omit** in outlier rows of the table.
6. Click **Analyze** to reanalyze the run data with any outliers removed.

Optimize display of negative controls in the Amplification Plot

1. In the **Result** tab, select **Amplification** in the horizontal scroll bar.
   The **Amplification Plot** is displayed for the selected wells in the **(Grid View)** .
2. Click **Targets** to configure what is displayed in the plot.
3. Click **(Settings)** , then make the following selections:
   - **Plot Type**: **ΔRn, Rn, or C_T**
   - **Graph Type**: **Log or Linear**
   - Select what to **Show**: **Legend**, **Cq Mark**, or **Unselected**
   - **Plot Color**: **Target**, **Sample**, **Well**, or **Flag_Status**
   - **Threshold**: Select **Auto** or specify a threshold, then select whether to **Show Threshold**.
   - **Baseline**: Select **Auto** or specify a threshold, then select whether to **Show Baseline**.
4. In either the **(Grid View)** or **(Table View)**, select the negative control wells (wells that should not have amplification for a particular target).

5. Adjust the display properties for individual wells. To configure the plot properties, select the well or wells of interest, then click **(Plot Properties)**.
   a. (*Optional*) In the **General** tab, add a **Plot Title**, adjust the **Font** and **Color**, then click **Apply**.
   b. In the **X Axis** or **Y Axis** tabs, you can:
      - Add a **Label**
      - Select whether you want **Tick Marks**
      - Select **Auto-adjust range** or enter minimum and maximum values

      Note: The minimum value must be greater than 0.

Evaluate results in the Table View

Table View overview

The **Table View** displays data for each well in the reaction plate. The data that are displayed depend on the specific experiment type and can include the following information:

- Sample name, target name, task, and dyes
- Values that are specific to particular stage of the method
  For example: C_t or melt temperature (T_m)
- Values that are specific to a particular experiment type
  For example: presence/absence calls, or quantities
- Omitted wells
- QC flags
- Comments

Group or sort in Table View

Possible options for grouping or sorting the **(Table View)** are described in the following table. Available grouping categories depend on the specific experiment type and analysis settings.

Note: You can select multiple columns when sorting, but you can only make one selection for grouping rows.

Group category	Description	Notes
Replicates[1,2]	Grouped by replicate	• Examine the C_t or quantity values for each replicate group to evaluate the precision of C_t values.
Flag	Grouped as flagged and unflagged wells	• A flag indicates that the software found a potential error in the flagged well.

Group category	Description	Notes
C_t[3]	Grouped by C_t value	• C_t value < 8—there may be too much template in the reaction. • C_t value > 35—there may be a low amount of target in the reaction; for C_t values > 35, expect a higher standard deviation.

[1] For standard curve experiments.
[2] For melt curve experiments.
[3] For genotyping experiments.

Review the dye signal profile using the Multicomponent Plot

Multicomponent Plot overview

The **Multicomponent Plot** displays the complete spectral contribution of each dye over the length of the PCR run.

Use the **Multicomponent Plot** to obtain the following information.

- Confirm that the signal from the passive reference dye remains unchanged throughout the run.
- Review reporter dye signal for spikes, dips, and/or sudden changes.
- Confirm that no amplification occurs in the negative control wells.

View and evaluate the Multicomponent Plot

You can view and evaluate the **Multicomponent Plot** in the **Result** tab.

If no data are displayed in the **Result** tab, click **Result Analysis**, then click **Analyze**.

1. In the **Result** tab, select **Multicomponent Plot** in the horizontal scroll bar.
2. Click to configure the plot, then make the following selections:
   - **Plot Color**: **Dye**, **Target**, or **Well**

   The **Multicomponent Plot** is displayed for the selected wells in the **(Grid View)**.
3. In the **(Grid View)**, select wells one at a time, then examine the **Multicomponent Plot** for the following plot characteristics.

Plot characteristic	Description
Passive reference dye	The passive reference dye fluorescence signal should remain relatively constant throughout the PCR process.
Reporter dye	The reporter dye fluorescence signal should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
Irregularities in the signal	Spikes, dips, and/or sudden changes in the fluorescence signal may have an impact on the data.
Negative control wells	The negative control wells should show little or no significant increase in fluorescence signal.

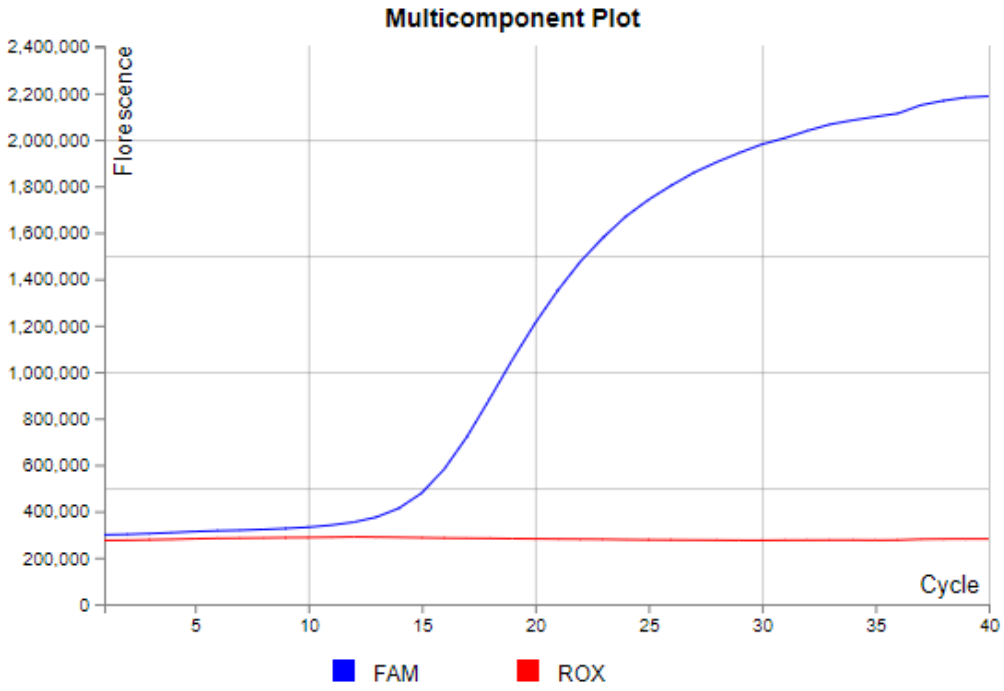


Figure 22 Example Multicomponent Plot (single well; TaqMan™ assays)

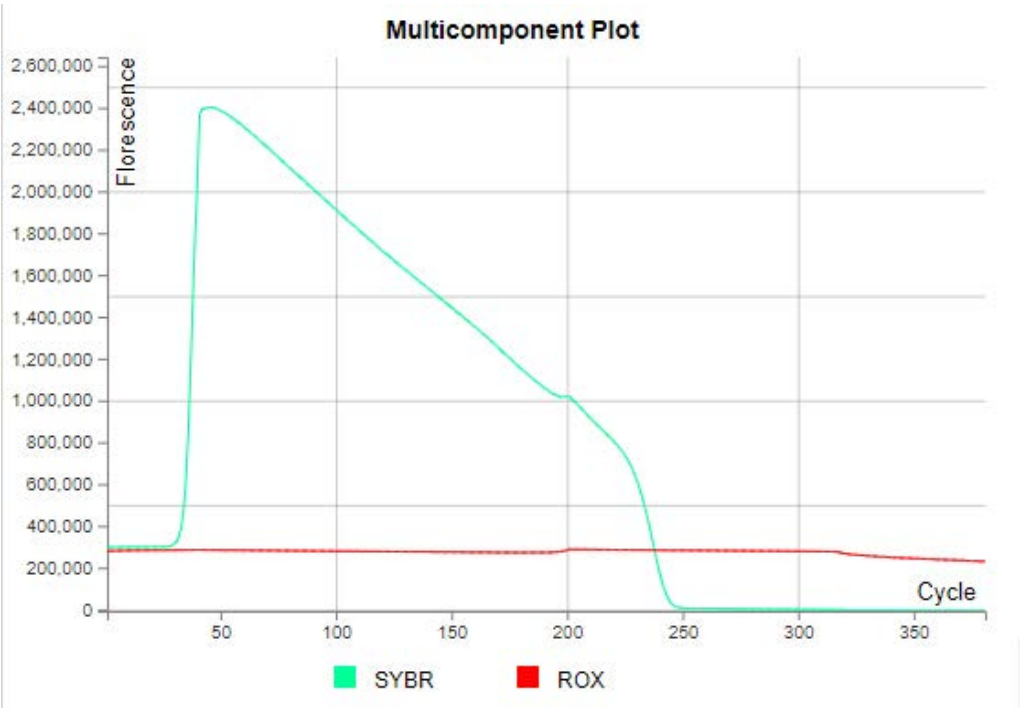


Figure 23 Example Multicomponent Plot (single well; SYBR™- MycoSEQ™ assays)

Review the signal profile using the Raw Data Plot

Raw Data Plot overview

The **Raw Data Plot** displays the raw fluorescence signal (not normalized) for each optical filter during each cycle of the real-time PCR.

View the Raw Data Plot to confirm a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View and evaluate the Raw Data Plot

You can view and evaluate the **Raw Data Plot** in the **Result** tab.

If no data are displayed in the **Result** tab, click **Analyze**.

1. In the **Result** tab, click **Result Analysis**, then select **Raw Data Plot** in the horizontal scroll bar.
   The **Raw Data Plot** is displayed for the selected wells in the **(Grid View)**.
2. Click to display the **Legend**.
3. Click-drag the **Show Cycle** pointer from cycle 1 to cycle 40, and confirm that each filter displays the characteristic signal increase.
   For more information on each filter set, see the instrument user guide (see Appendix C, "Documentation and support").

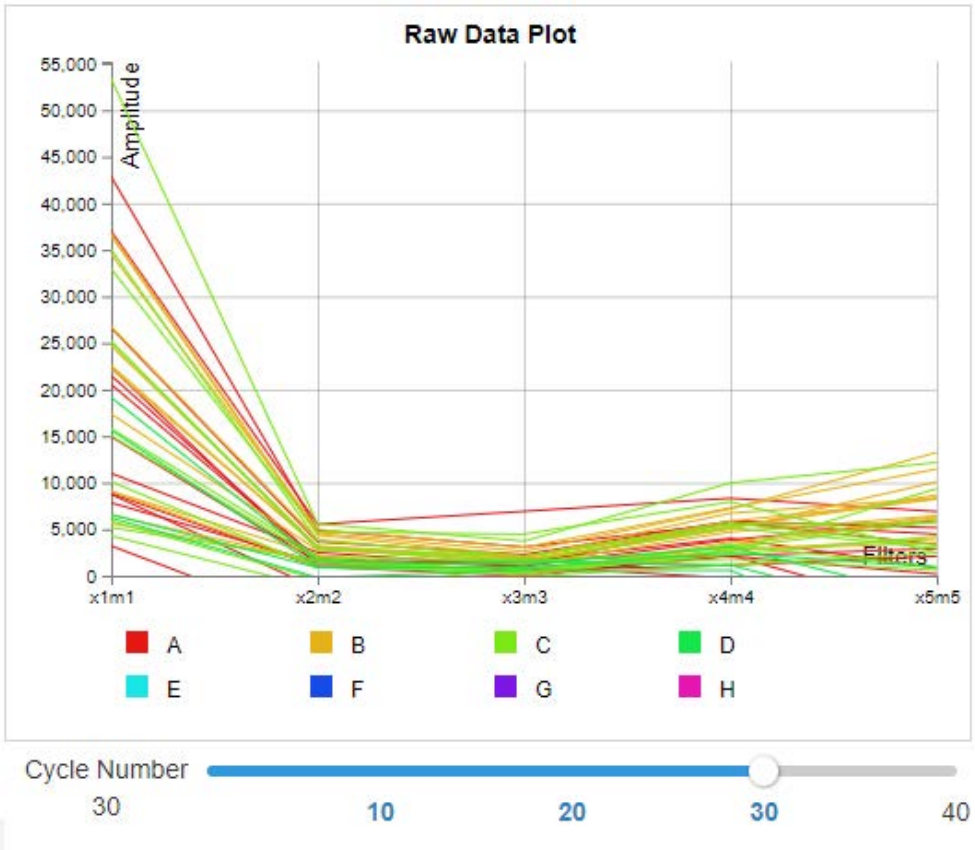


Figure 24 Example Raw Data Plot

Review the flags in the QC Summary

The **QC Summary** in the **Result** tab displays a list of the QC flags, including the flag frequency and location.

If no data are displayed in the **Result** tab, click **Analyze**.

1. In the **Result** tab, click **Result Analysis**, then select **QC Summary** in the horizontal scroll bar.
2. Review the **Flags**.
   The **Wells** column identifies wells that triggered a flag.
3. (*Optional*) Scroll through the flags to see a **Description** of the flag, its **Frequency**, and which **Wells** were flagged. Click the flag of interest for information text about the flag, including the **Flag** name, the **Flag Detail**, the **Flag Criteria**, and the **Flagged Wells**.

E-sign an experiment

1. Open an experiment (see "Open an experiment" on page 86).
2. Click the **Results** tab.
3. Click the **E-Sign** button, then enter your user name and password.
   Note: Users without **E-Signature** permissions will not be allowed to sign.

Table 7 E-signature status

If the experiment is	Icon displayed in Experiments screen[1]
Signed	**(Gray locked icon)**
Partially signed	**(Blue locked icon)**
Unsigned	**(Gray unlocked icon)**

[1] See "Experiments screen" on page 16.

If no additional e-signatures are required, the **E-Signature** status at the top of the screen and in the **Experiments** screen is set to **Signed**.

Revert an e-signature for a partially signed experiment

User accounts that have the **E-Signature** function enabled are allowed to revert the e-signature for partially signed experiments.

Experiments for which e-signature can be reverted have a **(Blue locked icon)** in the **Actions** field on the **Experiments** screen. If the **(Gray locked icon)** is displayed, the e-signature cannot be reverted. See "Experiments screen" on page 16.

1. Navigate to the **Experiments** screen from the **Home** screen.
2. Click the **(Blue locked icon)**.

View audit and e-sign reports for an experiment

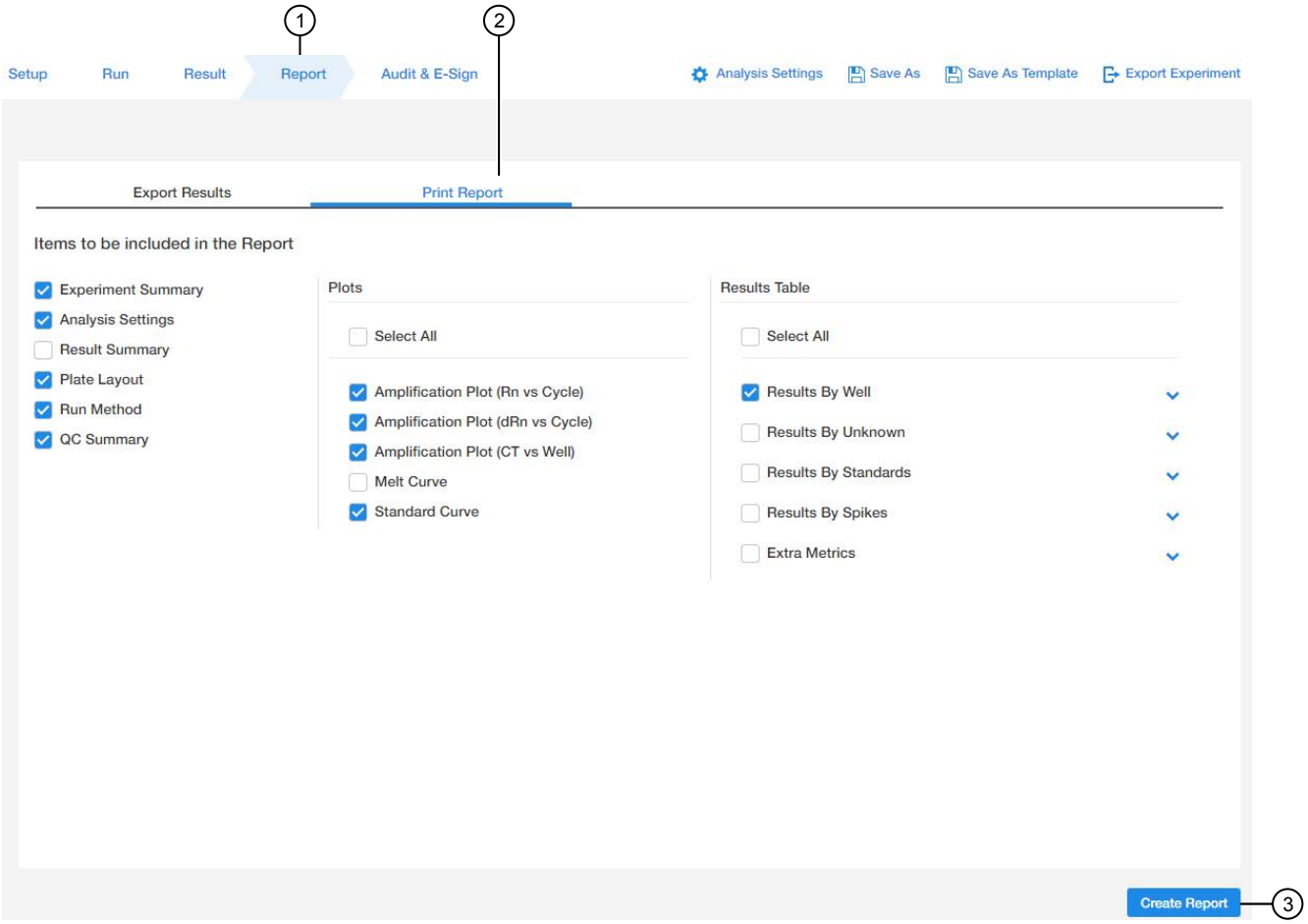
1. Open an experiment (see "Open an experiment" on page 86).
2. Click the **Audit & E-sign** tab at the top of the screen.
3. Click the **Audit Report** tab or the **E-Sign Report** tab to display the report.
4. If needed, enter a date range for the report.

Export data and print reports

Create and print a report

To create a report, an experiment must have a status of **Analysis**.

1. Open the experiment in the AccuSEQ™ Software. See "Open an experiment" on page 86.
2. Navigate to the **Report** tab, then the **Print Report** pane.



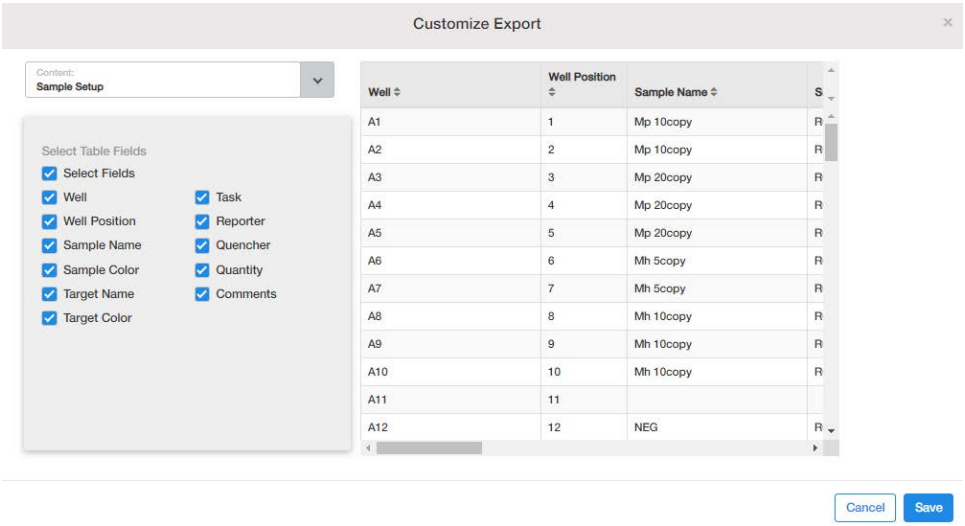
① **Report** tab
② **Print Report** pane
③ **Create Report** button

3. Select the **Items to be included in the Report**.
4. Click **Create Report**.
   The report opens with options to **Print**, **Download**, or**View PDF** the report.
5. (*Optional*) Click **Previous** to go back and make different selections for the report.

Export results

To export results, an experiment must have a status of **Analysis**.

1. Open the experiment in the AccuSEQ™ Software. See "Open an experiment" on page 86.
2. Navigate to the **Report** tab, then the **Export Results** pane.
3. Enter a **File Name**, then select the File Type (XLS or XLSX).
4. Select the **Content** to export, then click **Export**.
5. (*Optional*) Click **Customize Export** to select additional columns to include in the report.
   a. Select the **Content** to be customized, then **Select Table Fields** to be customized.
   b. Click **Save**.



6. Click **Export**.

   Note: The Results are exported in an XLS file to the Windows™ Downloads folder. You can change the Chrome™ browser settings to ask where to save each file before downloading.

Flags

QC flags overview

The following flags may be triggered by the run data.

Flag	Description
Pre-processing flags	
BADROX	Bad passive reference signal
OFFSCALE	Fluorescence is off-scale.
NOISE	Noise higher than others in plate
NOSIGNAL	No signal in well
NOAMP	No amplification
PRFDROP	Fluorescence signal from the passive reference changes significantly within defined range around the calculated C_q (C_T or C_{RT}) for a given well.
PRFLOW	For the replicate group of a given well, the average passive reference signal is below the percentage of the reference value defined in the analysis settings.
SPIKE	Noise spikes
Primary analysis flags	
BLFAIL	Automatic Baseline Algorithm failed to identify a baseline region for the amplification curve.
CQCONF	Calculated confidence for the C_q (C_T or C_{RT}) value is less than the defined minimum value.
CRTAMPLITUDE	PCR amplification curve amplitude is significantly lower than the other curves generated for the related target.
CRTNOISE	PCR amplification curve exhibits a significant amount of unexplained variability in comparison to the other curves generated for the related target.
CTFAIL	Baseline Threshold Algorithm failed to identify a C_t for the amplification curve.
DRNMIN	Normalized fluorescence (ΔRn) drops below the defined threshold.
EXPFAIL	Baseline Threshold Algorithm failed to identify an exponential region for the amplification curve.
THOLDFAIL	Automatic Threshold Algorithm failed to identify a threshold for the target.
Secondary analysis flags	
MTP	Melt curve analysis shows more than one peak.
AMPNC	Amplification in negative control
HIGHSD	High standard deviation in replicate group
OUTLIERRG	Outlier in replicate group

AMPNC flag

AMPNC—Amplification in negative control.

This flag indicates that a sequence in a negative control reaction amplified.

If a well is flagged, confirm the results:

1. Select the flagged wells in the plate layout or well table.
2. Ensure that the well corresponds to a negative control well (task = negative control or NTC).
3. View the amplification plot [ΔRn vs. Cycle (Linear) or ΔRn vs. Cycle (Log)], and confirm the fluorescence signal increased for the flagged negative control well.
4. If the fluorescence signal did not increase, consider omitting the well from analysis.

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

BADROX flag

BADROX—Bad passive reference signal.

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

If a well is flagged, confirm the results:

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

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

BLFAIL flag

BLFAIL—Baseline Threshold Algorithm failed to identify a baseline region for the amplification curve.

Note: The BLFAIL flag is only applicable when the Baseline Threshold Algorithm is used to analyze an experiment, though the flag column is always shown in the QC Summary.

This flag indicates that the Automatic Baseline Algorithm failed to identify a baseline region for the amplification curve, and the software cannot calculate the best-fit baseline for the data.

If a well is flagged, confirm the results:

1. Select the flagged wells in the plate layout or well table.
2. View the amplification plot [Rn vs. Cycle (Linear)], and note any late amplification or no amplification.
3. If the amplification is acceptable, set the baseline manually for the well in the analysis settings.
4. Reanalyze the data.
5. Evaluate the results and, if needed, make any additional changes to the baseline.

CQCONF flag

CQCONF—Calculated confidence in the C_q value is low.

This flag indicates that the calculated confidence for the C_q (C_t or C_{rt}) value of the well is less than the minimum value defined in the analysis settings.

Use the CQCONF flag to identify and (*optional*) to omit potentially poor results without manually inspecting every amplification curve. The minimum limit is set during the configuration of the flag settings.

If a well is flagged, confirm the results:

1. Select the flagged wells in the plate layout or well table.
2. View the amplification plot [ΔRn vs. Cycle (Linear) or ΔRn vs. Cycle (Log)] and check the shape of the curve.
3. If the curve is not typical, consider omitting the flagged wells from analysis.

CRTAMPLITUDE flag

CRTAMPLITUDE—Low relative amplitude.

Note: This flag is only applicable when the Relative Threshold Algorithm is used to analyze an experiment. The flag does not appear when the Baseline Threshold Algorithm is used.

This flag indicates that the amplitude of the amplification curve (generated from the data set that includes the given well) is significantly lower than the other curves (generated for the related target).

CRTNOISE flag

CRTNOISE—High relative noise.

Note: This flag is only applicable when the Relative Threshold Algorithm is used to analyze an experiment. The flag does not appear when the Baseline Threshold Algorithm is used.

This flag indicates that the amplification curve (generated from the data set that includes the given well) exhibited a significant amount of unexplained variability in comparison to the other curves (generated for the related target).

CTFAIL flag

CTFAIL—Baseline Threshold Algorithm failed to identify a C_t for the amplification curve.

Note: The CTFAIL flag is only applicable when the Baseline Threshold Algorithm is used to analyze an experiment, though the flag column is always shown in the QC Summary.

This flag indicates that the Baseline Threshold Algorithm failed to identify a C_t value for the given well, and the software cannot calculate the threshold cycle (C_t).

If a well is flagged, confirm the results:

1. Select the flagged wells in the plate layout or well table.
2. View the amplification plot [ΔRn vs. Cycle (Linear) or ΔRn vs. Cycle (Log)] and note:
   - Amplification too early
   - Amplification too late
   - Low amplification
   - No amplification
3. If the amplification is acceptable, set the threshold and baseline manually.
4. Reanalyze the data.
5. Evaluate the results. If the adjustments do not produce a valid C_t, consider omitting the well from analysis.

DRNMIN flag

DRNMIN—Detection of minimum ΔRn due to abnormal baseline.

This flag indicates that the normalized fluorescence (ΔRn) for a given well dropped below the threshold defined in the analysis settings.

Use the DRNMIN flag to easily identify and *(optional)* to omit potentially poor results without manually inspecting every amplification curve. The ΔRn threshold value is set during the configuration of the flag settings.

If a well is flagged, confirm the results:

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

EXPFAIL flag

EXPFAIL—Baseline Threshold Algorithm failed to identify an exponential region for the amplification curve.

Note: The EXPFAIL flag is applicable if the Baseline Threshold Algorithm is used to analyze an experiment, though the flag column is always shown in the QC Summary.

- It is a common flag for samples that do not amplify (C_t of 'Undetermined').
- The flag is not triggered for wells assigned the NTC task since they are not expected to have an identifiable exponential region.
  NTCs that do amplify will trigger the AMPNC flag to identify unexpected behavior.

Note: This flag indicates that the Baseline Threshold Algorithm failed to identify an exponential region of the amplification plot for the given well. The software set the baseline region to be the full length of the curve.

If a well is flagged, confirm the results:

1. Select the flagged wells in the plate layout or well table.
2. View the amplification plot [Rn vs. Cycle (Linear)], and note:
   - Amplification too early
   - Amplification too late
   - Low amplification
   - No amplification
3. If the amplification is acceptable, set the baseline manually for the well in the analysis settings.
4. Reanalyze the data.
5. Evaluate the results and, if needed, make any additional changes to the baseline in the analysis settings.

HIGHSD flag

HIGHSD—High standard deviation in replicate group.

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

If a replicate group is flagged, confirm the results:

1. Select the flagged replicate group in the plate layout or well table.
2. View the amplification plot [ΔRn vs. Cycle (Linear) or ΔRn vs. Cycle (Log)].
   a. Evaluate whether the signal varies significantly from others in the replicate group.
   b. If the signal varies significantly, consider omitting the outlier wells or omitting the entire replicate group from analysis.
3. If the amplification is acceptable, set the threshold manually.

   Note: Set the threshold manually only for experiments analyzed with the Baseline Threshold Algorithm.

4. Reanalyze the data.

5. Evaluate the results, and, if needed, make any additional changes to the threshold.

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

MTP flag

MTP—Melt curve analysis shows more than one peak.

Note: The MTP flag is present only in experiments with PCR methods that include a melting curve stage (a temperature ramp configured for data collection).

This flag indicates that the melt curve that is generated from the collected data exhibits multiple peaks. The multiple peaks indicate a possible PCR irregularities such as contamination or nonspecific amplification.

If a replicate group is flagged, confirm the results:

1. View the melt curve plot.

2. Note any peaks in the melt curve which can indicate the melting temperature (T_m) of a target nucleic acid or nonspecific PCR amplification.

NOAMP flag

NOAMP—No amplification.

This flag indicates that the sample did not amplify.

If a well is flagged, confirm the results:

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

Possible Cause	Recommended Action
Missing template.	• Repeat the experiment. Make sure to include all reaction components.
	• If this occurs in just one sample, it may be correct.

NOISE flag

NOISE—Noise is higher than other wells in plate.

This flag indicates that the well produced more noise in the amplification plot than the other wells on the same plate.

If a well is flagged, confirm the results:

1. Select the flagged wells and some unflagged unknown wells in the plate layout or well table.
2. View the amplification plot [ΔRn vs. Cycle (Linear)] and note any noisy amplification curves in the flagged wells.
3. In the multicomponent plot:
   a. From the dropdown list, select the dye option to color the data according to the dye.
   b. Note any drop in the ROX™ signal relative to the reporter dye, and compare flagged wells with unflagged wells.
   c. If there is a drop in the ROX™ signal relative to the reporter dye, consider omitting the flagged wells from analysis.

NOSIGNAL flag

NOSIGNAL —No signal in well.

This flag indicates that the well produced very low or no fluorescence signal.

If a well is flagged, confirm the results:

1. Select the flagged wells and some unflagged wells in the plate layout or well table.
2. View the multicomponent plot and compare the flagged wells to the unflagged wells:

Observation	Evaluation
The fluorescence signals for all dyes are low and similar to the background signal of the instrument.	The well is empty.
The fluorescence signals are higher than the background signal of the instrument and constant throughout the instrument run.	No amplification occurred.

3. If the flagged well produced no fluorescence signal, consider omitting the well from analysis.
4. On the reaction plate that was run, note the location for each flagged well, and examine each corresponding well in the reaction plate for low reaction volume.

OFFSCALE flag

OFFSCALE —Fluorescence signal is offscale.

This flag indicates that for one or more cycles, the fluorescence signal for one or more dyes in the well exceeds the maximum detectable range of the instrument.

If a well is flagged, confirm the results:

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

Possible Cause	Recommended Action
Fluorescent contaminant on the reaction plate, sample block, or adhesive cover	Perform a background calibration. If fluorescent contamination is detected, clean the block.
Fluorescent contaminant in the reaction	Replace the reagents.

OUTLIERRG flag

OUTLIERRG —Outlier detected in the replicate group.

This flag indicates that the C_q for the well deviates significantly from values in the associated replicate group (only the outlier is flagged).

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

If a well is flagged, confirm the results:

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

Possible Cause	Recommended Action
Pipetting errors	Repeat the reactions. Follow these guidelines to reduce pipetting errors: • Prepare enough master reaction mix for the entire replicate group, then transfer aliquots to all appropriate wells in the reaction plate. • Calibrate and service the pipettors regularly. • Pipette larger volumes. • Reduce the number of pipetting steps.
Contamination in that well	• Replace all reagents, then repeat the experiment. • Decontaminate the work area and pipettors.
Improper sealing or seal leaks	Repeat the reactions. Make sure to seal the reaction plate properly.

PRFDROP flag

PRFDROP—Passive reference signal changes significantly near the C_q (C_t or C_{rt}).

This flag indicates that the fluorescence signal from the passive reference changes significantly near the calculated C_q (C_t or C_{rt}) for a given well.

The flag is triggered when the covariance of the passive reference signal near the calculated C_q (C_t or C_{rt}) is above the detection threshold that is set in the flag settings. The algorithm includes up to 5 cycles on either side of the calculated C_q (C_t or C_{rt}) in the passive reference covariance calculation.

Use the PRFDROP flag to identify and optionally to omit potentially poor results without manually inspecting every amplification curve.

If a well is flagged, confirm the results:

1. Select the flagged wells in the plate layout or well table.
2. View the amplification plot [ΔRn vs. Cycle (Linear) or ΔRn vs. Cycle (Log)], then review the shape of the curve.
3. If the curve is not typical, consider omitting the flagged wells from analysis.

PRFLOW flag

PRFLOW —Average passive reference signal is below the threshold.

This flag indicates that for the replicate group of a given well, the average passive reference signal is below the percentage of the reference value defined in the analysis settings. The reference value for the PRFLOW flag is computed as a representational example from the set of curves.

Use the PRFLOW flag to easily identify and *(optional)* to omit potentially poor results without manually inspecting every amplification curve. The percentage of the reference value is set during the configuration of the flag settings.

If a well is flagged, confirm the results:

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

SPIKE flag

SPIKE—Noise spikes.

This flag indicates that the amplification curve for the given well contains one or more data points inconsistent with the other points in the curve.

If a well is flagged, confirm the results:

1. Select the flagged wells in the plate layout or well table.
2. View the amplification plot [ΔRn vs. Cycle (Linear) or ΔRn vs. Cycle (Log)] and evaluate whether the noise spike adversely affects the baseline or C_q.
3. If the baseline is adversely affected, set the baseline and threshold values manually.
4. Reanalyze the data.

5. Evaluate the results.

6. If the changes do not produce a valid C_q, consider omitting the well from analysis.

Possible Cause	Recommended Action
Bubbles in the reaction	Repeat the reactions. Make sure to centrifuge the plate at <1500 rpm for 2 minutes and confirm that the liquid in each well of the plate is at the bottom of the well.
Overall low signal for all dyes in the reaction	Repeat the reactions, pipetting a larger volume into all wells.
ROX™ dye not used as passive reference	Repeat the reactions, using ROX™ dye as the passive reference.
Evaporation due to improper sealing or seal leaks	Repeat the reactions. Make sure to seal the reaction plate properly.
No passive reference included in the experiment	In the analysis settings, reset the default SPIKE threshold value or deselect the use of the SPIKE flag.

THOLDFAIL flag

THOLDFAIL—Automatic Threshold Algorithm failed to identify a threshold for the target.

Note: The THOLDFAIL flag is only applicable when the Threshold Algorithm is used to analyze an experiment. The flag column is always shown in the QC Summary.

This flag indicates that the Automatic Threshold Algorithm failed to identify a threshold for the target. The software cannot calculate the threshold for the given set of wells associated with the target.

If a well is flagged, confirm the results:

1. Select the flagged wells associated with a particular target in the plate layout or well table.

2. View the amplification plot [ΔRn vs. Cycle (Linear) or ΔRn vs. Cycle (Log)], and note:
   - Amplification too early
   - Amplification too late
   - Low amplification
   - No amplification for all wells with this target

3. If the amplifications are acceptable, set the baselines and thresholds manually.

4. Reanalyze the data.

5. Evaluate the results and, if needed, make any additional changes to the threshold or baseline.

A Troubleshooting

Observation	Possible cause	Recommended action
Loss of internet connection during a run	Server malfunction.	Restart the server to reestablish a network connection. **Note:** After a connection is reestablished, the experiment is downloaded automatically by the AccuSEQ™ Software.
Only raw fluorescence readings collected (custom melt curve experiments only)	Optical filter for ROX™ is not selected.	Select the optical filter 4 when creating custom melt curve experiments. See "Select optical filters" on page 90.

B

Parts and materials

Kits, consumables, accessories, and reagents

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Store all calibration and RNase P plates at –20°C. All other items can be stored at 15–30°C. Use all materials by the expiration date on the packaging.

Consumables (96-well, 0.1-mL format)

Consumable	Amount	Cat. No.
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	20 plates	4346906
	200 plates	4366932

Instrument verification or calibration plate	Cat. No.
TaqMan™ RNase P Instrument Verification Plate, Fast 96-Well	4351979
Region of Interest (ROI) and Background Plates, Fast 96-Well 0.1-mL (2 plates) **Note:** For ROI/Uniformity calibrations.	4432426
QuantStudio™ 3/5 Spectral Calibration Plate 1, (FAM™, VIC™, ROX™, and SYBR™ dyes), 96-well, 0.1 mL	A26336
QuantStudio™ 3/5 Spectral Calibration Plate 3 (TAMRA™, NED™ and Cy®5 dyes) 96-well Fast (0.1-mL) Plate	A26340

Accessories

Item	Amount	Cat. No.
MicroAmp™ 96-Well Base	10 pieces	N8010531
MicroAmp™ Optical Adhesive Film	25 films	4360954
	100 films	4311971
MicroAmp™ Adhesive Film Applicator	5 applicators	4333183

C

Documentation and support

Related documents

The following related documents are available:

Document	Publication Number
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407
QuantStudio™ 3 and 5 Real-Time PCR Systems Site Preparation Guide	MAN0010405
AccuSEQ™ Real-Time PCR Software v3.0 Quick Reference	100084353
Security, Audit, and E-Signature (SAE) Administrator Console v1.0 User Guide	MAN0018760

Assay Document	Publication Number
MycoSEQ™ Mycoplasma Real-Time PCR Detection Kit User Guide	4482248
ProteinSEQ™ CHO Host Cell Protein Quantification Kit User Guide	MAN0010806
ProteinSEQ™ Protein A Quantification Kit User Guide	MAN0013524
resDNASEQ™ Quantitative DNA Kits User Guide	4469836

Note: For additional documentation, see "Customer and technical support" on page 126.

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support

- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

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

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**.

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