ProteinSEQ™ Protein A Quantification Kit

USER GUIDE

Catalog Number 4469343

Publication Number MAN0013524

Revision C.0

ThermoFisher SCIENTIFIC

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

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Revision	Date	Description
C.0	3 March 2020	Update for the AccuSEQ™ Real-Time PCR Software on the QuantStudio™ 5 Real-Time PCR System.
B.0	6 December 2015	Corrected qPCR well volume setting for standard PCR plates. Added “v2.0 or later” to AccuSEQ™ software references.

Contents

Product information

Product description

Use the ProteinSEQ™ Protein A Quantification Kit to quantify the amount of leached Protein A present in your bioprocess sample(s). The ProteinSEQ™ Protein A workflow consists of:

- Plate preparation
- A semi-automated sample processing run on the KingFisher™ Flex-96 Deep Well Magnetic Particle Processor
- A qPCR run on the QuantStudio™ 5 Real-Time PCR System or the Applied Biosystems™ 7500 Fast Real-Time PCR System
- Data analysis using the Applied Biosystems™ AccuSEQ™ software designed for your Real-Time PCR system or equivalent curve fitting software

Kit contents and storage

Table 1 ProteinSEQ™ Protein A Quantification Kit (Cat. no. 4469343)

Box Part No.	Contents	Cap color	Item Part No.	Quantity	Volume	Storage conditions
A25001	Repligen Protein A Standard (1 mg/mL)		4469347	1	0.1 mL	–20°C
	Protein A 5′ Probe		4469376	1	0.7 mL	
	Protein A 3′ Probe		4469381	1	0.7 mL	
	2X Protein A Sample Prep Reagent		4485137	1	10 mL	
	ProteinSEQ™ Ligation and Assay Mix		100024250	1	0.6 mL	
	ProteinSEQ™ Ligase		A25020	1	55 µL	
A25002	Fast Master Mix, 2X		4448622	1	6 mL	4°C
	Wash Buffer		4469398	1	100 mL	
	Protein A Capture Beads		4469368	1	1.2 mL	
	ProteinSEQ™ Elution Buffer		100024368	1	7 mL	
	ProteinSEQ™ Diluent		4469405	1	41 mL	

Materials and equipment not provided

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

"MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
One of the following instrument systems:	
QuantStudio™ 5 Real-Time PCR System	Contact your local sales office.
Applied Biosystems™ 7500 Fast Real-Time PCR System	4365464
Equipment	
Pharma KingFisher™ Flex-96 Deep Well Magnetic Particle Processor	A31508
Pharma KingFisher™ Flex Magnetic Head for PCR Plate	A31544
PCR Plate Adaptor (Fast or Standard)	Contact Technical Support
Heat block for 1.5/2-mL tubes	MLS
Benchtop microcentrifuge	MLS
Plate centrifuge	MLS
Benchtop vortexer	MLS
Reagents	
MabSelect SuRe™ Protein A, 2 mg/mL [1]	GE Healthcare 28-4018-60 (custom order)
0.5 M Sodium Phosphate Buffer, pH 7	MLS
Consumables	
Aerosol-resistant pipette tips	MLS
KingFisher™ Flex 96 tip comb for PCR magnets	97002514
Disposable gloves	MLS
25-mL reagent reservoir	VistaLab Technologies 3054-1002 or equivalent
15-mL conical tube	AM12500 or equivalent
Pipettors • P20, P200, and P1000 single-channel pipettors • P200 and P1000 multichannel pipettors, 8- or 12-channel	MLS

Item	Source
qPCR plates • MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (0.1 mL)[2] *or* • MicroAmp™ Optical 96-Well Reaction Plate with Barcode (0.2 mL)[3]	• 4346906 *or* • 4306737
Additional plates • Pharma KingFisher™ Flex 96 Standard Plate • PCR Plate, 96-well, low profile, skirted	• A43076 • AB0800
MicroAmp™ Optical Adhesive Film	4360954
Nonstick, RNase-free Microfuge Tubes, 1.5 mL	AM12450
Sterile Microcentrifuge Tubes with Screw Caps	Fisher Scientific 02-681-371 or equivalent
Aluminum foil for heat block	MLS
Software (one of the following)	
AccuSEQ™ Real-Time PCR Software (QuantStudio™ 5 Real-Time PCR System)	A40303
AccuSEQ™ Real-Time PCR software (7500 Fast Real-Time PCR System)	4443420
Microsoft™ Excel™ software	www.microsoft.com

[1] Optional, if not using Protein A provided in kit.
[2] For use with thermal cycler FAST sample blocks.
[3] For use with thermal cycler standard sample blocks.

Methods

Workflow

Pre-dilute the Protein A standard	5 minutes
▼	
Prepare serial dilutions of the diluted Protein A standard	5 minutes
▼	
Prepare samples	30–40 minutes, depending on sample size
▼	
Prepare the plates for the magnetic particle processor	15 minutes, can be performed concurrently with sample preparation
▼	
Run the plates in the magnetic particle processor	2 hours
▼	
Setup and run the qPCR reaction	50 minutes (10 minutes ligation and 40 minutes qPCR)
▼ ▼	
Setup, run, and analyze samples with AccuSEQ™ Software on the QuantStudio™ 5 Real-Time PCR Instrument Setup, run, and analyze samples with AccuSEQ™ software on the 7500 Fast Real-Time PCR Instrument	10 minutes

Important procedural guidelines

- **IMPORTANT!** The magnetic particle processor's Magnetic Head is very fragile. The magnetic rods are easily bent or broken. Handle with care.
- Use serially diluted standards when performing spiking studies.
- Run all reactions in triplicate.
- We recommend digital multi-channel pipettors for transfers into the magnetic particle processor plates.
- Working solutions and plates can be kept at room temperature during assay setup.

Important serial dilution guidelines

Pre-dilution guidelines— Pre-dilute the Protein A standard to obtain a concentration (40 ng/mL) to use as the starting point for the dilution series.

- Prepare the pre-dilutions in 1.5-mL non-stick RNase-free microfuge tubes (Cat. no. AM12450 or equivalent).
- If you are using:
  - Repligen Protein A Standard (1 mg/mL) provided with the ProteinSEQ™ Protein A Quantification Kit, see “Pre-dilute the Repligen Protein A standard” on page 11.
  - GE Healthcare MabSelect SuRe™ Protein A (2 mg/mL; GE Healthcare 28-4018-60, see “Pre-dilute the GE Healthcare MabSelect SuRe™ Protein A standard” on page 11.

Serial dilution guidelines — Prepare serial dilutions using the pre-diluted Protein A standard (D4).

- Prepare serial dilutions in a Pharma KingFisher™ Flex 96 Standard Plate (Cat. No. A43076 or equivalent polypropylene 96-well plate). If your standard dilution volume is >300 µL , prepare the serial dilutions in 1.5-mL non-stick RNase-free microfuge tubes (Cat. No. AM12450 or equivalent).
- Use a new pipette tip for each transfer.
- Pipet gently to minimize foaming and/or bubble formation.
- It is critical to mix standards during serial dilution. After each transfer,
  - **If preparing serial dilutions in microfuge tubes** — Invert the tube several times to mix.
  - **If preparing serial dilutions in a 96-well plate** — Gently pipet up and down 5–8 times to increase mixing efficiency.

Before you begin (first time use only)

- Contact your local sales or service representative to prepare your KingFisher™ Flex-96 Deep Well Magnetic Particle Processor for use with ProteinSEQ™ assays and to obtain the following items:
  - The ProteinSEQ™ Protein A script for the KingFisher™ Flex-96 Deep Well Magnetic Particle Processor (upload before you perform a ProteinSEQ™ assay for the first time)
  - The appropriate PCR Plate Adaptor (Fast or Standard)
  - If you are using GraphPad™ software for data analysis, the Protein A Master Template
- Ask your local representative if your KingFisher™ Flex-96 Deep Well Magnetic Particle Processor supports plate hold-downs. If supported, your local representative should install the plate hold-downs before you perform a ProteinSEQ™ assay for the first time.
- Calibrate your heat source. It is critical to heat samples to 100°C during incubation. Experiments performed at high altitude may require further assay development such as extended heating time or multiple heating and cooling cycles.

Before you begin each time

- Thaw the 2X Protein A Sample Prep Reagent at room temperature for an hour before preparing samples.
- Before preparing the plates for each assay, clean the pipettors, plate racks and the microcentrifuge (if using tubes for standard dilution) to avoid cross-contamination.

Pre-dilute the Protein A standard

Use the appropriate procedure for your Protein A standard.

Note: See “Important serial dilution guidelines” on page 9.

Pre-dilute the Repligen Protein A standard

If you are using the Repligen Protein A Standard provided with the kit, pre-dilute the standard from 1 mg/mL to 40 ng/mL:

1. Dispense the following to four labeled 1.5-mL microfuge tubes:

Dilution Tubes[1]	ProteinSEQ™ diluent	Repligen Protein A standard	Final volume	Final Protein A concentration
D1	480 µL	20 µL (1 mg/mL)	500 µL	40 µg/mL
D2	900 µL	100 µL from D1	1000 µL	4 µg/mL
D3	900 µL	100 µL from D2	1000 µL	0.4 µg/mL
D4	900 µL	100 µL from D3	1000 µL	40 ng/mL

[1] The pre-dilutions (D1 through D4) can be frozen at –20°C and thawed up to 5 times over one month.

2. After you add the Protein A standard to each tube, vortex the tube for 2 seconds, then briefly spin before making the next transfer.

Pre-dilute the GE Healthcare MabSelect SuRe™ Protein A standard

If you are using GE Healthcare MabSelect SuRe™ Protein A standard (not provided with kit), pre-dilute the standard from 2 mg/mL to 40 ng/mL:

1. Dispense the following to four labeled 1.5-mL microfuge tubes:

Dilution Tubes	ProteinSEQ™ diluent	MabSelect SuRe™ Protein A Standard	Final volume	Final Protein A concentration
D1	490 µL	10 µL (2 mg/mL)	500 µL	40 µg/mL
D2	900 µL	100 µL from D1	1000 µL	4 µg/mL
D3	900 µL	100 µL from D2	1000 µL	0.4 µg/mL
D4	900 µL	100 µL from D3	1000 µL	40 ng/mL

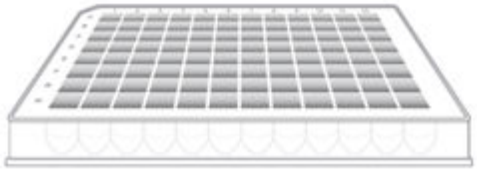
2. After you add the Protein A standard to each tube, vortex the tube for 2 seconds, then briefly spin before making the next transfer.

Prepare serial dilutions of the diluted Protein A standard

See “Important serial dilution guidelines” on page 9.

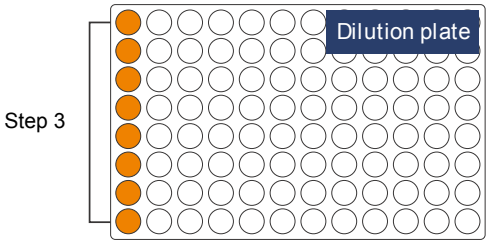
Prepare a dilution series using D4 from the previous step.

1. Label a Pharma KingFisher™ Flex 96 Standard Plate “Dilution”.



2. Aliquot approximately 2 mL of ProteinSEQ™ Diluent into a 25-mL reagent reservoir.

3. Dispense 200 µL ProteinSEQ™ Diluent to wells A1 through H1 of the labeled plate. Use a multi-channel pipette.



4. Add 50 µL diluted Protein A Standard (40 ng/mL; D4 from “Pre-dilute the Protein A standard” on page 11) to the A1 plate well, then pipet up and down 5 times to mix.

5. Transfer the remaining dilutions. Use a new pipette tip for each transfer. After each transfer, pipet up and down 5 times to mix. See Table 2.

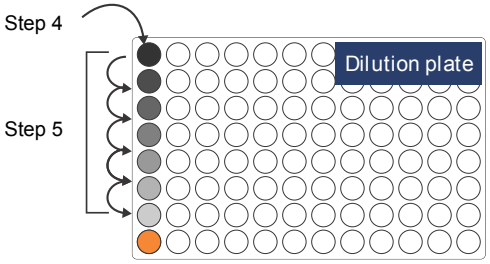


Table 2 Prepare serial dilutions

Serial dilutions	ProteinSEQ™ Diluent	Dilution transfer	Protein A concentration
SD1	200 µL	50 µL of diluted Protein A Standard (40 ng/mL)	8000 pg/mL
SD2	200 µL	50 µL from SD1	1600 pg/mL
SD3	200 µL	50 µL from SD2	320 pg/mL
SD4	200 µL	50 µL from SD3	64 pg/mL
SD5	200 µL	50 µL from SD4	12.8 pg/mL
SD6	200 µL	50 µL from SD5	2.56 pg/mL
SD7	200 µL	50 µL from SD6	0.51 pg/mL
NPC	200 µL	0	0

Prepare samples

The following procedures apply to the unknown samples and do not need to be performed for the Protein A standards used to generate the standard curve.

Adjust sample pH and/or concentration

For best results, adjust the sample pH, IgG concentration, and buffer concentration according to the table:

Sample type	Recommendation
pH < 6	Adjust the pH to > 6 with 0.5M Sodium Phosphate buffer pH 7
pH > 8	Adjust the pH to <8 with 0.5M Sodium Phosphate buffer pH 7
IgG > 25 mg/mL	Dilute to < 25 mg/mL with ProteinSEQ™ Diluent
Carboxylate buffer (Citrate)	Dilute to < 50 mM with ProteinSEQ™ Diluent

IMPORTANT! Do not dilute samples with 2X Protein A Sample Prep Reagent; the final concentration of the 2X Protein A Sample Prep Reagent must be 1X. If necessary to dilute samples, use the ProteinSEQ™ Diluent.

Treat samples with 2X Protein A Sample Prep Reagent

Heating the samples causes the IgG to denature and precipitate. The IgG pellets during centrifugation. The Protein A molecules remain in the supernatant.

Note: Use screw-cap tubes during heat incubation.

1. In a 1.5-mL screw cap or safe-lock tube, add 150 µL of sample to 150 µL of 2X Protein A Sample Prep Reagent.

   Note: 2X Protein A Sample Prep Reagent and ProteinSEQ™ Elution Buffer bottle are the same size and cap color. Confirm that you are using the correct reagent.

2. Vortex for 5–10 seconds, then quick spin to collect the contents at the bottom of the tube.

3. Place the sample tubes in a heat block, cover the tubes with aluminum foil, then incubate the tubes at 100°C for 10 minutes.

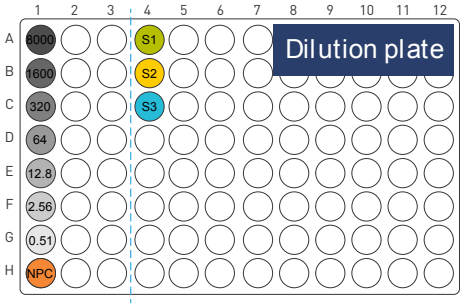
CAUTION! Adding water to heat blocks may create an electrical hazard. Refer to the manufacturer's guidelines for use of water in heat blocks.

4. Remove the tubes from the heat block and cool at room temperature for 5 minutes.

5. Vortex the tubes on a bench-top vortexer for 3 seconds at maximum speed.

6. Centrifuge the tubes for 5 minutes at 16,000 × g (~14,000 rpm).

7. Transfer the entire volume of supernatant to an empty well in the Dilution plate that contains the serially diluted standards.

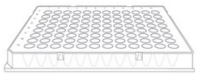
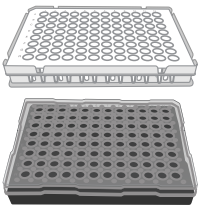
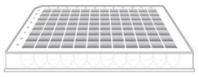


Prepare the plates for the magnetic particle processor

Note: When preparing wash, capture, qPCR, and probes plates, dispense at bottom of wells to prevent bubble formation (bubbles prevent effective mixing during the magnetic particle processor run). If bubbles form, quick-spin the plate at 560 × *g* (~2,000 rpm) in a plate centrifuge.

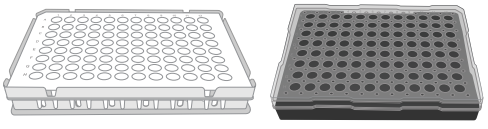
Label the plates

1. Label eight plates:

No. of plates	Plate type		Cat. No.	Label(s)
6	PCR Plate, 96-well, low profile, skirted		AB-0800	Capture Probes Wash 1 Wash 2 Wash 3 Wash 4
1	MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (0.1 mL)[1] *or* MicroAmp™ Optical 96-Well Reaction Plate with Barcode (0.2 mL)[2]		4346906 *or* 4306737	qPCR
1	Pharma KingFisher™ Flex 96 Standard Plate (200 µL)		A43076[3]	Comb

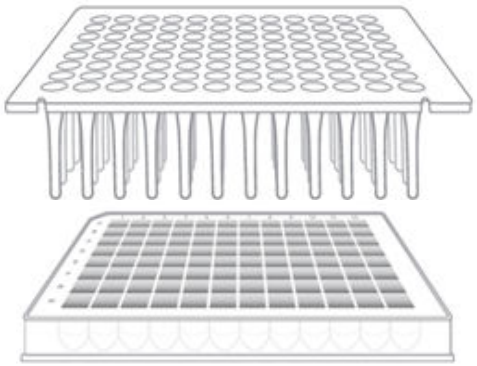
[1] For use with thermal cycler FAST sample blocks; shown in Fast PCR Plate Adaptor.
[2] For use with thermal cycler standard sample blocks; shown in Standard PCR Plate Adaptor.
[3] Or equivalent polypropylene plate.

2. (7500 Real-Time PCR System only) Insert the plate labeled “qPCR” into the appropriate PCR Plate Adaptor [Fast (on left) or Standard (on right); request from your local sales or service representative].



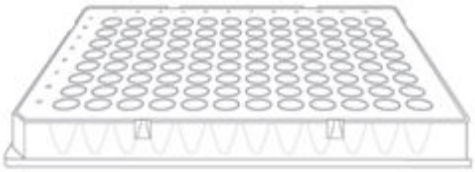
3. Insert the plate labeled “qPCR” into a standard or fast PCR Plate Adapter (request from your local sales or service representative).

4. Place a KingFisher™ Flex 96 tip comb for PCR magnets (Cat. No. 97002514) in the plate labeled "Comb".



Prepare wash plates

1. Pour approximately 30 mL of Wash Buffer into a fresh reagent reservoir.
2. Dispense 100 µL of Wash Buffer into each well of the 4 wash plates with a multi-channel pipette.



Prepare qPCR plate

Dispense ProteinSEQ™ Elution Buffer into each well of the qPCR plate:

- Fast PCR plate — 15 µL per well
- Standard PCR plate — 25 µL per well

Note: 2X Protein A Sample Prep Reagent and ProteinSEQ™ Elution Buffer bottle are the same size and cap color. Confirm that you are using the correct reagent.

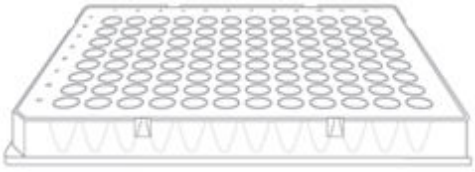
Prepare probes plate

1. Add the assay probe reagents to a 15-mL tube in the order shown in the table. Scale the volumes as needed for the number of reactions, including recommended overages. Vortex for 3 seconds at medium speed, then keep the 15-mL tube on ice.

Reagent	Cap color	Volume[1]		
		1 rxn	48 rxn	96 rxn
ProteinSEQ™ Diluent	Clear	56.3 µL	2700 µL	5400 µL
Protein A 5′ Probe	Grey	3.1 µL	150 µL	300 µL
Protein A 3′ Probe	Yellow	3.1 µL	150 µL	300 µL
Total		62.5 µL	3000 µL	6000 µL

[1] Includes 25% overage.

2. Invert the assay probe mix tube several times to mix, transfer to a reagent reservoir, then dispense 50 µL assay probe mix into each well of the Probes plate with a multi-channel pipette.



Prepare the capture plate

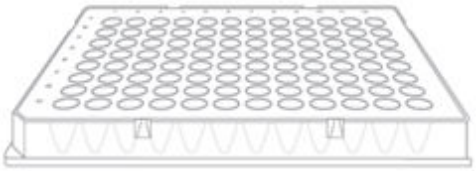
1. Vortex the Protein A Capture Beads for 3 seconds at medium speed 2 times.

2. In a 15-mL conical tube, mix the Protein A Capture Beads and the ProteinSEQ™ Diluent using the volumes from the table:

Reagent	Cap color	Volume[1]		
		1 rxn	48 rxn	96 rxn
ProteinSEQ™ Diluent	Clear	21.3 µL	1020 µL	2040 µL
Protein A Capture Beads	Clear	3.8 µL	180 µL	360 µL
Total		25.1 µL	1200 µL	2400 µL

[1] Includes 25% overage.

3. Immediately dispense 20 µL of diluted Protein A Capture Beads into each well of the Capture plate. Use a multi-channel pipette.



4. Transfer 50 µL of each standard and sample from the Dilution plate to the capture plate in triplicate. Use a multi-channel pipette.
   The final volume in the capture plate is 70 µL per well.

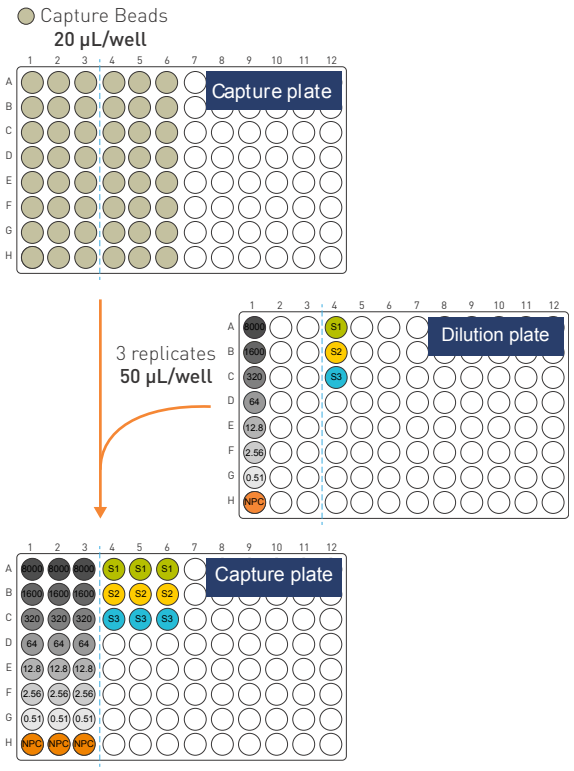


Figure 1 Transfer standards and samples to the capture plate

Example: Add 50 µL of each Protein A standard dilution and sample from the Dilution plate to the Protein A Capture Beads in the Capture plate to create 3 replicates of each standard dilution and sample.

Run the plates in the magnetic particle processor

1. Turn on the instrument, then select the **Protein A** program from the screen.

   Note: The instrument automatically resets each time that you turn it on.

2. Press **START** to initiate plate loading. Follow the prompts on the display screen to load each plate onto the turntable, starting with “Comb” (see Figure 2). Slide each plate into the plate hold-down (if present).

   IMPORTANT! When loading the **Tip Comb** in position 8, confirm that it rests in a Pharma KingFisher™ Flex 96 Standard Plate (200 µL; Cat. No. A43076), *not* a PCR Plate, 96-well, low-profile, skirted (Cat. No. AB-0800).

   For all plates, verify that A1 on the plate aligns with A1 on the instrument.

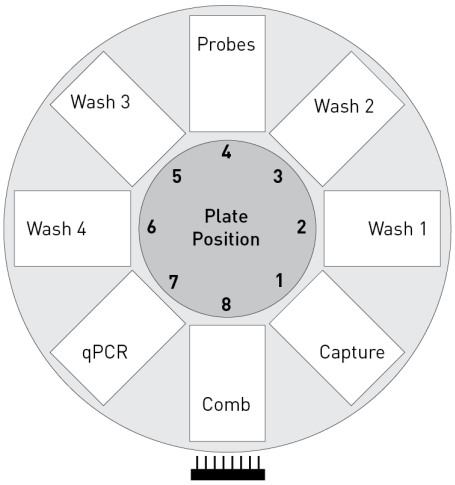


Figure 2 Plate positions in the turntable

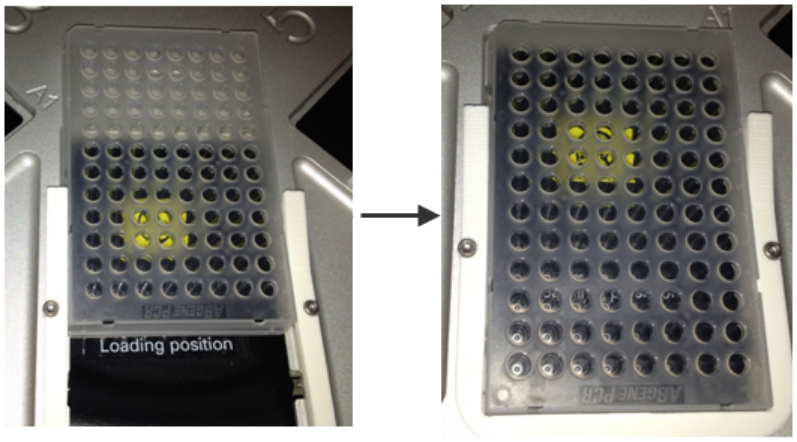


Figure 3 Load plate (with plate hold-down)

3. Load the last (Capture) plate, then press **START** to start the run.

   The run requires ~2 hours. When the run is complete, the screen displays "Proceed to qPCR".

Plate	Step
Capture	Protein A binds to Capture Beads
Wash 1	Capture Beads are washed
Wash 2	Capture Beads are washed
Probes	Probe binds to Protein A on Capture Beads
Wash 3	Capture Beads are washed
Wash 4	Capture Beads are washed
qPCR	Beads are released into qPCR plate

4. When the program is complete, carefully remove the qPCR plate. Discard the Capture, Wash, and Probes plates.

   IMPORTANT! Do not discard the plate adaptor.

   Note: Discard the PCR Head Tip Comb.

   The qPCR plate contains Protein A Capture Beads in ProteinSEQ™ Elution Buffer (total volume 15 µL (FAST plate) or 25 µL (standard plate).

Proceed immediately to "Setup and run the qPCR reaction" on page 20.

Setup and run the qPCR reaction

IMPORTANT! ProteinSEQ™ detection is based on qPCR, which is a highly sensitive technique with potential for cross-contamination. After the run completes, discard the qPCR plate. Do not remove the optical film from the qPCR plate. Removing the film introduces amplicon contamination into the local environment. See Appendix C, "Good laboratory practices for PCR and RT-PCR".

1. Prepare the Ligation/qPCR mix in a 15-mL tube according to the volumes shown in the appropriate table, including recommended overages, then briefly vortex to mix.

Table 3 Reagent volumes for FAST PCR plates.

Reagent	Cap color	Volumes[1]		
		1 rxn	48 rxn	96 rxn
Fast Master Mix, 2X	Clear	20 µL	960 µL	1920 µL
ProteinSEQ™ Ligation and Assay Mix[2]	Green	2 µL	96 µL	192 µL
ProteinSEQ™ Ligase	Orange	0.2 µL	9.6 µL	19.2 µL
Total		22.2 µL	1065.6 µL	2131.2 µL

[1] Includes 35% overage.
[2] Contains FAM™ dye and primers.

Table 4 Reagent volumes for standard (non-FAST) PCR plates.

Reagent	Cap color	Volumes[1]		
		1 rxn	48 rxn	96 rxn
Fast Master Mix, 2X	Clear	32.5 µL	1560 µL	3120 µL
ProteinSEQ™ Ligation and Assay Mix[2]	Green	3.25 µL	156 µL	312 µL
ProteinSEQ™ Ligase	Orange	0.26 µL	12.5 µL	25 µL
Total		36.01 µL	1728.5 µL	3457 µL

[1] Includes 30% overage. Volumes for 48 and 96 reactions are rounded to nearest tenth. For PCR on Fast PCR plates, reduce volumes accordingly.
[2] Contains FAM™ dye and primers.

2. Transfer the Ligation/qPCR mix to each bead-containing well of the qPCR plate that was prepared on the instrument.

- **For Fast PCR plates**—Use 15 µL per well
- **For Standard PCR plates**—Use 25 µL per well

Note: Dispense the mix to the sides of the well. Do not mix after dispensing.

3. Seal the qPCR plate with an optical film, centrifuge for 3 seconds at 500 rpm, then load the plate on your Real-Time PCR System.

4. Set up the qPCR run.

   - If you are using the AccuSEQ™ system software, follow the setup instruction in "Set up and run qPCR on the 7500 Fast instrument with AccuSEQ™ software v2.0 or later" on page 29.
   - If you are using other equivalent software (for example, SDS 1.4 software) use the following settings:

     –

Stage	Temperature	Time
Hold	37°C	10 minutes
Hold	95°C	20 seconds
40 cycles	95°C	3 seconds
	60°C	30 seconds

     –

Setting	FAST plates	Standard (non-FAST) plates
Protein A standards and sample wells volume	30 µL	50 µL
Detection dye set	FAM	FAM
Quencher	none	none

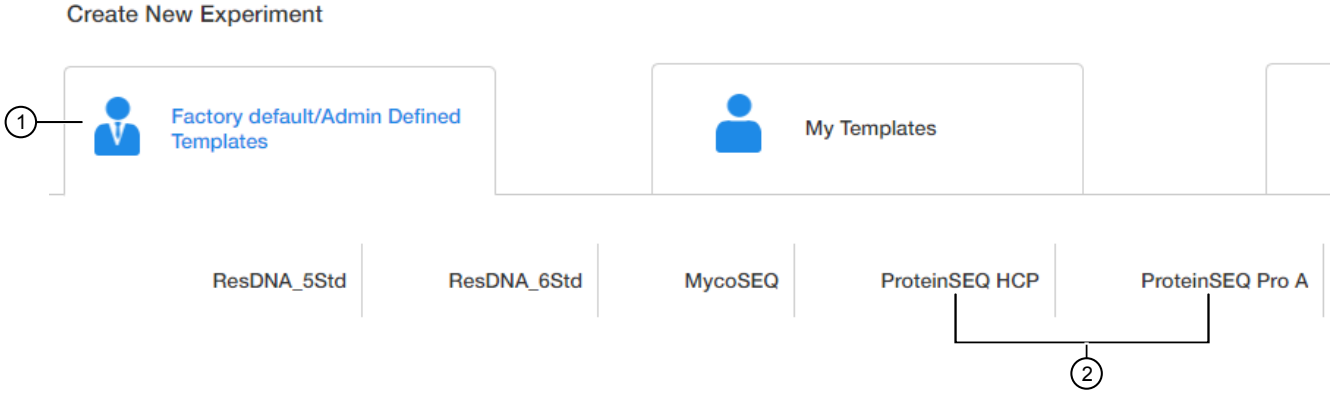
5. Start the run.

6. After the run completes, discard the qPCR plate. Do not remove the optical film from the qPCR plate. Removing the film introduces amplicon contamination into the local environment.

Setup, run, and analyze samples with AccuSEQ™ Software on the QuantStudio™ 5 Real-Time PCR Instrument

Create a ProteinSEQ™ experiment

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



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).

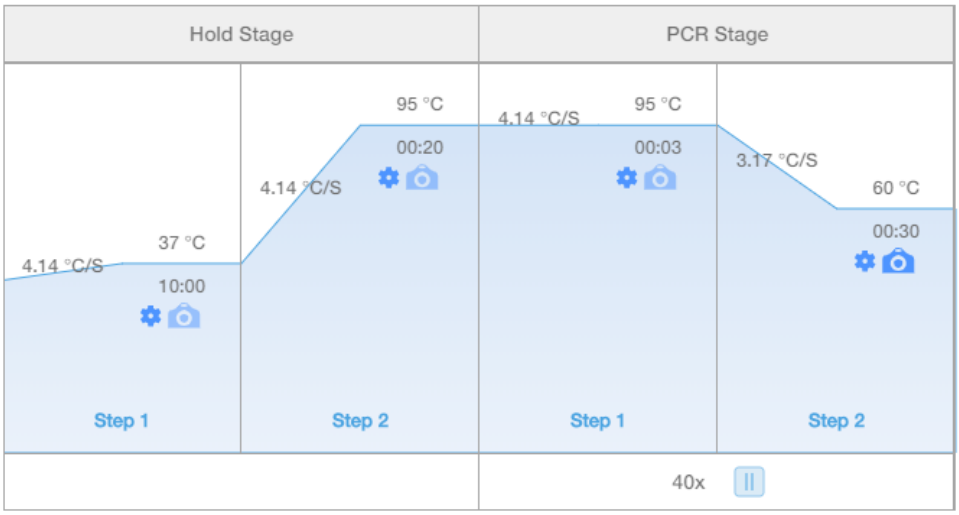
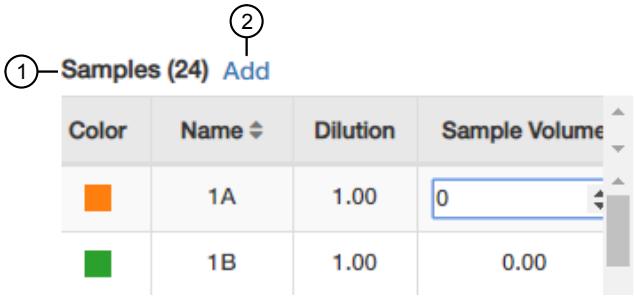


Figure 4 ProteinSEQ™ template default cycling conditions

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.

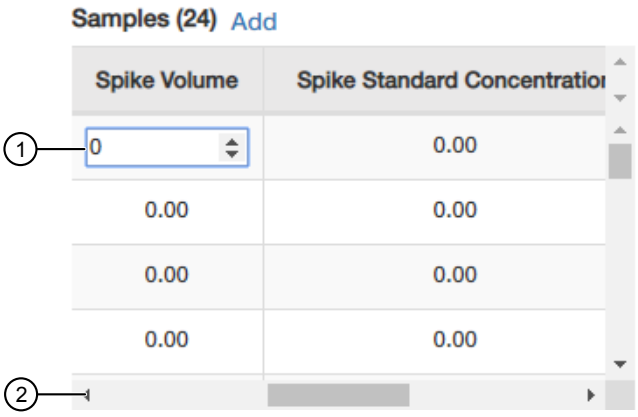
IMPORTANT! Do not change the **Targets**.



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

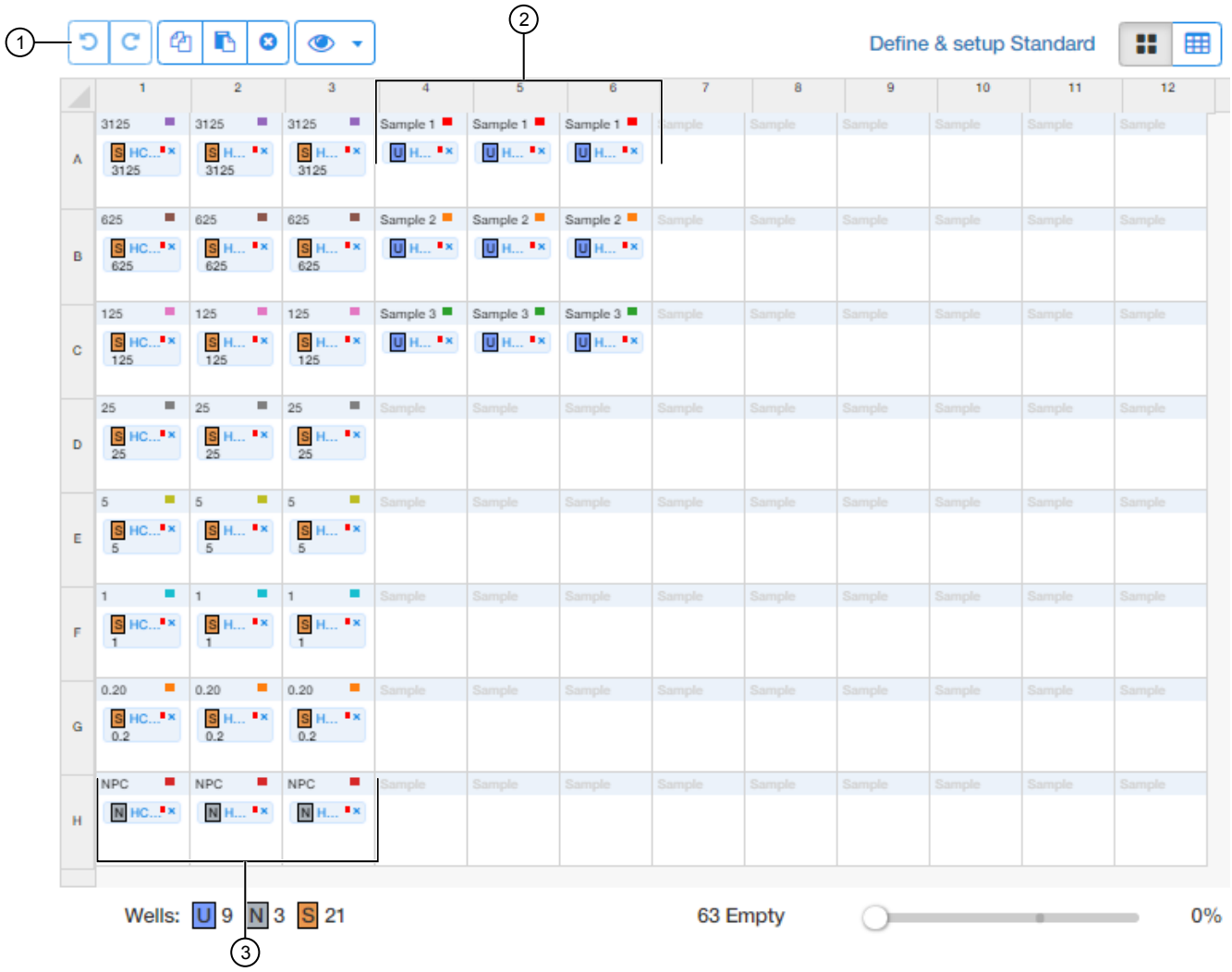


Figure 5 ProteinSEQ™ HCP template default sample plate layout

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**

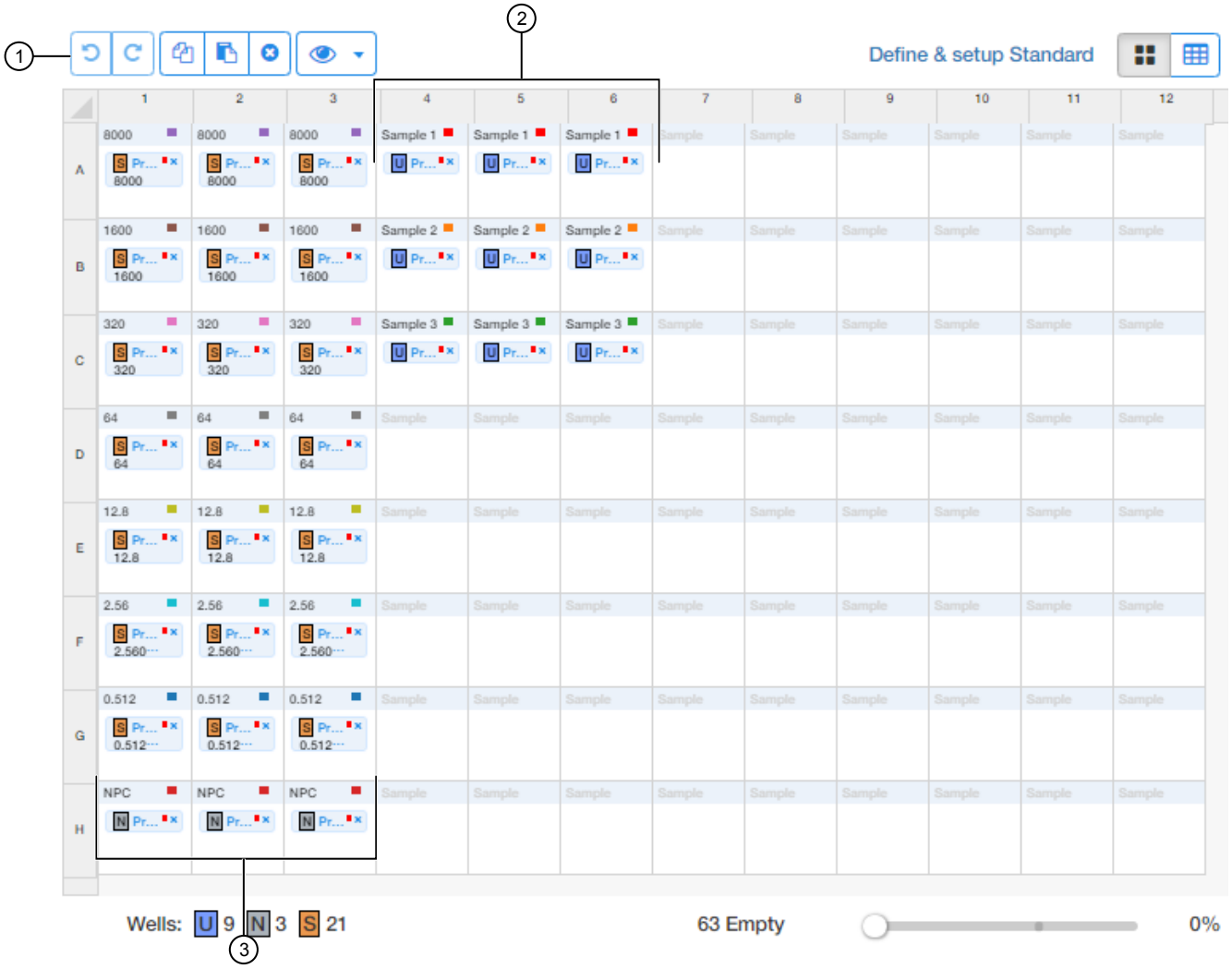


Figure 6 ProteinSEQ™ Pro A template default sample plate layout

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**.

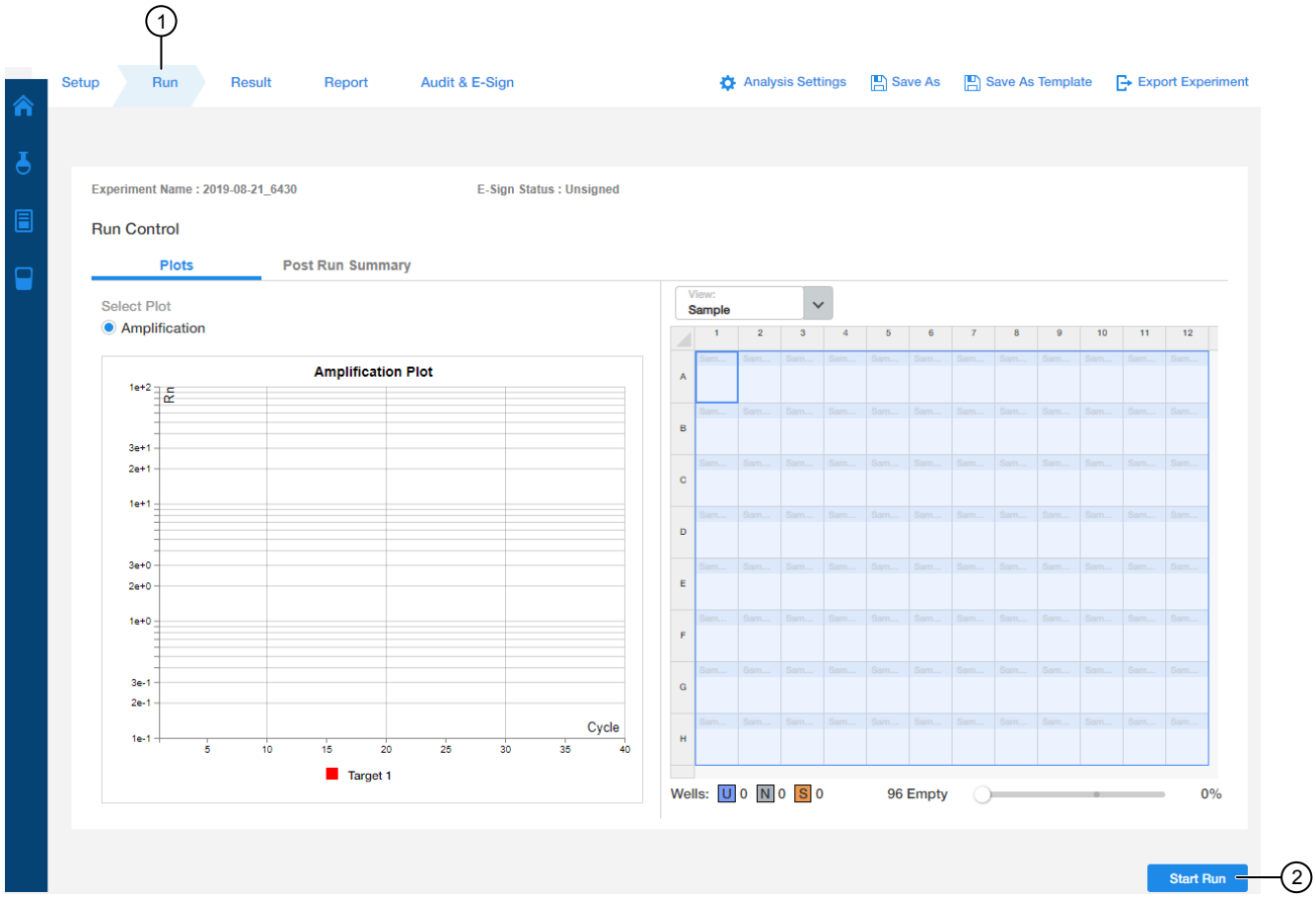
   Note: Clicking **Save As** will create a copy of the experiment.

9. Assemble the PCR reactions following the manufacturer's instructions for the reagents and following the plate layout set up in the template.

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. 2. Click the **Run** tab. 3. Click **Start Run**.



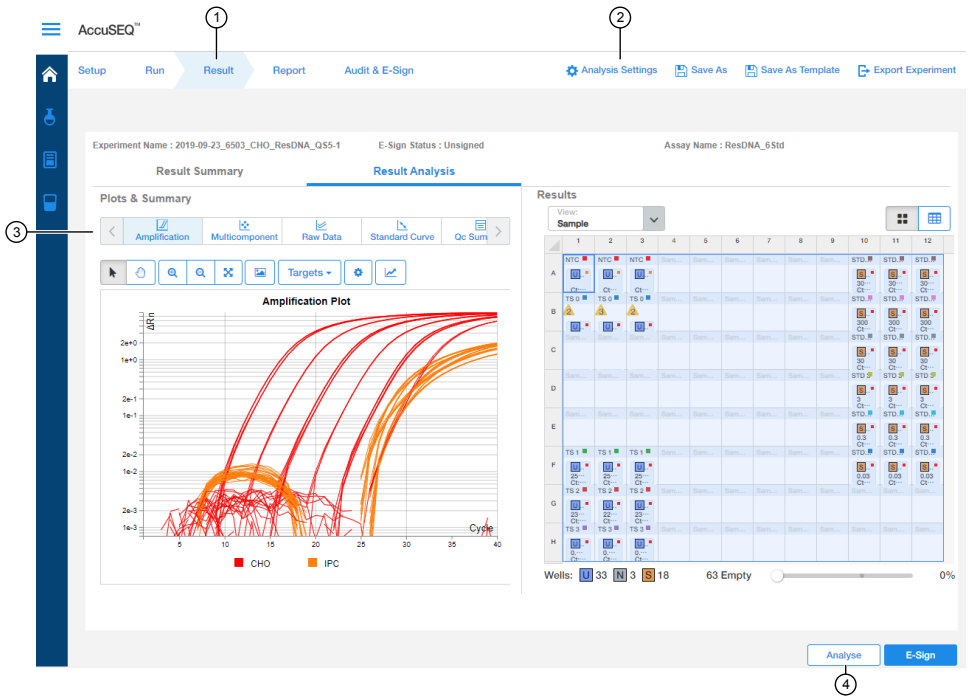
① **Run** tab
② **Start Run** button

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

Analyze the results

After the qPCR run is finished, use the following general procedure to analyze the results. For more detailed instructions see the *AccuSEQ™ Real-Time PCR Software v3.0 User Guide* (Pub. No. 100084348).

1. In the AccuSEQ™ Real-Time PCR Software, open your experiment, then navigate to the **Result** tab.



1. **Result** tab
2. **Analysis Settings**
3. Plot horizontal scrollbar
4. **Analyze** button

2. In the **Result Analysis** tab, review the Amplification Curve plots for amplification profiles in the controls, samples, and the standard curve.

3. In the **Result Analysis** tab, review the **QC Summary** for any flags in wells.

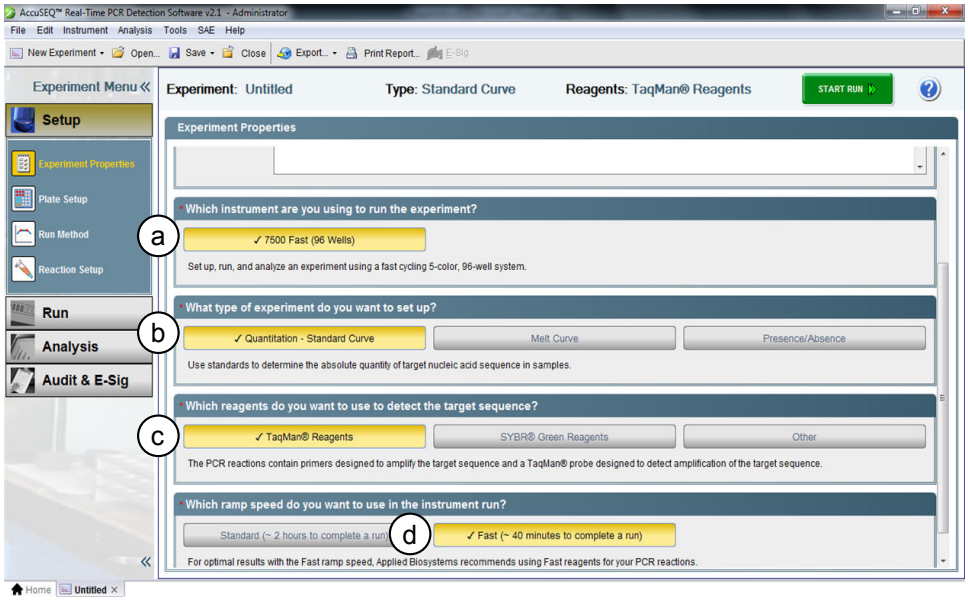
4. In the **Result Analysis** tab, review the **Standard Curve** plot. Verify the values for the Slope, Y-intercept, R2, and Efficiency.

5. (*Optional*) Navigate to the **Report** tab to generate a report of the experiment, or to export results.

Setup, run, and analyze samples with AccuSEQ™ software on the 7500 Fast Real-Time PCR Instrument

Set up and run qPCR on the 7500 Fast instrument with AccuSEQ™ software v2.0 or later

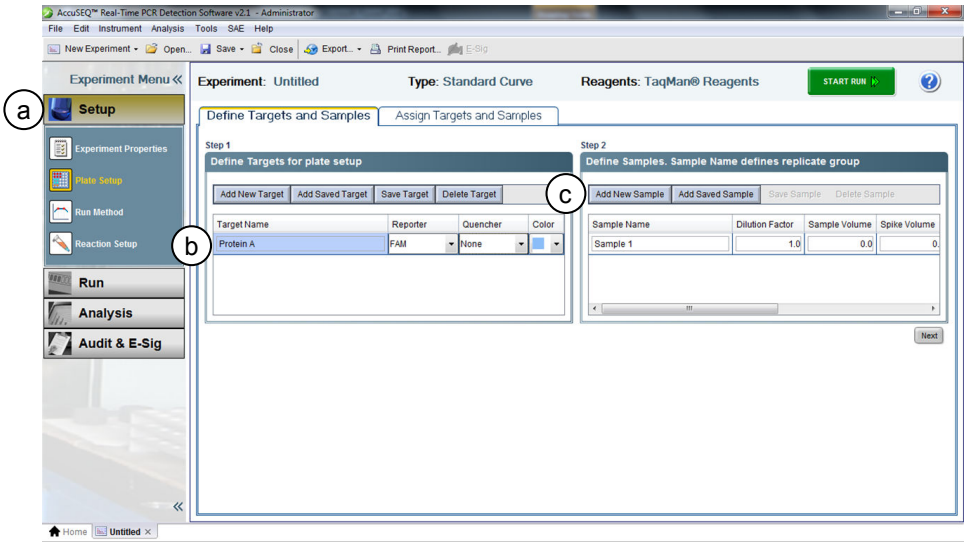
1. From the home screen click **Create Custom Experiment**.
2. Make the following selections in the Experiment Properties Pane:
   a. 7500 Fast (96 Wells)
   b. Quantitation – Standard Curve
   c. TaqMan™ Reagents
   d. Fast (~ 50 minutes to complete a run; 10 minutes for ligation and 40 minutes for qPCR)



3. Define Sample Number and Name.
   a. Click **Plate Setup** in the Experiment Menu Pane.
   b. Enter **Protein A** as the target name, select **FAM** as the reporter and **None** as the quencher.

c. Enter the number and name of your samples, excluding replicates. Click **Add New Sample** to enter the number of samples to be run.

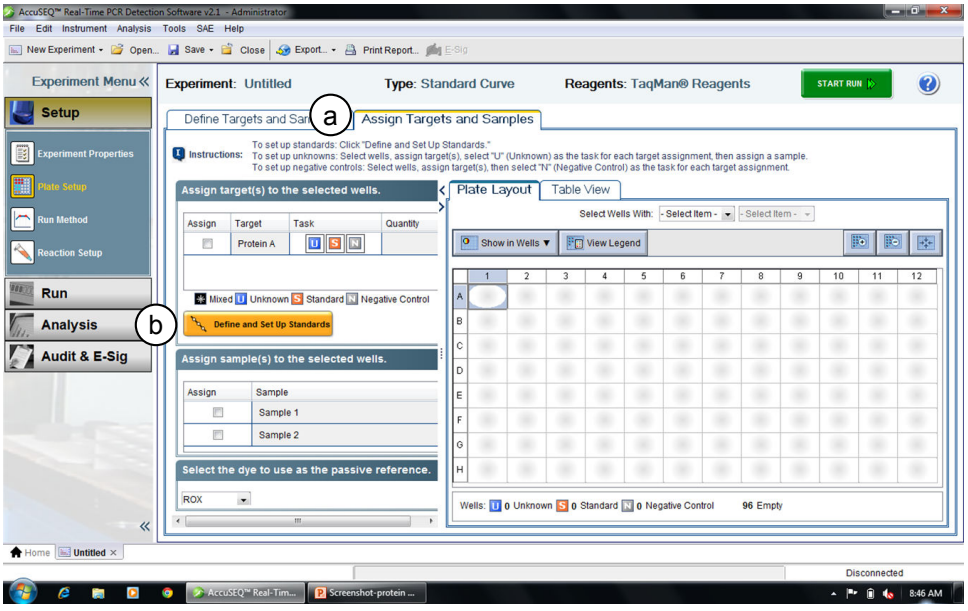
For example, if you have four samples run in triplicate, you would define four samples in this step. Replicates of those four samples will be defined in the next step.



4. Access the Standard Curve Dialog as follows:

   a. Select the **Assign Targets and Samples** tab.

   b. Click **Define and Setup Standards**.



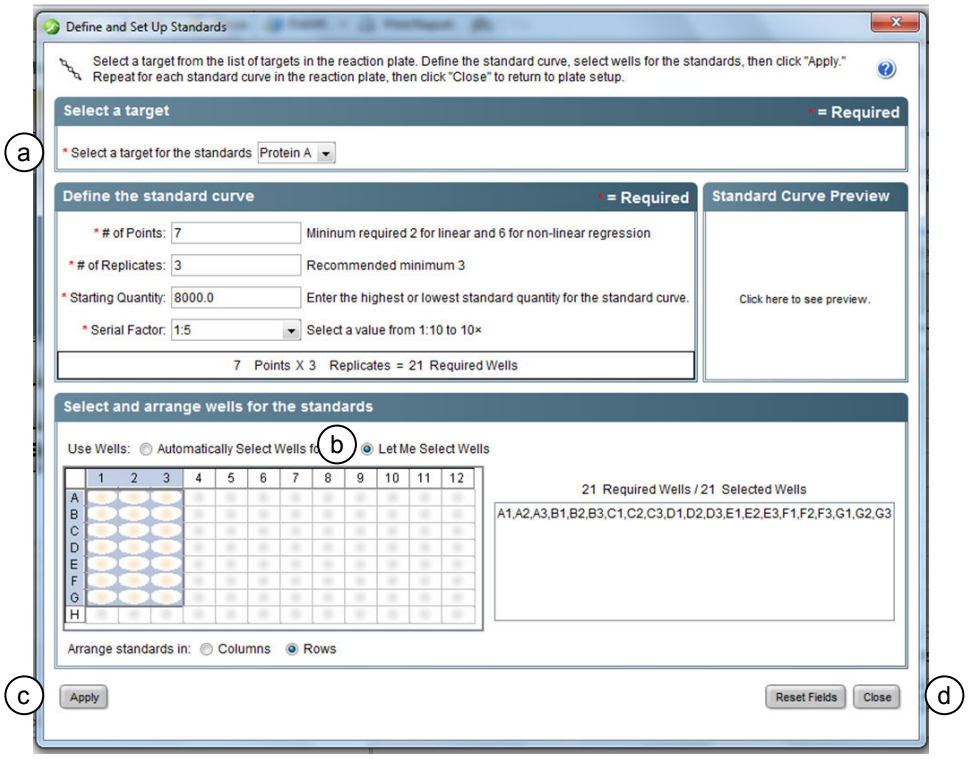
5. Setup the Standard Curve as follows:

   a. In the Define the standard curve tab, enter **7** for "# of Points", **3** for "# of Replicates", **8000** for "Starting Quantity", and **1:5** for "Serial Factor".

b. Click **Let Me Select Wells**. Click, hold, and drag the plate map to select the wells to be used as standards.

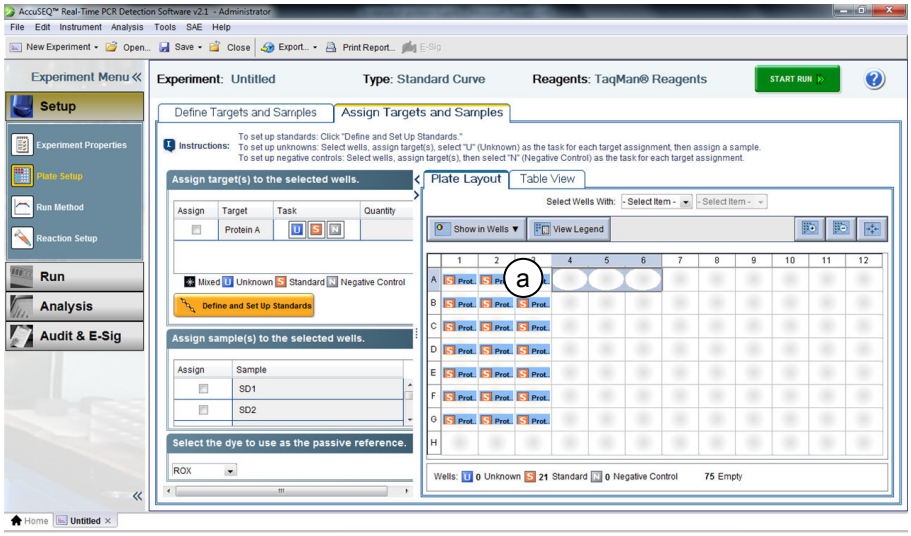
c. Click **Apply**.

d. Click **Close**.

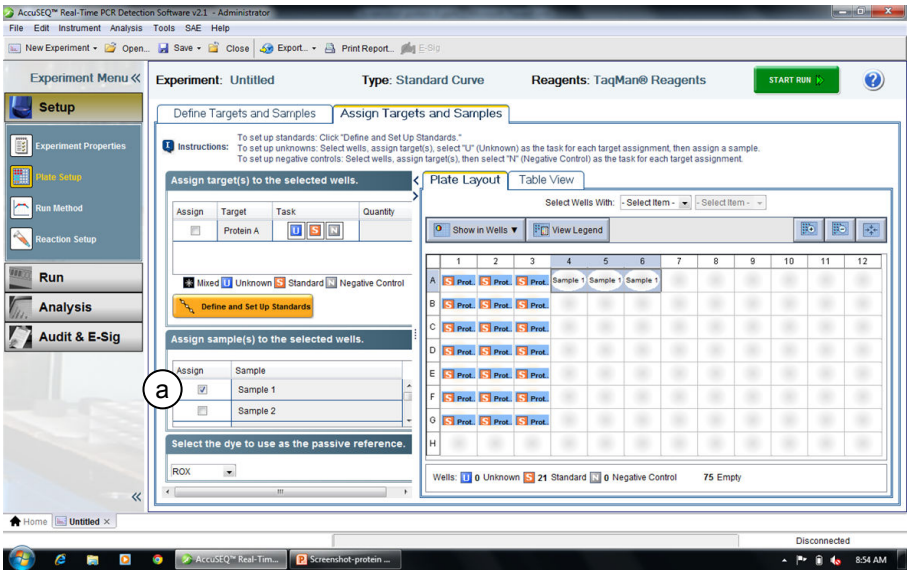


6. Assign sample name to wells.

   a. In Plate Layout, select all wells that will be assigned as replicates for Sample 1. In this example, A4, A5 and A6 are selected.

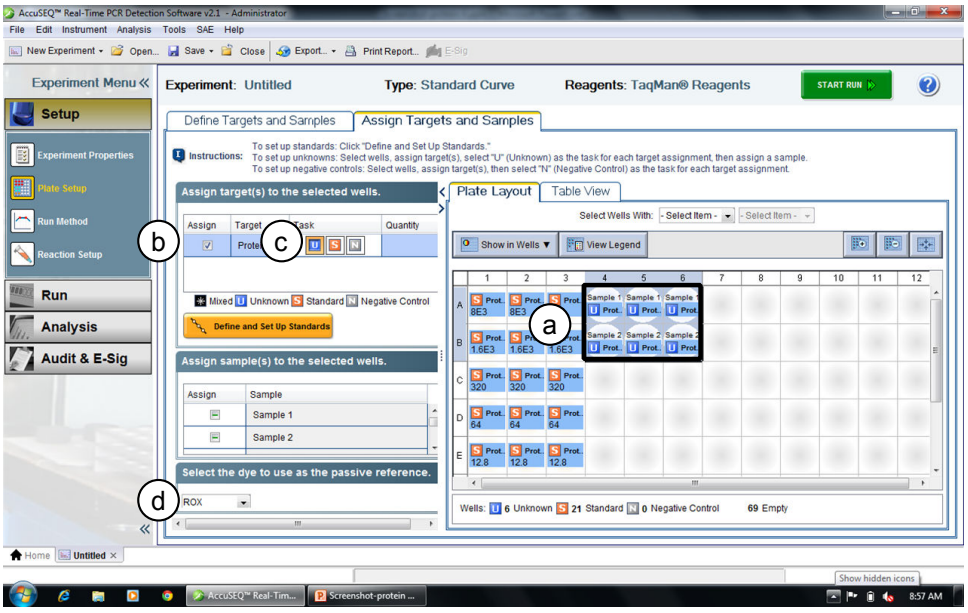


b. Click **Assign** next to the appropriate sample. Repeat for all unknown samples.

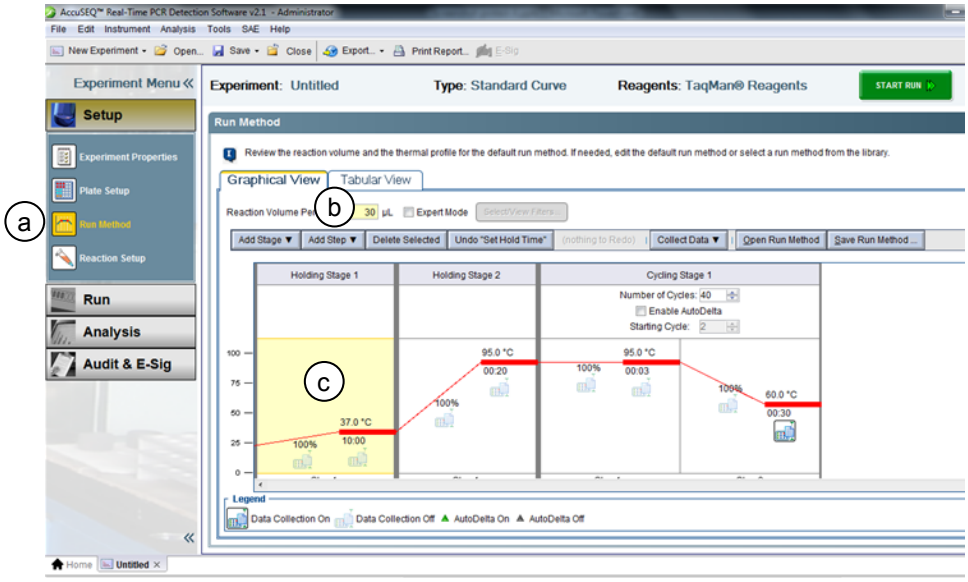


In this example, “Sample 1” is assigned to wells A4, A5 and A6.

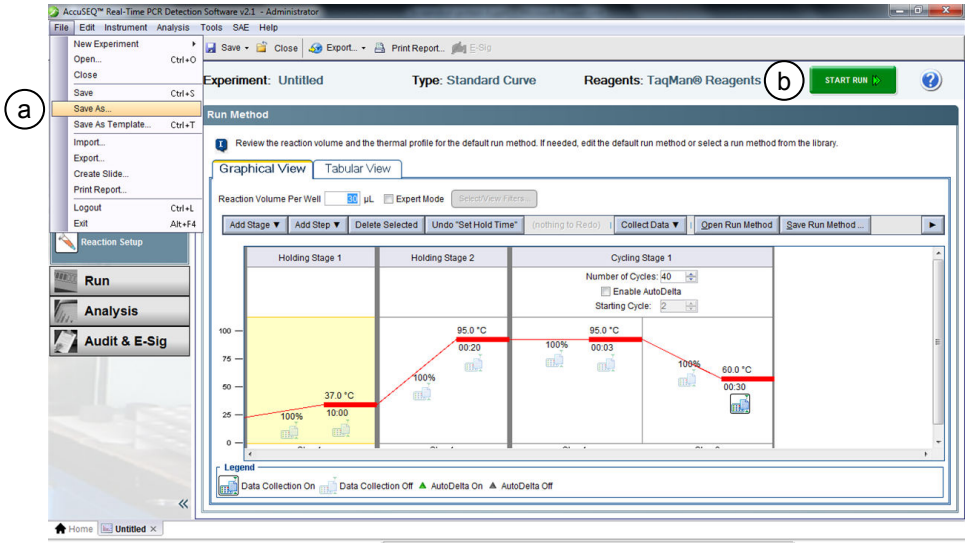
7. Assign Unknown Well Type as follows:
   a. Select all wells that will be designated as unknowns.
   b. Click **Assign** under Assign Targets to the Selected Wells.
   c. Click the blue **U** to assign the wells as unknowns.
   d. Verify that **ROX** is selected as the passive reference.



8. Setup qPCR parameters as follows:
   a. Click **Run Method**.
   b. Enter a reaction volume of **30 µL**.
   c. Verify reaction parameters match those shown:



9. Save the setup as follows:
   a. Select **File ▸ Save As...** (or select **File ▸ Save As Template...** to save this experimental setup for future use).
   b. Click **Start Run**.

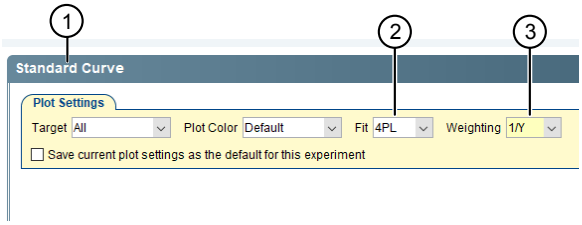


Perform data analysis with AccuSEQ™ software v2.1 or later

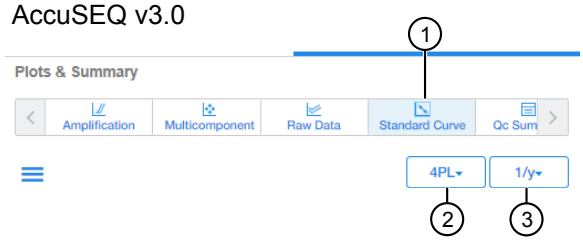
1. Ensure that the baseline is set to automatic and that the threshold is set to 0.2.

2. Use the AccuSEQ™ software to fit standards to a curve using a nonlinear method, then obtain interpolated values for the unknowns.
   - 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.

AccuSEQ v2.1



AccuSEQ v3.0



① Standard Curve
② 4PL curve fitting
③ 1/Y weighting

3. Export the data to a Microsoft™ Excel™ spreadsheet for custom statistical analysis if needed.

4. Evaluate the dynamic range using %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 LLOQ.
   - Common acceptance criteria for precision are %CV ≤20% throughout the curve and ≤25% at the LLOQ.

5. Obtain the final concentration for each sample by correcting for sample dilution and spike concentrations, if used.

Perform data analysis without AccuSEQ™ software

1. Select autobaseline **on**, then set the C_t threshold manually to **0.2**. Determine the C_t values.

2. Export the raw data from the qPCR software to a Microsoft™ Excel™ spreadsheet, then export from Microsoft™ Excel™ to your fitting program of choice. Transform the values to logarithmic values.

   Note: If you use GraphPad™, the Protein A Master Template (a Microsoft™ Excel™ template available from Technical Support) helps this process.

3. Fit standards to a curve using a non-linear method, then obtain interpolated values for the unknowns.
   - 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.

4. Transform concentration values from logarithmic to linear values.

5. Evaluate the dynamic range using %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 LLOQ.
   - Common acceptance criteria for precision are %CV ≤20% throughout the curve and ≤25% at the LLOQ.

6. Obtain the final concentration for each sample by correcting for sample dilution and spike concentrations, if used.

A Troubleshooting

Observation	Possible cause	Recommended action
Capture beads remain on comb	Misalignment of the magnetic head.	Contact your local Technical Support for re-alignment of the instrument.
	qPCR plate not placed in appropriate PCR Plate Adaptor during the magnetic particle processor instrument run.	Use the appropriate PCR Plate Adaptor (Fast or Standard; request from your local sales or service representative).
The standard curve plateaus at the lower standard concentrations and the NPC C_T is less than 28	Cross-contamination of Protein A or ligated product.	Decontaminate the bench and pipettors. Change gloves frequently and follow other good PCR practices. After the run completes, dispose of the qPCR plate. Do not remove the optical film from the qPCR plate; removing the film introduces amplicon contamination into the local environment. See Appendix C, "Good laboratory practices for PCR and RT-PCR". Before preparing the plates for each assay, clean the pipettors, plate racks and the microcentrifuge (if using tubes for standard dilution) to avoid cross-contamination.
		If prone to contamination, change the order of standards, sample, and plate preparation as follows: • Label plates • Prepare wash plates • Prepare probes plate • Move the prepared plates near the magnetic particle processor. • Prepare standards and samples. • Prepare capture plate
	The reagents are contaminated.	Use new reagents.
The C_T at 1600 pg/mL is above 20 and the NPC C_T is undetermined	Expired kit.	Check kit expiration date.
	Errors in reaction or run setup.	Repeat assay preparation. Make sure that the components are added in the recommended order.
Trending increase in C_T value for standard concentrations from run to run	Deterioration of standards.	Prepare fresh standards. Verify kit expiration date.

Observation	Possible cause	Recommended action
Random decrease in C_T during run	Cross-contamination of concentrated standards or samples with lower concentration samples.	Repeat experiment.
Random failures across the plate	Air bubbles introduced into plate wells during plate setup.	Dispense at bottom of wells to prevent bubble formation (bubbles prevent effective mixing during the magnetic particle processor run). If bubbles form, quick-spin the plate at 560 × g (~2000 rpm) in a plate centrifuge.
Poor recovery and/or efficiency during spike experiments	• Incorrectly designed spike amount. *or* • Sample concentration is higher than expected.	Use a spike amount 50–100% of the concentration in the unspiked sample. See Appendix B, "Design guidelines for ProteinSEQ™ System Protein A spike experiments".
Low spike efficiency	Salt concentration in sample well is too high.	Pre-dilute the sample so that final concentration of salt in the reaction well is <50 mM.
	Matrix interference from IgG or other components.	Evaluate the assay performance with higher sample dilutions.
Increased percent CV	Incorrect plate type used.	Use PCR Plates, 96-well, low profile, skirted, (Cat. No. AB-0800) for Capture, Probes, and Wash plates. See "Label the plates" on page 14.

B

Design guidelines for ProteinSEQ™ System Protein A spike experiments

About spike experiments

Spike recovery is an essential tool for evaluating the accuracy of a quantitation assay in relevant matrices.

A basic spike recovery experiment includes these steps.

1. If necessary, dilute the sample according to the experimental goal.
2. Split the starting sample matrix into two aliquots, one for spiking and a second for referencing.
3. Add a known amount of Protein A (e.g. stock from the standard curve dilution series) into the spike sample, and add a volume of sample diluent (e.g. buffer) equal to the spike volume to the reference sample.
4. Analyze the spike and the reference sample using the same method to generate a mean observed quantitation value.
5. In data processing, subtract the Mean Quantity (Reference) from the Mean Quantity (Spike) to calculate the Reference Adjusted Quantity (RAQ).
6. Divide the RAQ by the Spike Input and multiply by 100 to arrive at a Percent Recovery.

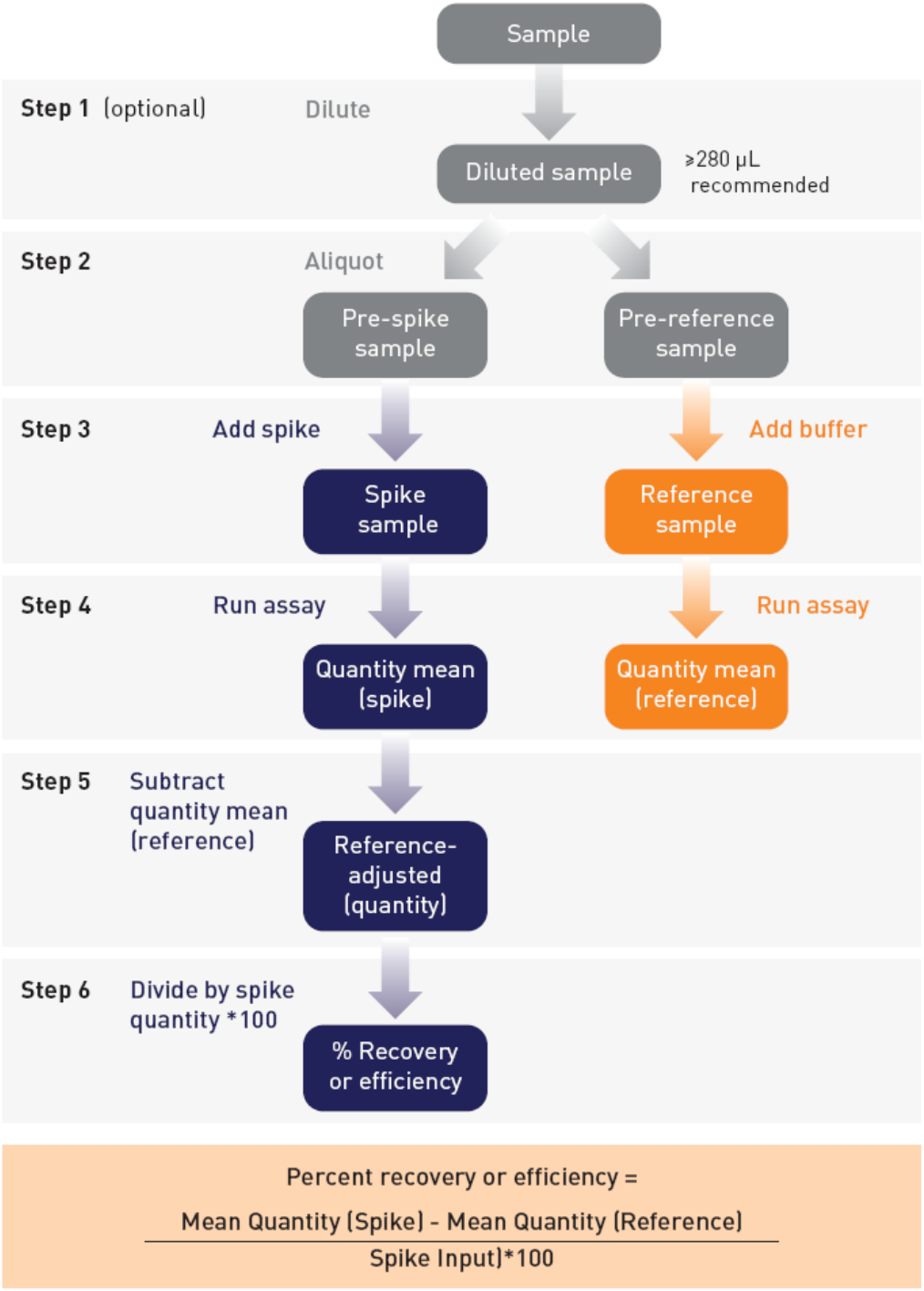


Figure 7 Use of a spike and reference sample to determine percent efficiency for a quantitation assay

Important experimental design considerations

To obtain informative and valid results, consider the following when designing the experiment:

- What concentration of analyte in the spike should be evaluated?
- At what ppm (drug product concentration) should the analyte be evaluated?
- What matrices are available for evaluation?

Guidelines for spike input concentration

The spike input concentration is defined by the goals of the experiment, the concentration of the analyte in the reference sample and the position of that concentration within the standard curve.

The concentration of the spike must be large enough to be differentiated from the analyte concentration already present in the reference sample. Typical spike concentrations range from 50–100% of the reference concentration. Therefore, the reference sample analyte concentrations must be known in order to select the proper spike input concentration.

- If the reference analyte concentration lies between the LLOQ and the mid-point of the standard curve, a 100% spike is recommended.
- If the reference analyte concentration is above the midpoint of the standard curve, a 50% spike is recommended. Note that working at the upper end of standard curve requires care that the final concentration after spiking does not exceed the ULOQ.

Guidelines for matrix selection

The choice of matrix directly affects the design of a spike experiment due to the fact that the ratio of analyte to drug (expressed as ppm in ng analyte/mg drug substance) is a fixed ratio that does not change with dilution. Therefore, practical limitations exist for working with all matrices and a priority must typically be assigned to evaluate either a specific analyte concentration (and the drug substance concentration that follows) or a specific drug substance concentration (and the analyte concentration that follows). For this reason, it is recommended that the goal of the experiment be established followed by procurement of a matrix rather than vice versa.

Volume of sample		250	250	250
Volume of spike		5	10	15
Stock concentrations for spiking	400,000	7,843.14	15,384.62	22,641.50
	40,000	784.31	1,538.46	2,264.15
	8,000	156.86	307.69	452.83
	1,600	31.37	61.54	90.56
	320	6.27	12.30	18.11
	64	1.25	2.46	3.62
	12.8	0.25	0.49	0.72
	2.56	0.05	0.10	0.14
	0.512	0.010	0.019	0.029

Figure 8 Final Protein A concentration of spike using various standard curve stock concentrations and volumes. Green cells = concentrations recommended for spiking studies. Yellow cells = concentrations within the dynamic range but not recommended for spiking studies. Red cells = concentrations out of the ProteinSEQ™ standard curve dynamic range.

Protein A quantitation example

Experimental goals: Evaluate Protein A quantitation at ~150 pg/mL in the presence of ~5 mg/mL IgG. An evaluation matrix containing an estimated 800 pg/mL of residual Protein A and 100 mg/mL IgG is procured.

1. Dilute 5X by mixing 60 µL sample with 240 µL sample diluent to reach a volume of 300 µL.
2. Dilute an additional 2X by adding 150 µL of 2X Sample Prep reagent to reach a final volume of 600 µL. The expected concentration is 160 pg/mL Protein A and 20 mg/ mL IgG.
3. Generate a pre-reference aliquot of 250 µL and a pre-spike aliquot of 250 µL in microcentrifuge tubes, enabling subsequent sample preparation steps. The remaining volume may be discarded.
4. Spike the sample. Figure 8 indicates that the closest spike to 100% can be achieved by spiking 250 µL of sample with 15 µL of the 1600 pg/mL stock solution. In order to have a matching reference, 250 µL of matrix is also combined with 15 µL of sample diluent to prepare the reference sample. Proceed with the described protocol by boiling, cooling and centrifuging the sample followed by complete transfer of the supernatant to the dilution plate.
5. Run the assay to obtain values for Mean Quantity (Spiked) and Mean Quantity (Reference). In this example, the observed values for Mean Quantity (Spiked) and Mean Quantity (Reference) are 160 pg/mL and 73.6 pg/mL respectively.
6. Calculate the Mean Quantity (Adjusted) for the spiked sample by subtracting Mean Quantity (Reference) from Mean Quantity (Spiked).
7. Calculate Percent Recovery by dividing the Mean Quantity (Adjusted) by the Spike Input and multiplying by 100.

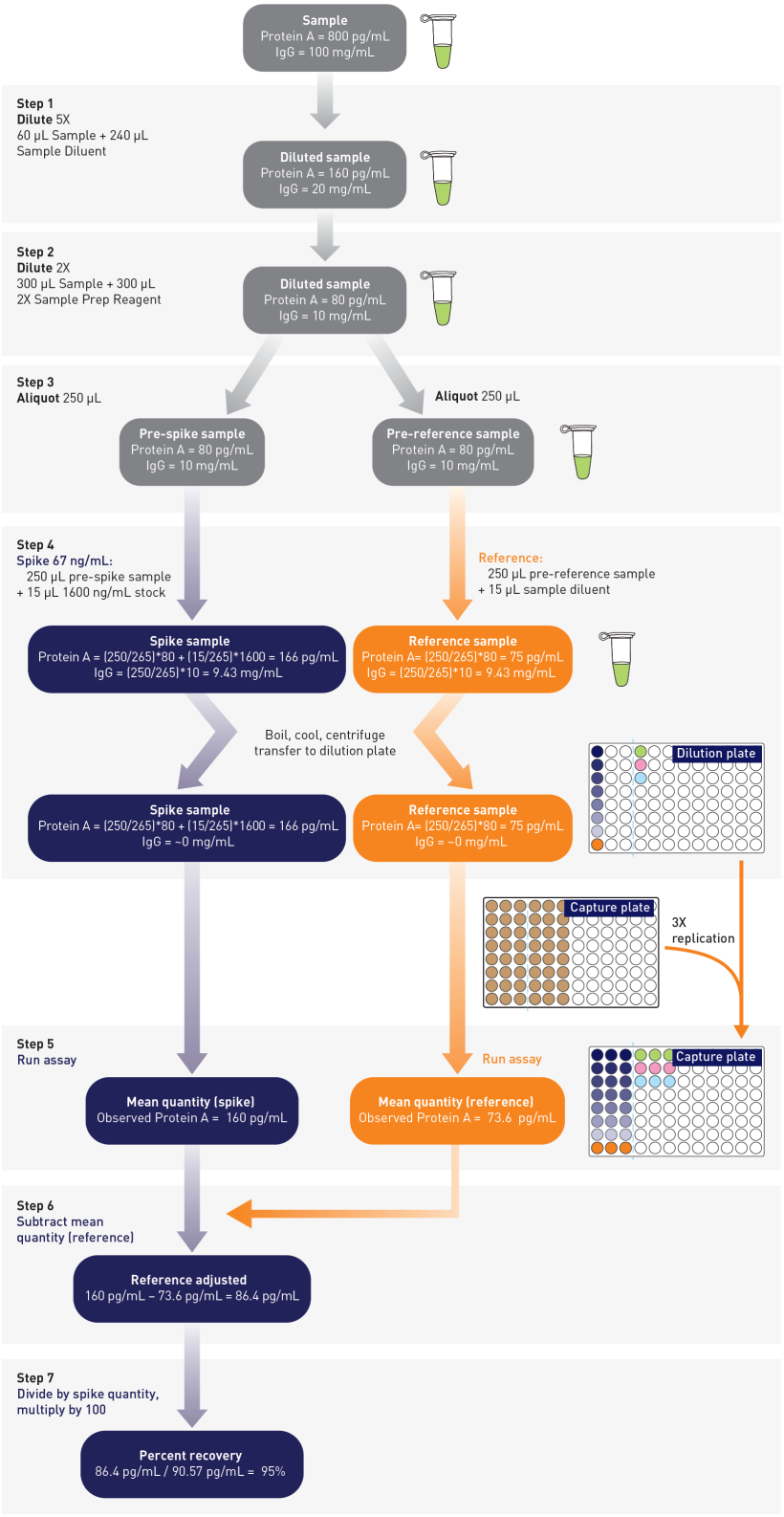


Figure 9 Example Protein A spike experiment with calculations.

Good laboratory practices for PCR and RT-PCR

- Wear clean gloves and a clean lab coat.
  - Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples.
- Change gloves if you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA decontamination solution.

D

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

Related documentation

Portable document format (PDF) versions of this guide and the following related documents are available from **thermofisher.com/support**:

Document	Publication number	Description
ProteinSEQ™ Protein A Quantification Kit Quick Reference — Workflow for FAST PCR plates	MAN0013525	Provides information on preparing and running assays using FAST PCR plates.
ProteinSEQ™ Protein A Quantification Kit Quick Reference — Workflow for Standard (non-FAST) PCR plates	MAN0013526	Provides information on preparing and running assays using Standard (non-FAST) PCR plates.

Note: To open the user documentation, use the Adobe Reader™ software available from **www.adobe.com**

Note: For additional documentation, see “Customer and technical support” on page 48.

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**.