applied biosystems

ViralSEQ™ Lentivirus Titer Kits

USER GUIDE

for use with:
ViralSEQ™ Lentivirus Proviral DNA Titer Kit
ViralSEQ™ Lentivirus Physical Titer Kit

Catalog Numbers A53561, A53562, A52597, and A52598

Publication Number MAN0026126

Revision C

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Revision history: MAN0026126 C (English)

Revision	Date	Description
C	15 September 2023	Update to include run and analysis information for AccuSEQ™ Real-Time PCR Software v3.2 on the QuantStudio™ 5 Real-Time PCR System and AccuSEQ™ Real-Time PCR Software v2.2 on the 7500 Real-Time PCR System.
B.0	2 September 2022	The ViralSEQ™ Lentivirus Physical Titer Kit was added to the guide, including: • Updates to the product description. kit contents and storage, and workflows • "Serial dilutions and sample preparation for the ViralSEQ™ Lentivirus Physical Titer Kit" on page 19 • Chapter 4, "Set up, run, and review ViralSEQ™ Lentivirus Physical Titer Kit experiments" • Appendix B, "Use the ViralSEQ™ Lentivirus Physical Titer Kit with the 7500 Fast Real-Time PCR Instrument and AccuSEQ™ software v2.x"
A.0	17 July 2022	New document for the ViralSEQ™ Lentivirus Proviral DNA Titer Kit (Cat. No. A53561).

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

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1 Product information

Product description

The Applied Biosystems™ ViralSEQ™ Lentivirus Titer Kits are TaqMan™-based, real-time PCR assays for process development and characterization of lentivirus vectors. The ViralSEQ™ Lentivirus Physical Titer Kit quantitates the amount of lentivirus in the supernatants of cell-based, bioproduction systems. The ViralSEQ™ Lentivirus Proviral DNA Titer Kit quantitates genome copies integrated into cells post-transduction. Both kits use provided controls to generate standard curves used in quantitation. With specific quantitation of viral and proviral copy numbers, qPCR data from physical and infectious titers can be easily compared. Together, the ViralSEQ™ Lentivirus Titer Kits provide a convenient method to correlate total and infectious lentiviral particles during process development and optimization.

Both the ViralSEQ™ Lentivirus Physical Titer Kit and the ViralSEQ™ Lentivirus Proviral DNA Titer Kit are compatible with the PrepSEQ™ Nucleic Acid Sample Preparation Kit (Cat. A50485), which offers both manual and automated sample preparation workflows. For real-time PCR, both kits have been validated on the Applied Biosystems™ 7500 Fast Real-Time PCR System and the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System. Data analysis is streamlined using AccuSEQ™ Real-Time PCR Software that provides accurate quantitation. The software has security, audit, and e-signature capabilities to enable 21 CFR Pt 11 compliance.

- The ViralSEQ™ Lentivirus Physical Titer Kit can be used to quantitate viral titers through the measurement of lentivirus RNA in the supernatants of cell-based production systems.
- The ViralSEQ™ Lentivirus Proviral DNA Titer Kit is used to quantitate the number of proviral copies incorporated into host cells following transduction.

Contents and storage

ViralSEQ™ Lentivirus Titer Kits

Table 1 ViralSEQ™ Lentivirus Physical Titer Kit (Cat. No. A52597)

Contents	Cap color	Amount	Storage
Box 1, ViralSEQ™ Lentivirus Physical Titer qPCR Reagents			
Physical Titer Assay Mix	(Gold)	275 µL	Protect from light. –25°C to –15°C
25× RT-PCR Enzyme Mix	(Green)	110 µL	–30°C to –10°C
2× RT-PCR Buffer	(Red)	1.375 mL	
Nuclease Free Water	(White)	1.0 mL	–25°C to –15°C until first use. After first use, store at 2–8°C.
Box 2, ViralSEQ™ Lentivirus Physical Titer RNA Control			
Physical Titer RNA Control (2 ×10^{10} copies/µL)	(Gray)	44 µL	–25°C to –15°C
RNA Dilution Buffer	Clear bottle	7.0 mL	

Table 2 ViralSEQ™ Lentivirus Physical Titer Kit with PrepSEQ™ Nucleic Acid Sample Preparation Kit (Cat. No. A52598)

Contents	Cat. No.	Amount
ViralSEQ™ Lentivirus Physical Titer Kit	A52597	1 kit
PrepSEQ™ Nucleic Acid Sample Preparation Kit	A50485	1 kit
DNase I, RNase-free (1 U/µL)	EN0531	1 kit

Table 3 ViralSEQ™ Lentivirus Proviral DNA Titer Kit (Cat. No. A53561)

Contents	Cap color	Amount	Storage
Box 1, ViralSEQ™ Lentivirus Proviral DNA Titer qPCR Reagents			
Proviral DNA Titer Assay Mix	(Purple)	660 µL	Protect from light. –25°C to –15°C
2× Environmental Master Mix 2.0	(Orange)	2 ×0.75 mL	–25°C to –15°C
Nuclease Free Water	(White)	1.0 mL	–25°C to –15°C until first use. After first use, store at 2–8°C.
Box 2, ViralSEQ™ Lentivirus Proviral DNA Titer Control			
Proviral DNA Titer Control (2×10^8 copies/µL)	(Blue)	44 µL	–25°C to –15°C **IMPORTANT!** Do not store below –25°C.
DNA Dilution Buffer	Clear bottle	7.0 mL	–25°C to –15°C

Table 4 ViralSEQ™ Lentivirus Proviral DNA Titer Kit with PrepSEQ™ Nucleic Acid Sample Preparation Kit (Cat. No. A53562)

Contents	Cat. No.	Amount
ViralSEQ™ Lentivirus Proviral DNA Titer Kit	A53561	1 kit
PrepSEQ™ Nucleic Acid Sample Preparation Kit	A50485	1 kit

PrepSEQ™ Nucleic Acid Sample Preparation Kit (included with Cat. Nos. A52598 and A53562)

Table 5 PrepSEQ™ Nucleic Acid Sample Preparation Kit

Contents	Amount	Storage
Box 1, PrepSEQ™ Nucleic Acid Extraction Kit		
Lysis Buffer	2 × 50 mL	Room temperature
Binding Solution (Isopropanol), empty bottle	1	
Wash Buffer Concentrate	2 × 26 mL	
Elution Buffer	25 mL	
Proteinase K (PK) Buffer Can be used for existing validated manual protocols.	50 mL	

Table 5 PrepSEQ Nucleic Acid Sample Preparation Kit *(continued)*

Contents	Amount	Storage
Proteinase K (PK) Buffer II[1] Recommended for new manual protocols. Required for automated protocols.	11 mL	Room temperature
Box 2, PrepSEQ™ Nucleic Acid Extraction Kit		
Magnetic Particles	2 × 1.5 mL	Room temperature
Box 3, PrepSEQ™ Nucleic Acid Extraction Kit		
Proteinase K, 20 mg/mL	1.25 mL	–20°C or below
PrepSEQ™ Residual DNA Sample Preparation Kit		
Proteinase K, 20 mg/mL	1.25 mL	–20°C or below
Yeast tRNA, 10 mg/mL	0.5 mL	
Glycogen, 5 mg/mL	2 × 1.0 mL	

[1] Also sold separately (Cat. No. 4415320).

Required materials not supplied

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.

Catalog numbers that appear as links open the web pages for those products.

Required for the ViralSEQ™ Lentivirus Titer Kits

Item	Source
Real-time PCR Instrument and software	
QuantStudio™ 5 Real-Time PCR System with AccuSEQ™ software v3.1 or later	Contact your local sales representative.
Equipment	
(If using automated sample preparation) KingFisher™ Flex Purification System with 96 Deep-Well Head	5400630
Microcentrifuge	MLS
Laboratory mixer, vortex, or equivalent	MLS
Plate centrifuge	MLS
VeritiPro™ 96-well Thermal Cycler	A48141

(continued)

Item	Source
Consumables	
Disposable gloves	MLS
Aerosol-resistant pipette tips	MLS
Nuclease-free microcentrifuge tubes	MLS
Pipettors: • Positive-displacement • Air-displacement • Multichannel	MLS
MicroAmp™ Optical 96-Well Reaction Plate, 0.2 mL	4316813
Consumables for the QuantStudio™ 5 Real-Time PCR System	
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	4346906
MicroAmp™ 96-Well Tray/Retainer Set	403081
MicroAmp™ Optical Adhesive Film	4360954
MicroAmp™ 96-Well Base	N8010531
MicroAmp™ Adhesive Film Applicator	4333183
Recommended for sample extraction	
PrepSEQ™ Nucleic Acid Sample Preparation Kit[1]	A50485
5 M NaCl	MLS
Recommended for DNase treatment (ViralSEQ™ Lentivirus Physical Titer Kit only)	
DNase I, RNase-free (1 U/µL)[2]	EN0531
50 mM EDTA	MLS

[1] Included with Cat. Nos. A52598 and A53562.
[2] Included with Cat. No. A52598.

Required for manual sample preparation

Item	Source
Equipment	
Magnetic stand, 16-position	12321D
Block heater for use with 2-mL tubes. Manual DNA/RNA extraction involves two incubations at different settings, so two heaters may be convenient.	MLS
Benchtop microcentrifuge for 1.5-mL and 2-mL tubes	MLS
Vortex	MLS
Vortex Adapter-60, for use with the Vortex-Genie™	AM10014
Consumables	
Disposable gloves	MLS
Aerosol-resistant micropipette tips	MLS
Pipettors, P1000, P200, P20 and P10: • Positive-displacement • Air-displacement • Multichannel	MLS
Nonstick, RNase-free Microfuge Tubes, 1.5-mL (1 box; 250 tubes/box)	AM12450
Safe-Lock Tube, 2.0-mL	VWR™ 62111-754
Reagents	
Ethanol, 95% **IMPORTANT!** Do not use denatured ethanol. It contains components that are not compatible with the protocol.	MLS
Isopropanol, 100%	MLS
1X PBS (free of Mg and Ca)	MLS
0.5 M NaCl	MLS

Required for automated sample preparation

Table 6 Pharma KingFisher™ Flex Purification System with 96 Deep-Well Head (Cat. No. A31508) accessories

Item	Source
Pharma KingFisher™ Flex 96 Deep-Well Tip Combs	A43074
Pharma KingFisher™ Flex 96 Deep-Well Plates	A43075
Pharma KingFisher™ Flex Magnetic Head for 96 Deep-Well Plate	A31542
Pharma MagMAX™ 96 PCR Well Magnetic Head	4472991
Pharma KingFisher™ Flex 96 Standard Plate	A43076
(*Optional*) Pharma Magnetic Stand-96	A31543

Table 7 Additional materials

Item	Source
Consumables	
Disposable gloves	MLS
Aerosol-resistant micropipette tips	MLS
Pipettors, P1000, P200, P20 and P10: • Positive-displacement • Air-displacement • Multichannel	MLS
Nonstick, RNase-free Microfuge Tubes, 1.5-mL (1 box; 250 tubes/box)	AM12450
Reagents	
Ethanol, 95% **IMPORTANT!** Do not use denatured ethanol. It contains components that are not compatible with the protocol.	MLS
Isopropanol, 100%	MLS
1X PBS (free of Mg and Ca)	MLS
0.5 M NaCl	MLS

Lentivirus production

The ViralSEQ™ Lentivirus Physical Titer Kit is used to quantitate viral particles produced using cell-based systems. We recommend using the LV-MAX™ Lentiviral Production System (Cat. No. A35684) to produce high titer lentiviral vectors. The system is based on the transient transfection of high-density, suspension HEK293F cells adapted to a chemically defined, serum-free and protein free medium. For more information, see the *LV-MAX™ Lentiviral Production System User Guide* (Pub. No. MAN0017000).

Kit controls

Table 8 ViralSEQ™ Lentivirus Physical Titer Kit

Control	Use
Internal Positive Control (IPC) (Included in the Physical Titer Assay Mix)	Lack of amplification Indicates PCR inhibition.
Physical Titer RNA Control (2×10^{10} copies/µL)	Amplification indicates that the PCR was successful.
Negative control (RNA Dilution Buffer)	Amplification indicates contamination.

Table 9 ViralSEQ™ Lentivirus Proviral DNA Titer Kit

Control	Use
Internal Positive Control (IPC) (Included in the Proviral DNA Titer Assay Mix)	Lack of amplification Indicates PCR inhibition.
Proviral DNA Titer Control (2×10^{8} copies/µL)	Amplification indicates that the PCR was successful.
Negative control (DNA Dilution Buffer)	Amplification indicates contamination.

Workflow: ViralSEQ™ Lentivirus Physical Titer Kit

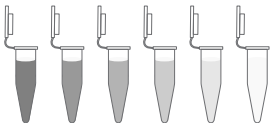
ViralSEQ™ Lentivirus Physical Titer Kit Workflow

varies

Produce lentivirus with the LV-MAX™ Lentiviral Production System

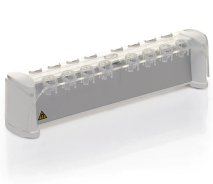
1h

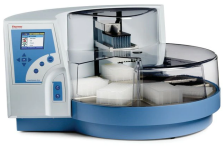
Prepare reagents and serial dilutions



1h30min

Sample extraction with the PrepSEQ™ Nucleic Acid Sample Preparation Kit



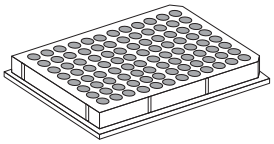


45min

Treat samples with DNase I, RNase-free (1 U/µL)

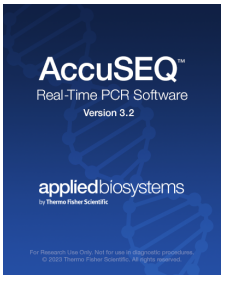
15min

Prepare the PCR reactions



2h

Start the run (QuantStudio™ 5 Real-Time PCR Instrument)



20min

Review the results

Workflow: ViralSEQ™ Lentivirus Proviral DNA Titer Kit

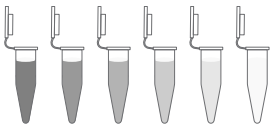
ViralSEQ™ Lentivirus Proviral DNA Titer Kit Workflow

varies

Lentivirus transduction of target cells

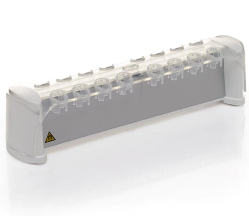
1h

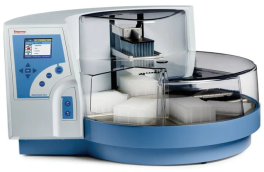
Prepare reagents and serial dilutions



1h30min

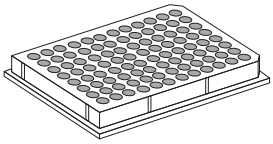
Sample extraction with the PrepSEQ™ Nucleic Acid Sample Preparation Kit





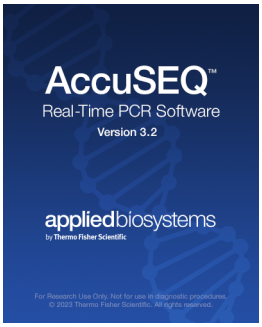
15min

Prepare the PCR reactions



2h

Start the run (QuantStudio™ 5 Real-Time PCR Instrument)



20min

Review the results

2 Prepare reagents and serial dilutions

We recommend using the PrepSEQ™ Nucleic Acid Sample Preparation Kit (Cat. No. A50485) for sample preparation. The kit uses chemical lysis and magnetic beads to extract nucleic acid from recombinant lentiviruses and integrated proviruses.

Prepare the PrepSEQ™ reagents: before first use of the kit

Magnetic beads

1. Set a block heater to 37°C.
2. Incubate the Magnetic Particle suspension at 37°C for a minimum of 10 minutes with intermittent vortexing at 900 rpm, or until the particles are completely suspended.

Binding Solution

1. Add 45 mL of 100% isopropanol to the Binding Solution bottle.
2. Label the bottle to indicate that it contains isopropanol, then store the bottle at room temperature.

Wash Buffer Concentrate

1. Add 74 mL of 95% ethanol to one bottle of PrepSEQ™ Wash Buffer Concentrate, then mix completely.
2. Label the bottle to indicate that it contains ethanol, then store the bottle at room temperature.

Prepare PrepSEQ™ reagents: before each use of the kit

Proteinase K (PK) mix

- Use Proteinase K (PK) Buffer II for all protocols.

  Note: Proteinase K (PK) Buffer is provided in the kit for use by laboratories that had previously validated with this buffer. If required, use exactly as described for Proteinase K (PK) Buffer II.

- Prepare a fresh mix before each use of the kit.
- Include a 10% overage to account for pipetting losses.

Component	Number of extractions				
	1	7	10	13	25
Proteinase K, 20 mg/mL	10 µL	70 µL	100 µL	130 µL	250 µL
Proteinase K (PK) Buffer II	60 µL	420 µL	600 µL	780 µL	1,500 µL

Lysis solution

- Prepare a fresh mixture immediately before use or during Proteinase K incubation.
- Prepare 360 µL (amount required) of lysis solution mix per sample.

Reagent	Volume for 1 extraction	Volume for ~20 extractions
Glycogen, 5 mg/mL	8.32 µL	180 µL
Yeast tRNA, 10 mg/mL	0.18 µL	4 µL
Lysis Buffer	351.5 µL	7,600 µL
Total	**360 µL**	**7,784 µL**

Guidelines for optimal yields

- Maintain a homogenous suspension of the magnetic beads to maximize the surface area to which the DNA/RNA can bind. The appearance of the mixture should be homogenous after mixing.
- After drying, the DNA/RNA remains bound to the magnetic beads. Do not allow the magnetic beads to over-dry because this reduces the elution efficiency; over-dried beads are not easily resuspended.
- During manual elution, vortex every 2 minutes to assist elution. This will result in better yield during recovery.

Note: Some test samples cause the beads to adhere very firmly to the tube wall, while others form loose pellets that detach during the vortex steps. All pellets should dissolve with vortexing during heated elution. If vortexing does not result in full resuspension, then wash the beads off the tube by pipetting.

Note: White or brown precipitate may form in the Magnetic Particles tube if it is stored at 2–8°C. The precipitate will dissolve when it is heated to 37°C for a minimum of 10 minutes with intermittent vortexing. Make sure the precipitate is completely dissolved before using the beads.

Serial dilutions and sample preparation for the ViralSEQ™ Lentivirus Physical Titer Kit

Collect lentivirus particles for RNA extraction and sample preparation. For test samples that contain levels of RNA above the highest point of the assay standard curve (10^9 copies per reaction), dilute these samples (from 1:100 up to 1:10,000) with a solution of 1 X PBS (free of Mg and Ca) or 50 mM Tris, pH 8.0, 0.5 M NaCl.

Note: Use sample dilution buffer as the negative extraction control or dilute extracted RNA with elution buffer before running the PCR reaction. Diluting samples in water or TE reduces extraction efficiency.

Prepare the Lentivirus RNA control serial dilutions

Guidelines for standard dilutions

- Prepare the standard curve and the test samples in different areas of the lab.
- Use different sets of pipettors for test sample preparation and for standard curve preparation and aliquoting to avoid cross-contamination of test samples.

Prepare the serial dilutions

Thaw the Physical Titer RNA Control (2 ×10^{10} copies/µL) on ice. Vortex at medium speed for 5 seconds, briefly centrifuge, then place on ice until use.

1. Label nonstick 1.5-mL microfuge tubes: **NTC**, **SD1**, **SD2**, **SD3**, **SD4**, **SD5**, and **SD6** [used for limit of detection (LOD)].
2. Add 35 µL of RNA Dilution Buffer (RDB) to the NTC (no template control) tube. Place the tube on ice.
3. Perform the serial dilutions.

   When dispensing RNA, pipette up and down gently. After each transfer, vortex for 7 seconds, then centrifuge briefly.

Table 10 Standard curve dilutions (ViralSEQ™ Lentivirus Physical Titer Kit)

Serial dilution (SD) tube	Dilution	Dilution factor	Copies/ PCR reaction (5 µL of diluted RNA)
SD1	5 µL RNA control + 495 µL RDB	100	1.00E+09
SD2	5 µL SD1 + 495 µL RDB	100	1.00E+07
SD3	5 µL SD2 + 495 µL RDB	100	1.00E+05
SD4	5 µL SD3 + 495 µL RDB	100	1,000
SD5	10 µL SD4 + 190 µL RDB	20	50
SD6 (LOD)	40 µL SD5 + 160 µL RDB	5	10

Store the standard curve dilution tubes at 4°C or on ice. Use the dilutions within 6 hours for RT-qPCR.

Extraction control guidelines

We recommend that you use the following extraction controls:

Type of control	Contains	Number to run	Used to
Negative (NEG)[1]	1X PBS (free of Mg and Ca)	1 per batch of extractions	To monitor for contamination of the extraction reagents.
Extraction/recovery (ERC)	Positive control from the kit	3 per sample	• Evaluate the efficiency of DNA or RNA extraction, recovery, and quantification from test samples. • Verify reagent and system performance.

[1] Optional during routine testing.

Table 11 Starting volumes for sample extraction

Sample type	Sample (µL)	SD1 (µL)	1 x PBS (µL)	Total (µL)
Sample	5	0	95	100
Sample ERC	5	5	90	100
NEG	0	0	100	100

Note: To calculate the efficiency of nucleic acid recovery and quantification from the test samples,subtract the amount of nucleic acid measured in the sample without the addition of DNA or RNA control from the amount of nucleic acid measured in the ERC sample.

Spike recovery calculations

For spiked samples the percent spike recovery can be calculated from the mean quantity of replicates and the input spike quantity:

$$\text{Spike Recovery \%} = \frac{\text{Mean quantity of replicates}}{\text{Input spike quantity}} \times 100$$

Percent spike recovery can also be calculated with respect to spiked test replicates from the mean quantity of spiked replicates, the mean quantity of non-spiked replicates, and the input spike quantity:

$$\text{Spike Recovery \%} = \frac{\text{(Spiked mean quantity - non-spiked mean quantity)}}{\text{Input spike quantity}} \times 100$$

The total DNA in the test samples can be calculated using the percent spike recovery with respect to spiked test replicates and the mean quantity of non-spiked replicates:

$$\text{DNA in test sample} = \frac{\text{Non-spiked mean quantity}}{\text{Spike recovery}}$$

Prepare the extraction/ recovery control (ERC) for the ViralSEQ™ Lentivirus Physical Titer Kit

We recommend spiking the positive control RNA into the test sample so that the total control RNA amount is 2- 10 times the amount of RNA measured in the test sample.

1. Add the appropriate control RNA dilution amount to the test sample.
2. Add PBS to a total volume of 100 uL.
3. Perform the sample extraction.

For example, if the test sample contains 1 x 10^8 copies in 5 µL:

1. Add 5 µL of SD1 (1 x 10^9) to 5 µL of the test sample.
2. Add 90 µL of PBS (final volume of 100 µL).
3. Perform sample preparation with the PrepSEQ™ Nucleic Acid Sample Preparation Kit (on the 100 µL final volume). Elute volume is 200 µL.
4. Perform the DNase treatment and qPCR.

Note: When calculating the total sample dilution factor, account for the 1:2 dilution from DNase pre-treatment step .

The sample dilution is 1:80, so the expected copy number following qPCR is ~1.25 x 10^7.

Triplicate extractions

Triplicate extractions are required for post-PCR analysis calculation of mean quantity, standard deviation, and coefficient of variation.

In addition to test samples, we recommend triplicate extractions for the negative control and the extraction/recovery control (ERC).

Perform a single PCR reaction for each extraction.

The table below illustrates the total number of extractions required based on the 1, 2, and 3 samples extracted in a batch.

Table 12 Total number of extractions per batch of test samples

Number of test samples		Total number of extractions for the batch
1	3 extractions required for each: • Test sample • Test sample extraction/recovery control (ERC) • Negative extraction control[1]	9
2		15
3		21

[1] Optional during routine testing.

Serial dilutions and sample preparation for the ViralSEQ™ Lentivirus Proviral DNA Titer Kit

Count and transduce host cells with the lentiviral vector. The number of cells seeded for transduction (day 1) is used to calculate infectious titer of the vector.

Harvest cells when ready. Quantitate the number of cells extracted either by the counting them or by using the nucleic acid quantity equivalence (1 cell = 6.6 pg for diploid cells). For cells that are non-diploid, establish the appropriate nucleic acid quantity equivalence. The number of cells extracted is used to calculate vector copy number.

Extract DNA and prepare samples for provirus quantitation. For test samples that are suspected to contain levels of proviral DNA above the highest point of the assay standard curve (10^7 copies per reaction), dilute these samples (from 1:100 up to 1:10,000) with a solution of 1 X PBS (free of Mg and Ca) or 50 mM Tris, pH 8.0, 0.5 M NaCl.

Note: Use sample dilution buffer as the negative extraction control or dilute extracted RNA with elution buffer before running the PCR reaction. Diluting samples in water or TE reduces extraction efficiency.

Prepare the Lentivirus DNA control serial dilutions

Guidelines for standard dilutions

- Prepare the standard curve and the test samples in different areas of the lab.
- Use different sets of pipettors for test sample preparation and for standard curve preparation and aliquoting to avoid cross-contamination of test samples.

Prepare the serial dilutions

1. Thaw the Proviral DNA Titer Control (2×10^8 copies/µL) and the DNA Dilution Buffer (DDB) at room temperature for **30 – 60 minutes**. Confirm that the Proviral DNA Titer Control and DDB are completely thawed before proceeding.

2. Vortex the Proviral DNA Titer Control at high speed for 20–60 seconds, then centrifuge for 3–5 seconds.

   Note: When vortexing, ensure the Proviral DNA Titer Control is moving in a circular motion in the tube. Complete thawing and resuspension is required in order to fully homogenize the Proviral DNA Titer Control.

3. Label nonstick 1.5-mL microfuge tubes: **NTC**, **SD1**, **SD2**, **SD2.1**, **SD3**, **SD4**, **SD5**, and **SD6** [used for limit of detection (LOD)].

4. Add 35 µL of DNA Dilution Buffer (DDB) to the NTC (no template control) tube. Place the tube on ice.

5. Perform the serial dilutions.

   When dispensing DNA, pipette up and down gently. After each transfer, vortex for 7 seconds, then centrifuge briefly.

Table 13 Standard curve dilutions (ViralSEQ™ Lentivirus Proviral DNA Titer Kit)

Serial dilution (SD) tube	Dilution[1]	Dilution factor	Copies/ PCR reaction (5 µL of diluted DNA)
SD 1	5 µL DNA control + 495 µL DDB	100	1.00E+07
SD 2	5 µL SD1 + 495 µL DDB	100	1.00E+05
SD2.1	**20 µL SD2 + 180 µL DDB**	**10**	**Not used in standard curve**
SD 3	20 µL SD2.1 + 180 µL DDB	10	1.00E+03
SD 4	20 µL SD3 + 180 µL DDB	10	100
SD 5	20 µL SD4 + 60 µL DDB	4	25
SD 6 (LOD)	20 µL SD5 + 30 µL DDB	2.5	10

[1] Dilution volumes are sufficient for 1 standard curve in triplicates. Scale volumes accordingly for more replicates.

Store the standard curve dilution tubes at 4°C or on ice. Use the dilutions within 6 hours for qPCR.

Extraction control guidelines

We recommend that you use the following extraction controls:

Type of control	Contains	Number to run	Used to
Negative (NEG)[1]	1X PBS (free of Mg and Ca)	1 per batch of extractions	To monitor for contamination of the extraction reagents.
Extraction/recovery (ERC)	Positive control from the kit	3 per sample	• Evaluate the efficiency of DNA or RNA extraction, recovery, and quantification from test samples. • Verify reagent and system performance.

[1] Optional during routine testing.

Table 14 Starting volumes for sample extraction

Sample type	Sample (µL)	SD1 (µL)	1 x PBS (µL)	Total (µL)
Sample	5	0	95	100
Sample ERC	5	5	90	100
NEG	0	0	100	100

Note: To calculate the efficiency of nucleic acid recovery and quantification from the test samples,subtract the amount of nucleic acid measured in the sample without the addition of DNA or RNA control from the amount of nucleic acid measured in the ERC sample.

Spike recovery calculations

For spiked samples the percent spike recovery can be calculated from the mean quantity of replicates and the input spike quantity:

$$\text{Spike Recovery \%} = \frac{\text{Mean quantity of replicates}}{\text{Input spike quantity}} \times 100$$

Percent spike recovery can also be calculated with respect to spiked test replicates from the mean quantity of spiked replicates, the mean quantity of non-spiked replicates, and the input spike quantity:

$$\text{Spike Recovery \%} = \frac{\text{(Spiked mean quantity - non-spiked mean quantity)}}{\text{Input spike quantity}} \times 100$$

The total DNA in the test samples can be calculated using the percent spike recovery with respect to spiked test replicates and the mean quantity of non-spiked replicates:

$$\text{DNA in test sample} = \frac{\text{Non-spiked mean quantity}}{\text{Spike recovery}}$$

Prepare the extraction/ recovery control (ERC) for the ViralSEQ™ Lentivirus Proviral DNA Titer Kit

We recommend spiking the positive control DNA into the test sample so that the total control DNA amount is 2- 10 times the amount of DNA measured in the test sample.

1. Add the appropriate control DNA dilution amount to the test sample.
2. Add PBS to a total volume of 100 uL.
3. Perform the sample extraction.

For example, if test sample contains 1 x 10^6 copies in 5 µL:

1. Add 5 µL of SD1 (1 x 10^7) to 5 µL of the test sample.
2. Add 90 µL of PBS (final volume of 100 µL).
3. Perform sample preparation with the PrepSEQ™ Nucleic Acid Sample Preparation Kit (on the 100 µL final volume). Elute volume is 200 µL.
4. Perform the qPCR.

Note: To calculate the efficiency of nucleic acid recovery and quantification from the test samples, subtract the amount of nucleic acid measured in the sample without the addition of DNA or RNA control from the amount of nucleic acid measured in the ERC sample.

The sample dilution is 1:40, so the expected copy number following qPCR is 2.5 x 10^5.

Triplicate extractions

Triplicate extractions are required for post-PCR analysis calculation of mean quantity, standard deviation, and coefficient of variation.

In addition to test samples, we recommend triplicate extractions for the negative control and the extraction/recovery control (ERC).

Perform a single PCR reaction for each extraction.

The table below illustrates the total number of extractions required based on the 1, 2, and 3 samples extracted in a batch.

Table 15 Total number of extractions per batch of test samples

Number of test samples		Total number of extractions for the batch
1	3 extractions required for each: • Test sample • Test sample extraction/recovery control (ERC) • Negative extraction control[1]	9
2		15
3		21

[1] Optional during routine testing.

3 Sample extraction with the PrepSEQ™ Nucleic Acid Sample Preparation Kit

We recommend using the PrepSEQ™ Nucleic Acid Sample Preparation Kit (Cat. No. A50485) for sample preparation. The kit uses chemical lysis and magnetic beads to extract nucleic acid from recombinant lentiviruses and integrated proviruses.

Manual protocol for DNA/RNA extraction

Digest the test samples and controls

1. Set a block heater to 56°C. If available, set a second block heater to 70°C.
2. Label the Safe-Lock Tubes, 2.0-mL:
   - 3 for each sample
   - 3 for each sample + ERC
   - 3 for NEG
3. Add 100 µL of sample, sample + ERC, or 1X PBS (free of Mg and Ca) into each tube.
4. Add 10 µL of 5 M NaCl and 70 µL Proteinase K/Proteinase K Buffer II mix.
5. Briefly vortex and centrifuge.
6. Incubate at 56°C for 30 minutes.

   If only one block heater is available, after this incubation step is complete, reset the block heater to 70°C for the elution step.

   Note: For samples with high protein concentration, extending the incubation time to 60 minutes can increase recovery.
7. Cool samples to room temperature.
8. Add 360 µL freshly made Lysis solution mix to each tube.

Bind the DNA/RNA

1. Vortex the Magnetic Particles to resuspend the particles.

   Note: The appearance of the mixture should be homogeneous.

2. Add 30 µL of the Magnetic Particles to each tube.
3. Add 400 µL **Binding Solution** to the first sample, then close the cap and invert twice to mix.
4. Repeat step 3 for each additional sample.

   Note: Low DNA recovery can be observed if the sample and the **Binding Solution** are not immediately mixed.

5. Vortex all the tubes in the vortex adaptor for 5 minutes at 900 rpm.
6. Briefly centrifuge the tubes for 15 seconds at top speed (>15,000 × *g*) to collect the Magnetic Particles at the bottom of the tubes.
7. Place the tubes in the magnetic stand with the pellet against the magnet, then let the tubes stand for 5 minutes or until the solution is clear.
8. Without disturbing the magnetic beads, remove the supernatant using a pipette or by aspiration.

Wash the DNA/RNA

1. Remove the tube rack (with tubes) from the magnetic stand, then add 300 µL of Wash Solution to the tubes. Vortex the tubes for 5 seconds at room temperature at 900 rpm.
2. Centrifuge the tubes in a microcentrifuge at top speed (>15,000 × *g*) for a maximum of 20 seconds. Do not centrifuge for >20 seconds.
3. Place the tubes in the magnetic stand, then let the tubes stand for 1 minute.

   Note: The Magnetic Particles with the bound DNA/RNA are magnetically captured after approximately 1 minute.

4. Without disturbing the Magnetic Particles, remove the supernatant by pipette or by aspiration.
5. Remove the tube rack (with tubes) from the magnetic stand, then add 300 µL of Wash Solution to each tube for a second wash. Vortex the tubes for 5 seconds at room temperature at 900 rpm.
6. Centrifuge the tubes in a microcentrifuge at top speed (>15,000 × *g*) for a maximum of 20 seconds. Do not centrifuge for >20 seconds.
7. Place the tubes in the magnetic stand, then let the tubes stand for 1 minute.

   Note: The Magnetic Particles with the bound DNA/RNA are magnetically captured after approximately 1 minute.

8. Open all tubes, then start the 5-minute timer.

9. Without disturbing the Magnetic Particles, remove the supernatant by pipette or aspiration.
   Use a P200 to remove the remaining solution from the bottom of the tube.

10. With the tube lid open, air-dry the Magnetic Particles pellet in the magnetic stand for no more than 5 minutes at room temperature.

IMPORTANT! Air-dry to remove ethanol from the Wash Solution. Once dry, the DNA/RNA stays bound to the magnetic beads. Do not over-dry; over-dried beads are not easily resuspended.

Elute the DNA/RNA

1. Add Elution Buffer to each tube.
   - ViralSEQ™ Lentivirus Proviral DNA Titer Kit: Add 50 µL of Elution Buffer
   - ViralSEQ™ Lentivirus Physical Titer Kit: Add 200 µL of Elution Buffer

2. Vortex the tubes for 20 seconds at high speed, then incubate the tubes at 70°C for 7 minutes. Vortex the tubes two to three times during the incubation to help resuspend the beads.

3. Centrifuge the tubes in a microcentrifuge at top speed (>15,000 × *g*) for a maximum of 20 seconds. Do not centrifuge for >20 seconds.

4. Place the tubes in the magnetic stand, then let the tubes stand for 1 minute.

5. Without disturbing the Magnetic Particles, transfer the liquid phase containing the eluted DNA/RNA to a new nonstick 1.5-mL microcentrifuge tube.

6. Centrifuge the tube at top speed (>15,000 × *g*) for 3 minutes to collect the Magnetic Particles at the bottom of the tube, then place the tubes in the magnetic stand for 1 minute.

7. Without disturbing the Magnetic Particles, transfer the liquid phase containing the eluted DNA/RNA to a new nonstick 1.5-mL microcentrifuge tube.

Note: Magnetic Particles can inhibit PCR.

Store eluted DNA/RNA for up to 6 hrs on ice, or up to 24 hrs at –20°C.

Automated protocol for DNA/RNA extraction

You can use the KingFisher™ Flex platform to automate the extraction of DNA/RNA. For all chemicals, read the Safety Data Sheet (SDS) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Before each use of the kit

Ensure that you have the correct plates

The KingFisher™ Flex or the MagMAX™ Express require 5 plates.

Plate name	Plate type
Lysis	96 deep-well plate
Wash 1	96 deep-well plate
Wash 2	96 deep-well plate
Elution	96 deep-well plate
Comb loading plate	96 deep-well tip comb combined with 96 standard plate

Prepare the plates

Prepare the Wash 1, Wash 2, and Elution plates:

Plate name	Plate type	Volume of buffer to add
Wash 1	96 deep-well plate	300 µL of Wash buffer
Wash 2	96 deep-well plate	300 µL of Wash buffer
Elution	96 deep-well plate	200 µL of Elution buffer

Prepare the lysis plate

In all steps that require pipetting, dispense liquid at bottom center of the wells.

1. Add 100 µL to the appropriate wells of the 96 deep-well Lysis plate:
   - 3 wells for each sample
   - 3 wells for each sample + ERC
   - 3 wells for NEG
2. Add 10 µL of 5 M NaCl to each sample well.
3. Add 70 µL Proteinase K/Proteinase K (PK) Buffer II mix to each sample well.

Process samples on the instrument

1. Select the script or program for the instrument and kit that you are using:

Kit	Instrument	Script
PrepSEQ™ Nucleic Acid Sample Preparation Kit	KingFisher™ Flex	**PrepSEQ_NucleicAcid_v1**

2. Load the plates into the instrument in the order listed below. After loading each plate, press **START** to move the turntable.
   a. Comb loading plate
   b. Elution plate with 200 µL of Elution Buffer
   c. Wash 2 plate with 300 µL of wash buffer
   d. Wash 1 plate with 300 µL of wash buffer
   e. Lysis plate
3. Press **START** to begin the PK digestion process.

   The instrument mixes the samples for 10 seconds at fast speed, then incubates the samples at 56°C for 30 minutes, mixing at slow speed. When digestion is complete, the instrument pauses and returns the Lysis plate to the loading position.
4. After the digestion step is complete, add additional components to the Lysis plate:
   a. Remove the Lysis plate from the instrument.
   b. Add 360 µL of Lysis Solution to each sample well.
   c. Add 30 µL of Magnetic Particle suspension to each sample well.
   d. Add 400 µL of Binding Solution to the first sample well, then immediately pipet up-and-down three times to mix. Repeat for each additional sample well.

      Note: Low DNA recovery can be observed if the sample and the **Binding Solution** are not immediately mixed.

   e. Place the plate back into the instrument loading position, then press **START** to begin binding.
5. When DNA/RNA extraction is finished, the instrument returns the Elution plate to the loading position.

Store eluted DNA/RNA for up to 6 hrs on ice, or up to 24 hrs at –20°C.

4 Set up, run, and review ViralSEQ™ Lentivirus Physical Titer Kit experiments

This section describes the general procedures to prepare PCR reactions, set up a template in the AccuSEQ™ Real-Time PCR Software v3.1 or later, run the samples on the QuantStudio™ 5 System, then review the results. For more information, see the *AccuSEQ™ Real-Time PCR Software v3.2 User Guide* (Pub. No. MAN0029199).

Note: Check all settings, including qPCR method and optical filter selection, for correctness and alignment with your laboratory's standard operating procedures.

Treat samples with DNase I, RNase-free (1 U/µL)

DNase I, RNase-free (1 U/µL) treatment is used to digest double-stranded DNA.

Thaw all reagents on ice. Invert the DNase I, RNase-free (1 U/µL) several times to mix, then centrifuge briefly. All other reagents should be vortexed, then centrifuged briefly before use.

1. Set up the DNase I, RNase-free (1 U/µL) reactions in a MicroAmp™ Optical 96-Well Reaction Plate (0.2 mL).

Component	Volume for one reaction
Nuclease-free water	4.4 µL
10x DNase I Buffer	1.8 µL
Extracted RNA sample[1]	10 µL
DNase I, RNase-free (1 U/µL)	1.8 µL
Total	**18 µL**

[1] Mix gently by pipetting 3-5 times when adding.

2. Mix the reactions by gently pipetting up and down 5 times, then seal the reaction plate with MicroAmp™ Clear Adhesive Film.

3. Centrifuge the plate at 1,000 x g for 2 minutes.

4. Load the reactions onto the VeritiPro™ 96-well Thermal Cycler, then start the DNase I treatment.

   Set cover temperature: 105°C

   Set reaction volume: 18 µL

Step	Temperature	Time
DNase I treatment	37°C	10 minutes
Hold	4°C	3-5 minutes[1]

[1] Do not hold for more than 5 minutes. Proceed immediately to DNase I inactivation.

5. Centrifuge the plate at 1,000 x g for 2 minutes.

CAUTION! The plate is in contact with the heated lid. Remove carefully.

6. Gently remove the MicroAmp™ Clear Adhesive Film, then discard.

IMPORTANT! Do not touch wells when removing the MicroAmp™ Clear Adhesive Film. Contamination can lead to inaccurate results.

7. Add 2 µL of 50mM EDTA to each reaction well. Mix by gently pipetting 5 times with a P10/P20 pipettor set to 10 µL.

8. Seal the reaction plate with MicroAmp™ Clear Adhesive Film, then centrifuge the plate at 1,000 x g for 2 minutes.

9. Load the reactions onto the VeritiPro™ 96-well Thermal Cycler, then start the DNase I inactivation.

   Set cover temperature: 105°C

   Set reaction volume: 20 µL

Step	Temperature	Time
DNase I inactivation	75°C	10 minutes
Hold	4°C	3-5 minutes[1]

[1] Do not hold for more than 5 minutes.

10. Centrifuge the plate at 1,000 x g for 2 minutes.

CAUTION! The plate is in contact with the heated lid. Remove carefully.

IMPORTANT! Do not vortex.

Place the plate on ice until use.

Prepare the kit reagents and premix solution

Thaw all kit reagents on ice. Vortex the reagents for 5 seconds, briefly centrifuge, then place the reagents on ice until use.

1. Label a microcentrifuge tube for the Premix Solution.

2. Prepare the Premix Solution according to the following tables.

   IMPORTANT! Use a separate pipette tip for each component.

Table 16 Premix Solution

Component	Volume for one 25-µL reaction	Volume for four 25-µL reactions[1]
2× RT-PCR Buffer	12.5 µL	55.0 µL
25× RT-PCR Enzyme Mix	1.0 µL	4.4 µL
Physical Titer Assay Mix	2.5 µL	11 µL
Nuclease Free Water	4.0 µL	17.6 µL
Total Premix Solution Volume	**20.0 µL**	**88.0 µL**

[1] Includes 10% excess to compensate for pipetting loss.

3. Vortex the Premix Solution for 10 seconds to mix, then briefly centrifuge.
   Store the Premix Solution at 4°C or on ice until use.

Prepare the PCR reactions

Plate setup differs slightly for each AccuSEQ™ System. See your software user guide for specific instructions. Place samples, NTCs, and standards in different quadrants of the plate.

A factory default template is available for the **ViralSEQ Lentivirus Physical Titer Kit** in AccuSEQ™ Real-Time PCR Software v3.2 or later.

Place the plate containing DNase I-treated samples on a MicroAmp™ 96-Well Base, then gently remove the MicroAmp™ Clear Adhesive Film. Gently pipette up and down 3 times to mix the samples.

1. Dispense the following into the appropriate wells of a MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL, gently pipetting at the bottom of the well.

To prepare…	Combine in each tube or well…
No template control (NTC) reaction	• 20 µL of Premix Solution • 5 µL of RNA Dilution Buffer
Unknown sample reaction	• 20 µL of Premix Solution • 5 µL of DNase I-treated RNA sample
Standard curve reaction	• 20 µL of Premix Solution • 5 µL of standards diluted from the RNA Control (see “Prepare the serial dilutions” on page 20)

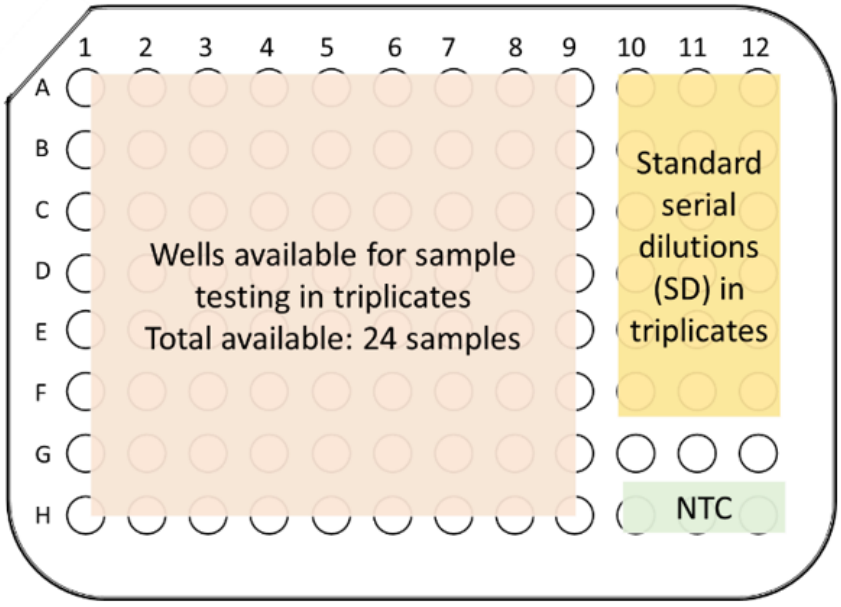


Figure 1 Recommended plate layout

2. Seal the plate with MicroAmp™ Optical Adhesive Film.
3. Vortex the reaction plate for 10 seconds, then centrifuge at 1,000 x g for 2 minutes.

Note: Ensure there are no bubbles in the reaction wells. If present, tap the well gently to remove bubbles, then recentrifuge.

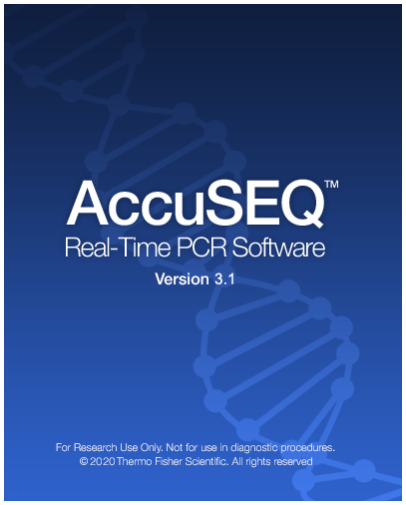
Set up, run, and analyze samples with AccuSEQ™ Software v3.1 or later on the QuantStudio™ 5 Instrument

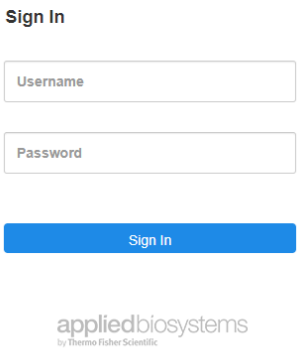
Sign in to the AccuSEQ™ Real-Time PCR Software

Thermo Fisher Scientific recommends configuring the Windows™ 10 screen save feature to require sign in when the screensaver is activated. This prevents other users from accessing the AccuSEQ™ Real-Time PCR Software and making changes.

1. Launch the AccuSEQ™ Real-Time PCR Software by double-clicking the AccuSEQ icon .
2. Enter the **Username**, then **Password**.

   (First login only) The default username is **Administrator** and the default password is **Administrator**.
3. Click **Sign in**.





The following restrictions may be seen in the software:

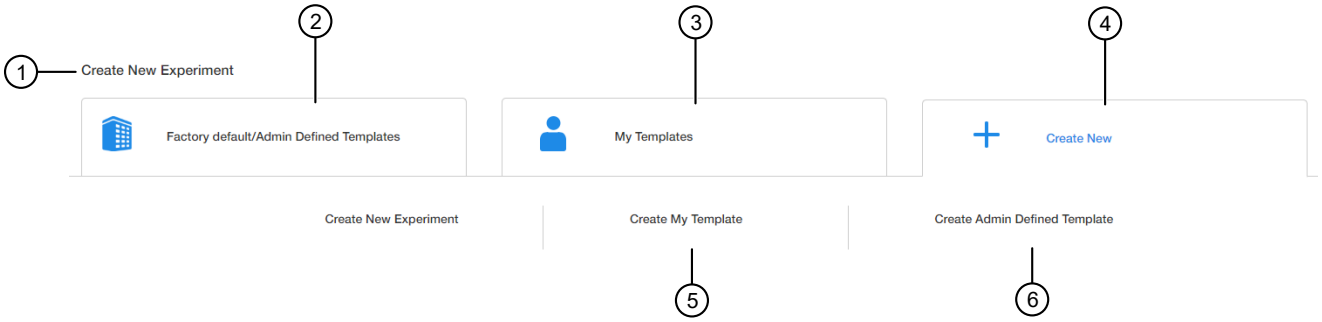
- Access to functions in the software is based on the permissions associated with the individual user account.
- If a user account does not have permission to perform a function, the function is grayed out in the software.
- If the system is configured for password expiration, you will be periodically prompted to change your password. If the 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.

Create a ViralSEQ™ template in AccuSEQ™ Real-Time PCR Software v3.1

Create a new template in the **(Home)** screen of the AccuSEQ™ Real-Time PCR Software v3.1.

Note: AccuSEQ™ Real-Time PCR Software v3.2 or later has a **Factory default/Admin Defined Template** for the ViralSEQ™ Lentivirus Physical Titer Kit. See “Create a ViralSEQ™ Lentivirus Physical Titer Kit experiment in AccuSEQ™ Real-Time PCR Software v3.2 or later” on page 42.

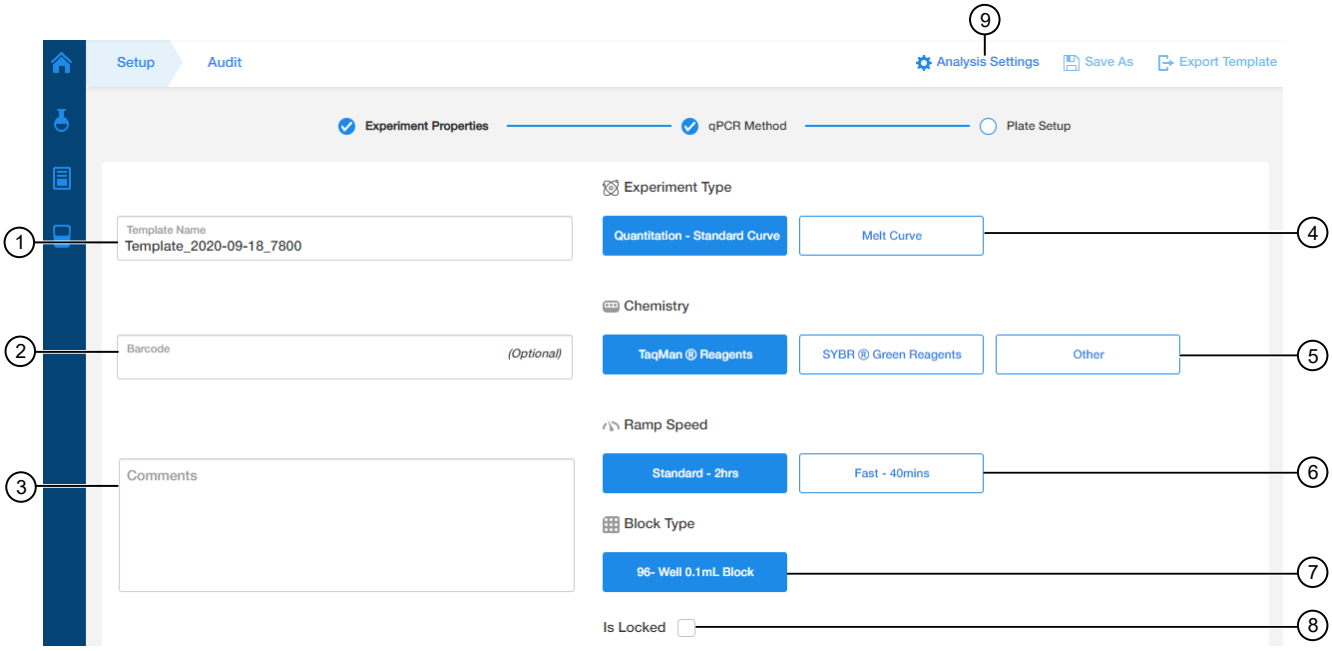
1. Click + **Create New** on the home screen.



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).

2. Select **Create My Template** or **Create Admin Defined Template**.

3. Edit the **Experiment Properties** as required.



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**

a. In the **Template Name** field, modify the template name. For example, LV Titer template.

b. (*Optional*) Enter information in the **Comments** field.

c. In the **Setup** tab, select:
   - **Experiment Type—Quantitation-Standard Curve**
   - **Chemistry—TaqMan ® Reagents**
   - **Ramp Speed—Standard-2hrs**
   - **Block Type—96-Well 0.1mL Block**

d. (*Optional*) Select **Is Locked** to lock the template. If locked, users are unable to edit the template.

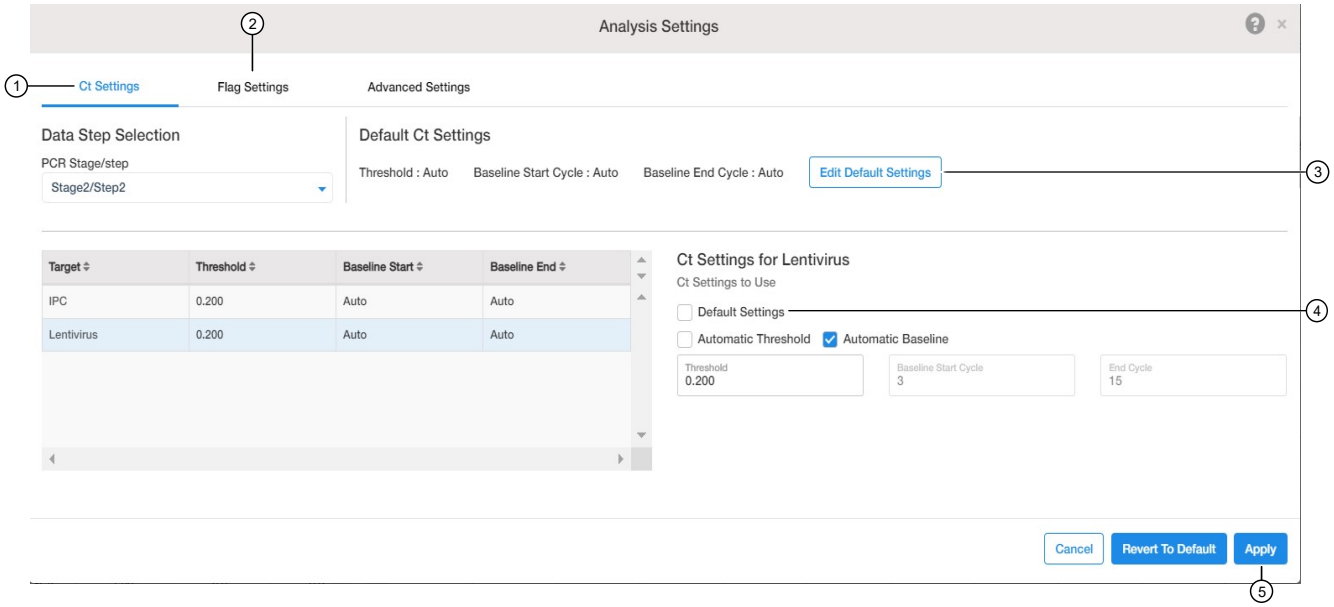
4. Click **Analysis settings** to change the default C_t Settings and Flag Settings.
   a. In the **C_t Settings** tab, click **Edit Default Settings**.

   b. Deselect **Automatic Threshold**, then enter **0.200**.

   c. Ensure that **Automatic Baseline** is selected.

   d. Click **Save Changes**.

e. Deselect **Default Settings**, then click **Apply** to save any changes before closing the window.



① **C_t Settings**
② **Flag Settings**
③ **Edit Default Settings** button
④ **Default Settings** checkbox
⑤ **Apply** button

f. In the **Flag Settings** tab, deselect the following flags.

- **CQCONF**—Low Cq confidence
- **EXPFAIL**—Exponential algorithm failed
- **NOAMP**—No amplification
- **NOSIGNAL**—No signal in well

Note: Use the scrollbar on the right to scroll down the list of flags.

g. Click **Apply** to save any changes before closing the window.

5. Click **Next**.

Template name cannot be changed after this step.

The qPCR Method screen is displayed.

Edit the run method and optical filter selection

This section provides general procedures to edit the run method and optical filter selection in the qPCR Method. To edit the default run method, see the *AccuSEQ™ Real-Time PCR Software v3.1 User Guide* (Pub. No. 100094287).

1. Set the reaction volume to **25 µL**.
2. Edit **Step 1** of the **Hold Stage** to 45℃ for 30 minutes.
3. Set **Step 2** of the **Hold Stage** to 95℃ for 10 minutes.
4. Set **Step 1** of the **PCR Stage** to 95℃ for 15 seconds.
5. Edit **Step 2** of the **PCR Stage** to 60℃ for 45 seconds.
6. Set the cycle number to **40**.
7. Ensure that **Data Collection** occurs after **Step 2**.

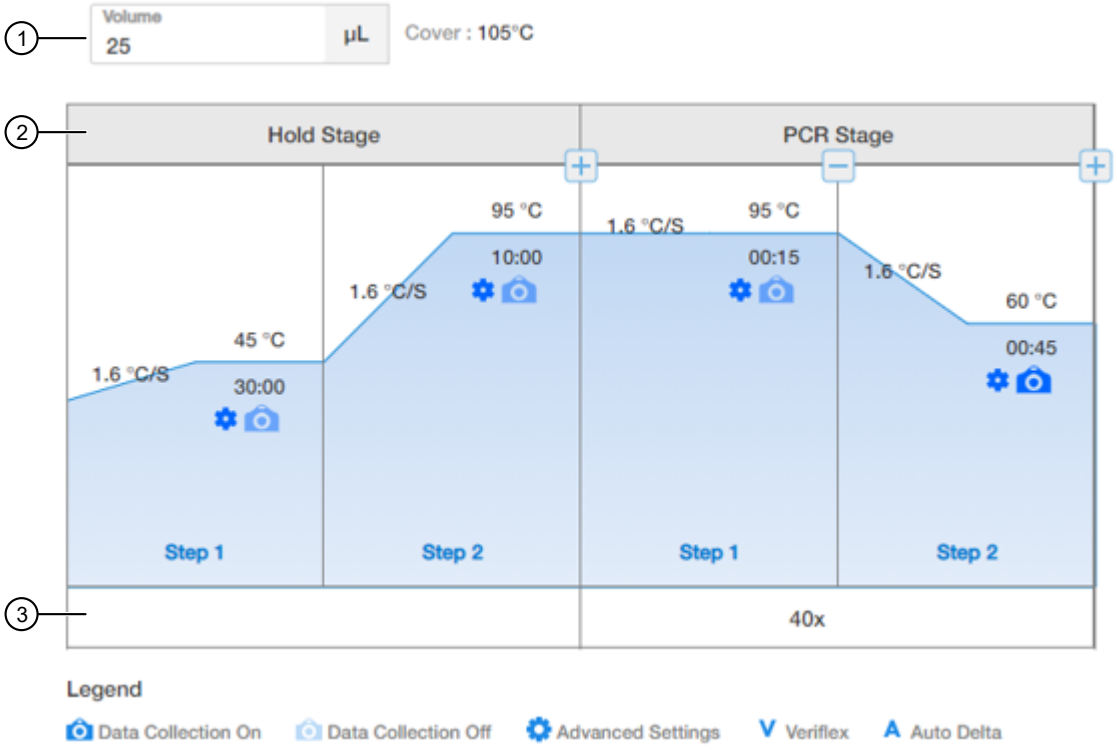


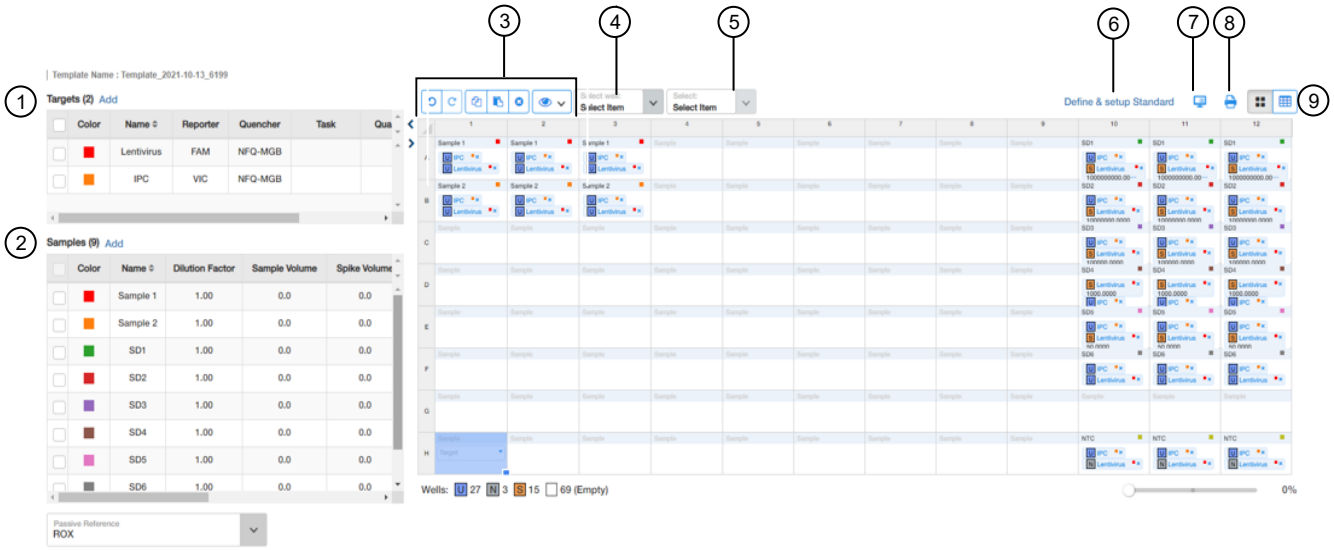
Figure 2 Lentivirus Physical Titer Run Method

① Reaction volume- set to 25µL
② Stage
③ Cycle number- set to 40 cycles

8. (*Optional*) Click **(Optical Filter Settings)** to view the default filter settings.
   - The default optical filter selection is suitable for the ViralSEQ™ Lentivirus Physical Titer Kit.
   - The ViralSEQ™ Lentivirus Physical Titer Kit requires the QuantStudio™ 5 System to be calibrated for FAM™, VIC™, and ROX™.
   - 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).
9. Click **Next**.

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. Select Item to highlight (Sample, Target, or Task).
5. Select Item. For example, Sample 1. Sample 1 replicates are highlighted.
6. Define & setup Standard
7. (View Legend)
8. (Print Preview)
9. 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. Each well should have a Sample Name under **Samples**, as well as the appropriate Targets under **Targets**. Reporters should be **FAM™** dye for Lentivirus Physical Titer, and **VIC™** dye for internal positive control (IPC).

   a. 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.

   b. For each sample (e.g. DNase-treated lentivirus sample, standard curve dilution, or NTC), two targets should be included.
      - Select the **FAM™** dye reporter for Lentivirus Physical Titer detection.
      - Select the **VIC™** dye for IPC detection.

   c. Select **NFQ-MGB** as the quencher for both targets.

   d. For standard curve dilution samples (SD1 to SD5), the **Task** for Lentivirus Physical Titer target should be indicated as "**S**" for Standard, with the appropriate copy number written under **Quantity**. For instance, the quantity for SD1 is 1E9 copies. Change the **Task** by clicking on the field and using the drop-down menu. Copy numbers can be indicated using scientific notation (e.g. "1E9") and the program will convert it to numerical format.

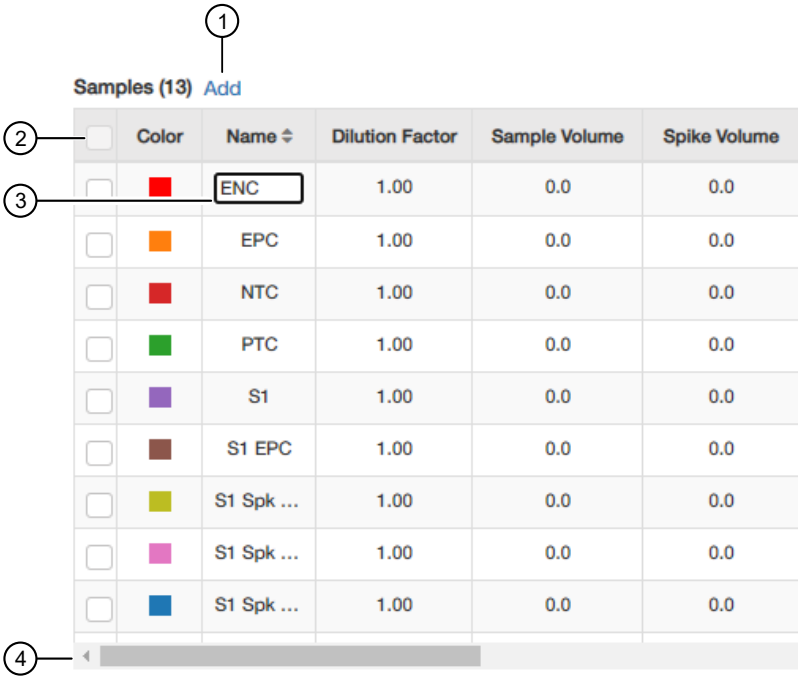
   e. For DNase-treated samples and SD6, set the **Task** for Lentivirus Physical Titer target to **U** for Unknown.

   f. For NTC wells, set the **Task** for Lentivirus Physical Titer target to **N** for NTC.

   g. For IPC wells, set the **Task** for Lentivirus Physical Titer target to **U** for Unknown.

h. 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.

When a **Sample** or **Target** name are edited, two entries are added to the Audit trail (one for Delete, and another for Create).



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.

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

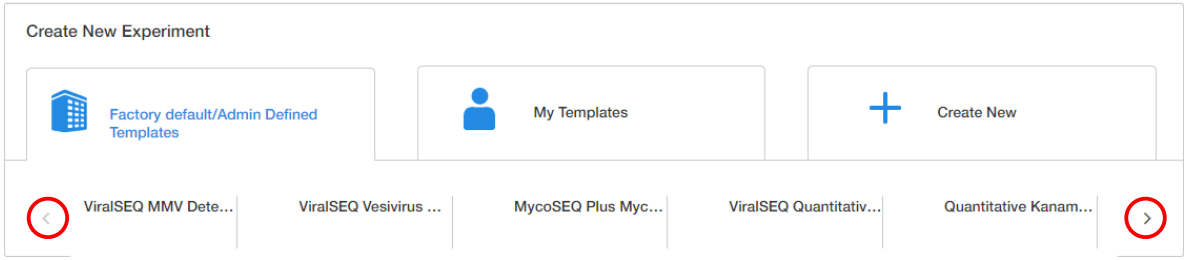
4. Select ROX™ dye from the **Passive Reference** drop-down list (bottom left of screen).

5. Click **Save** to save the template.
This template can then be used to create experiments.

Create a ViralSEQ™ Lentivirus Physical Titer Kit experiment in AccuSEQ™ Real-Time PCR Software v3.2 or later

1. In the **Home** screen, click the **Factory default/Admin Defined Template** tab, then select a **ViralSEQ Lentivirus Physical Titer Kit** template.

To navigate to through the available templates click the arrows to either side of the available templates.



To create an experiment from an existing ViralSEQ™ Lentivirus Physical Titer Kit experiment, see *AccuSEQ™ Real-Time PCR Software v3.2 User Guide* (Pub. No. MAN0029199).

2. In the **Experiment Properties** pane of the **Setup** screen:
   a. (*Optional*) Change the system-generated name of the experiment.

   Note: Names must be unique. Deleted experiment names can not be reused.

   b. (*Optional*) Enter the plate barcode in the **Barcode** field, then add comments in the **Comments** field.

   Note: Comments are not editable post analysis.

   Default ViralSEQ™ Lentivirus Physical Titer Kit settings (cannot be changed)
   - **Experiment Type—Quantitation-Standard Curve**
   - **Chemistry—TaqMan™ Reagents**
   - **Ramp Speed—Standard - 2hrs**

   c. Click **Next**.

   Note: Experiment names cannot be changed after this step.

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

Note: This assay is run with a **Standard** ramp speed (2hrs).

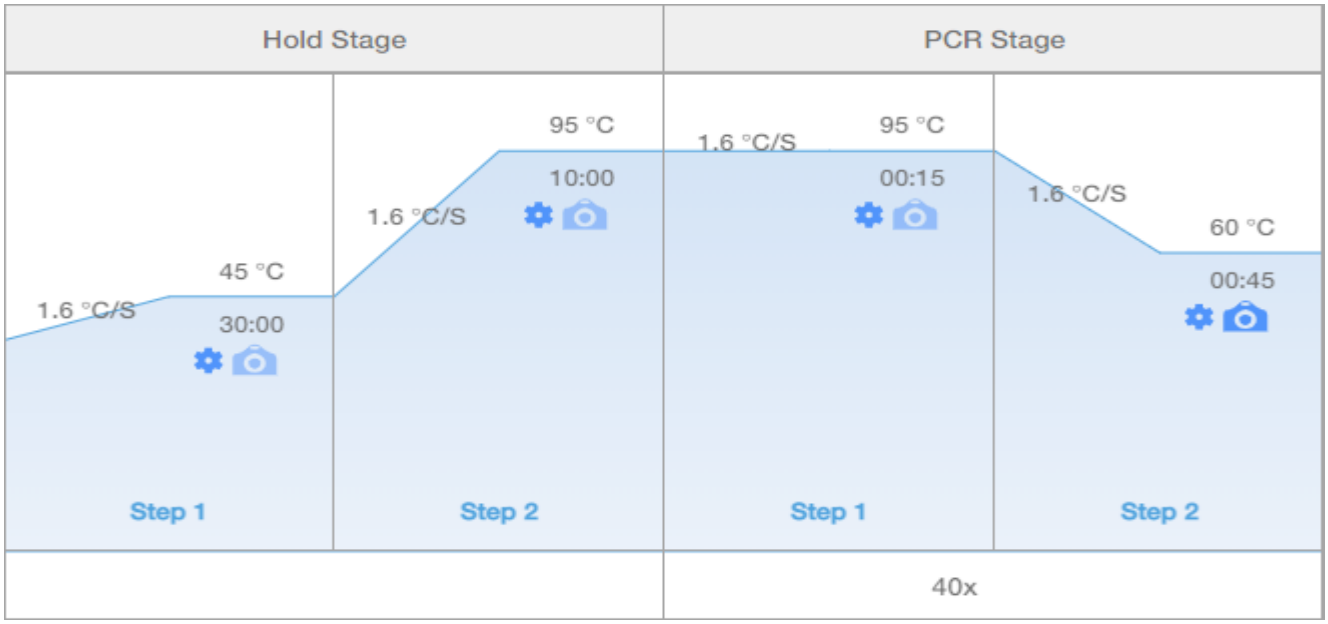


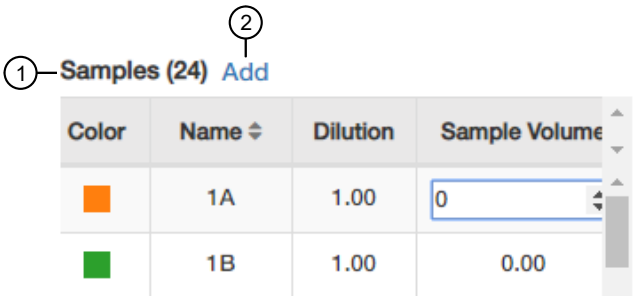
Figure 3 ViralSEQ™ Lentivirus Physical Titer Kit template default cycling conditions

4. Click **Next**.

5. In the **Samples** table in the **Plate Setup** pane, enter the sample name and sample volume. Click **Add** to add more samples if needed.

Note: The software calculates viral particles per mL if the sample volume was entered.

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



① **Samples** table
② **Add**—adds more samples

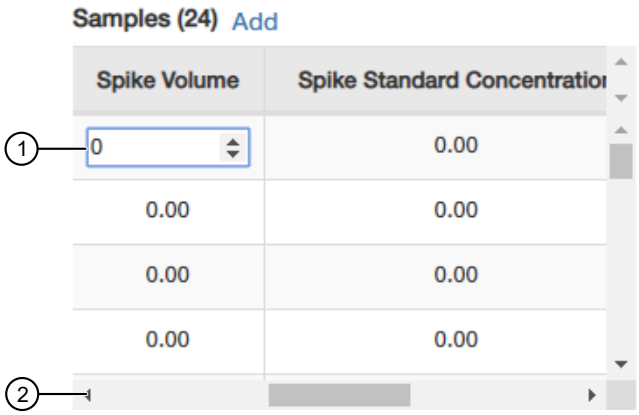
For more information about plate setup, see "Assign plate and well attributes" on page 40.

- **Sample Volume**—add sample volume to calculate viral particles per mL in the results.
- **Spike Volume**—volume of DNA added to the PCR (set to 10).
- **Spike Standard Concentration**—expected spike amount per reaction (for example, 10 copies).
- **Reference**—the non-spiked sample; the mean quantity of reference is subtracted during % recovery calculation.

- **Spike Input**—calculated (double check if correct).

Note: If not correct, ensure **Spike Volume** is set to 10 and **Spike Standard Concentration** is the expected copies spike per PCR reaction.

- **Protein Concentration**—Drug substance protein concentration (if total DNA in copies RNA/mg protein is needed).
- (Optional) **Comments**



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

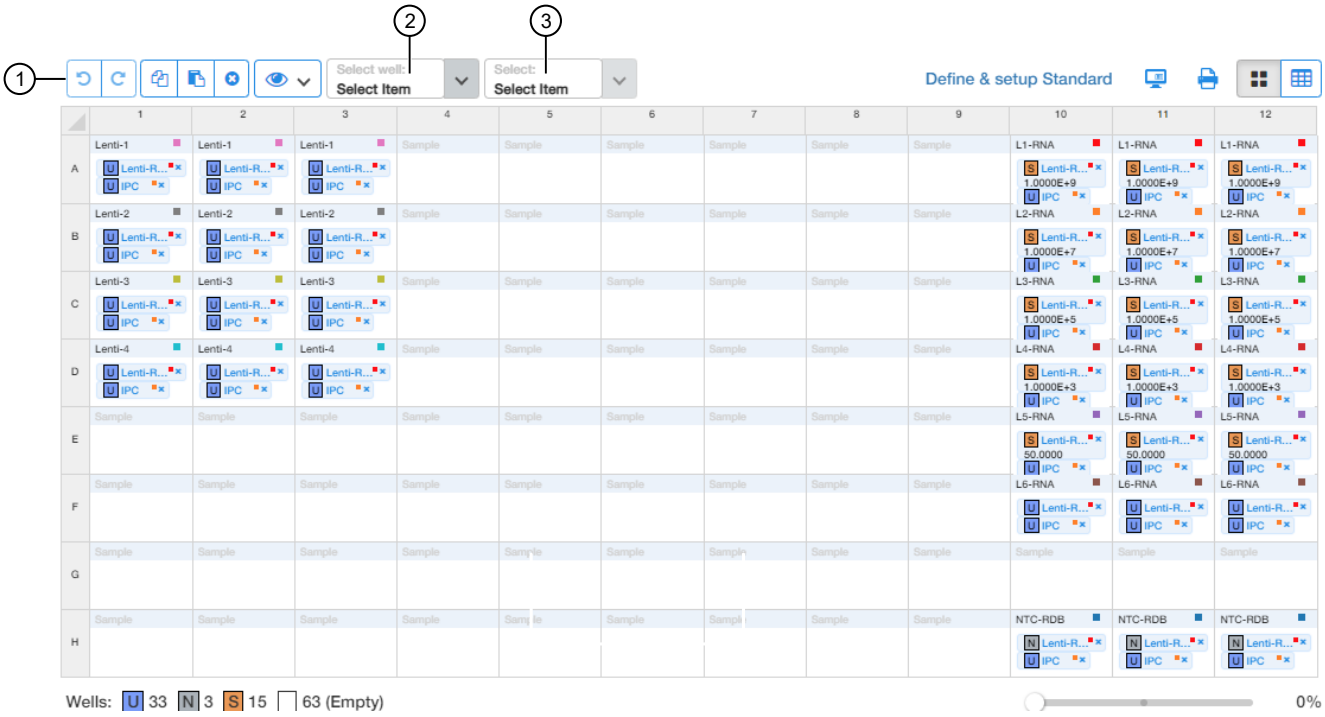


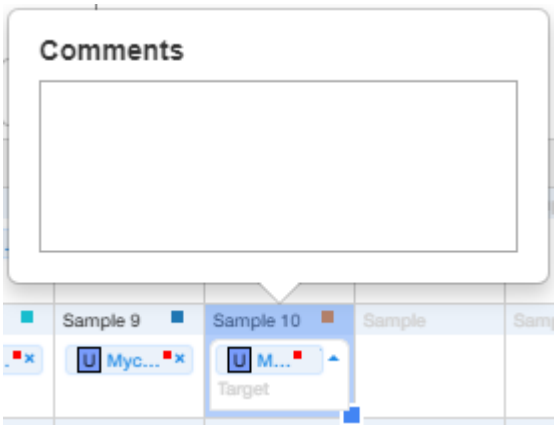
Figure 4 ViralSEQ™ Lentivirus Physical Titer Kit template default sample plate layout

① Toolbar (in order: Undo, Redo, Copy, Paste, Delete, View)
② Select type (Sample, Target, or Task) to highlight.
③ Select specific items of that type. For example, Sample 1. Sample 1 replicates are highlighted.

Table 17 Plate well descriptions

Name	Description
Lenti-1 to Lenti-4	4 samples in triplicate
L1-RNA to L6-RNA	Standard curve dilutions in triplicate
NTC-RDB	No template control in triplicate

6. (*Optional*) Double-click wells to add comments. Comments can also be added post-analysis.



7. Click **Next**.
   The **Run** screen 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** creates a copy of the experiment.

9. (*Optional*) Click **Print** to print the plate layout for use in preparing the reactions.

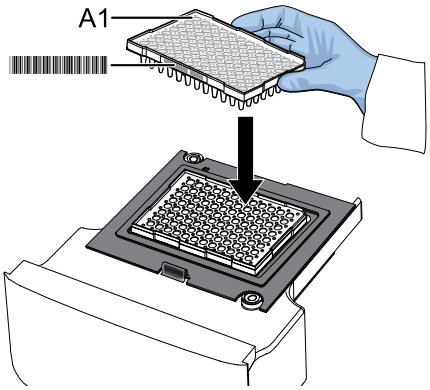
10. Assemble the PCR reactions following the instructions of the manufacturer for the reagents and following the plate layout set up in the template.

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.

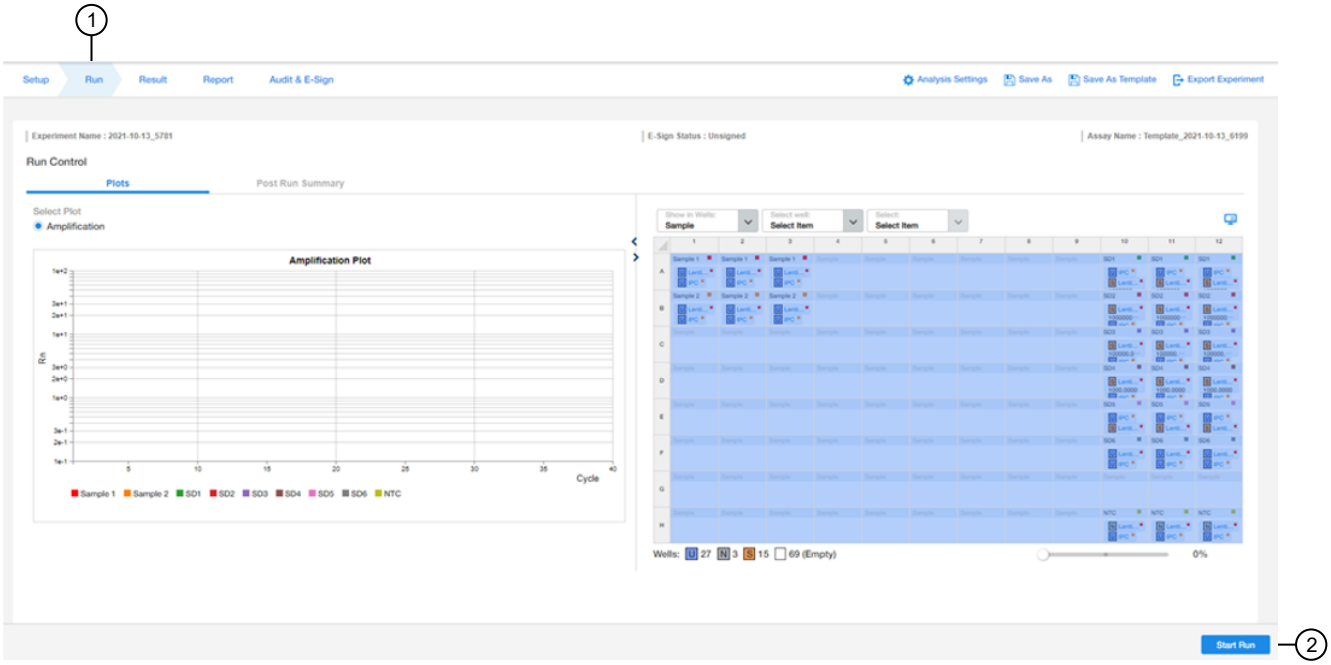
3. Touch ⏏ to close the instrument drawer.

Start the run (QuantStudio™ 5 Real-Time PCR Instrument)

Ensure that the plate is loaded in the QuantStudio™ 5 Real-Time PCR Instrument.

Start the run in the AccuSEQ™ Software v3.1 or later.

Option	Description
If the experiment is open	Click **Start Run**.
If the experiment is closed	a. Open the experiment. b. Click the **Run** tab. c. 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:

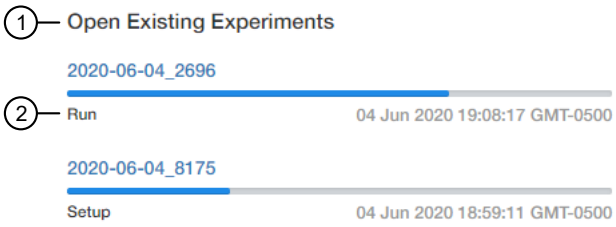
- On the instrument touchscreen.
- In the **Monitor the Run** pane of the AccuSEQ™ Software **(Home)** screen.

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: You cannot start another run while the instrument status is **Running**.

When the run is complete, the status changes to **Analysis** and the status bar displays as complete.



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.

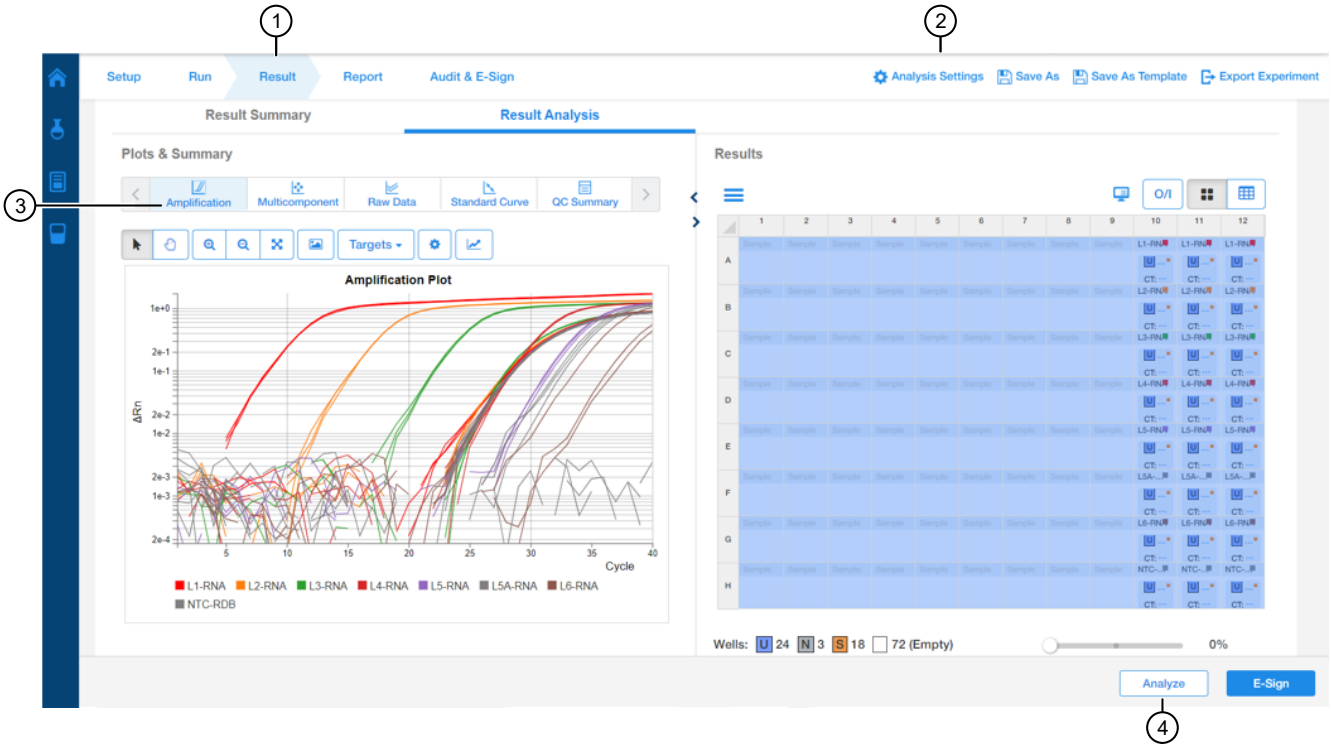
On run completion, the **Post Run Summary** displays the run length, user and instrument information, and a list of any errors that occurred.

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

Review 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 v3.2 User Guide* (Pub. No. MAN0029199).

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



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

2. In the **Result Analysis** tab, select individual targets, then review the Amplification Curve plots for amplification profiles in the controls, samples, and the standard curve. Ensure that threshold is set to 0.200 with an automatic baseline.

Note: If any settings are changed, click the **Analyze** button to apply the new settings to the results. Target names and Sample names (under the **Setup** tab) can also be changed after the run is completed.

---

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, R^2, and Efficiency are within acceptable limits.

① Slope, R^2, and Efficiency values

Note: The **Standard Curve** efficiency should be between 90-110% and the R^2>0.99. If these criteria are not met, up to two points, not in the same triplicate, can be removed from the standard curve data, and the analysis repeated.

5. In Table View, ensure that C_t values are within the standard curve range.
   - Samples with C_t values that exceed the upper limit of quantitation (10^9 copies) of the standard curve should be diluted and run again.
   - Samples with C_t values that exceed the lower limit of detection (LoD of 10 copies) and IPC shows no signs of PCR inhibition, suggests the absence of lentivirus.

6. (*Optional*) Outliers can be excluded from the results. To exclude, select the well, then click **Omit/Include**, then reanalyze by clicking **Analyze**.

7. (*Optional*) Select **File ▸ Print Report** to generate a hard copy of the experiment, or click **Print Preview** to view and save the report as a PDF or HTML file.

8. Export the results.
    a. Navigate to the **Report** screen.
    b. Check all boxes under **Contents**.
    c. Select **Export Data in One File**.
    d. Select the **XLS** format, then click **Export**.

Calculate the titer (VP/mL)

Note: After analysis, AccuSEQ™ Software v3.2 or later provides the titer in viral particles per mL in the table view of the **Results** screen. Use of the **Physical Titer Calculation Tool** is not needed.

1. Download the **Physical Titer Calculation Tool**.
    a. Go to **thermofisher.com**.
    b. Search for the ViralSEQ™ Lentivirus Physical Titer Kit.
    c. Download the tool from the **Documents** section.
2. Open the tool, then follow the instructions in the tool to calculate the titer.

Analyze the data without AccuSEQ™ Real-Time PCR Software

1. Set the threshold to 0.2 and use automatic baseline.
2. Export the C_t values to a Microsoft™ Excel™ spreadsheet.
3. For the standard curve, plot $\log_{10}$ of the standard quantity versus the mean C_t.
4. Determine the equation of the standard curves by adding a trendline and displaying the equation.
5. Determine the quantity of the unknown samples using the C_t values and the equation generated from the trendline.

Calculate lentivirus titers from qPCR data

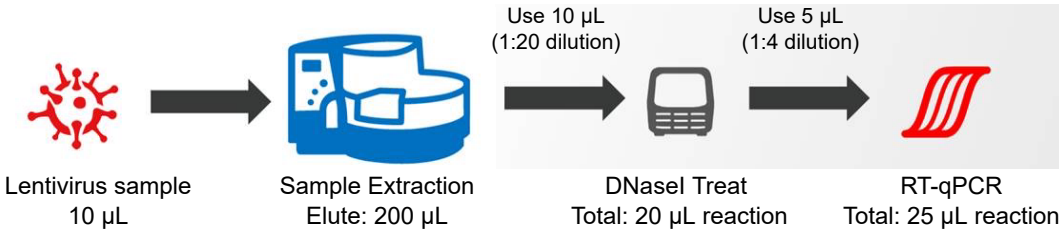
To determine the number of lentivirus RNA copies per mL in the original sample, the copy numbers obtained from the qPCR must be multiplied by the dilution factor of the sample during extraction and DNase I treatment. Since there are 2 copies of RNA/target per lentivirus particle, the number of viral particles per mL (VP/mL) is 0.5x the number of lentivirus RNA copies.

$$\text{Viral particles per mL} = \frac{\text{qPCR copies x sample dilution factor x 0.5}}{\text{Volume of sample used (mL)}}$$

For help in determining the qPCR copy numbers, see the *QuantStudio™ Design and Analysis Desktop Software User Guide* (Pub. No. MAN0010408).

For example, if the following parameters were used,

- 10 µL of lentivirus culture was extracted with the KingFisher™ Flex Purification System with 96 Deep-Well Head and eluted in 200 µL
- 10 µL of this eluate (20x dilution) was treated with DNase I, RNase-free (1 U/µL) in a total volume of 20 µL.
- 5 µL of the DNase-treated sample (4x dilution) was used for the qPCR reaction.



then, the calculation would be:

$$\text{Viral particles per mL} = \frac{\text{qPCR copies x (20x4) x 0.5}}{\text{0.01 (mL)}}$$

Note: qPCR can only determine the number of physical particles in a virus culture. To determine the numbers of infectious units, cell-based transduction experiments must be carried out. The titers of physical particles are often higher than infectious titers by 10-1000 fold, depending on the purity of the lentivirus preparation and the levels of infectious particles within the culture.

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5 Set up, run, and review ViralSEQ™ Lentivirus Proviral DNA Titer Kit experiments

This section describes the general procedures to prepare PCR reactions, set up a template in the AccuSEQ™ Real-Time PCR Software v3.1 or later, run the samples on the QuantStudio™ 5 System, then review the results. For more information, see the *AccuSEQ™ Real-Time PCR Software v3.2 User Guide* (Pub. No. MAN0029199).

Note: Check all settings, including qPCR method and optical filter selection, for correctness and alignment with your laboratory's standard operating procedures.

Prepare the kit reagents and premix solution

Thaw all kit reagents on ice. Vortex the reagents for 5 seconds, briefly centrifuge, then place the reagents on ice until use.

1. Label a microcentrifuge tube for the Premix Solution.
2. Prepare the Premix Solution according to the following tables.

   IMPORTANT! Use a separate pipette tip for each component.

   Table 18 Premix Solution

Component	Volume for one 30-µL reaction	Volume for four 30-µL reactions[1]
2× Environmental Master Mix	15.0 µL	66.0 µL
Proviral DNA Titer Assay Mix	6.0 µL	26.4 µL
Nuclease Free Water	4.0 µL	17.6 µL
Total Premix Solution Volume	**25.0 µL**	**110.0 µL**

[1] Includes 10% excess to compensate for pipetting loss.

3. Vortex the Premix Solution for 10 seconds to mix, then briefly centrifuge.

Prepare the PCR reactions

Plate setup differs slightly for each AccuSEQ™ System. See your software user guide for specific instructions. Place samples, NTCs, and standards in different quadrants of the plate.

A factory default template is available for the **ViralSEQ Lentivirus Proviral DNA Titer Kit** in AccuSEQ™ Real-Time PCR Software v3.2 or later.

1. Dispense the following into the appropriate wells of a MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL, gently pipetting at the bottom of the well.

To prepare…	Combine in each tube or well…
No template control (NTC) reaction	• 25 µL of Premix Solution • 5 µL of DNA Dilution Buffer
Unknown sample reaction	• 25 µL of Premix Solution • 5 µL of sample
Standard curve reaction	• 25 µL of Premix Solution • 5 µL of standards diluted from the DNA Control (see "Prepare the serial dilutions" on page 23)

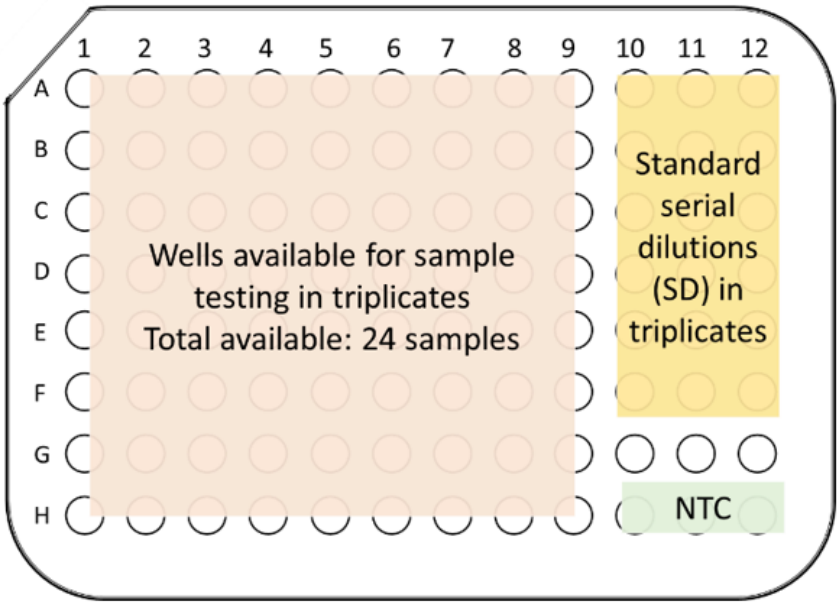


Figure 5 Recommended plate layout

2. Seal the plate with MicroAmp™ Optical Adhesive Film.

3. Vortex the reaction plate for 10 seconds, then centrifuge at 1,000 × *g* for 2 minutes.

Note: Ensure there are no bubbles in the reaction wells. If present, tap the well gently to remove bubbles, then recentrifuge.

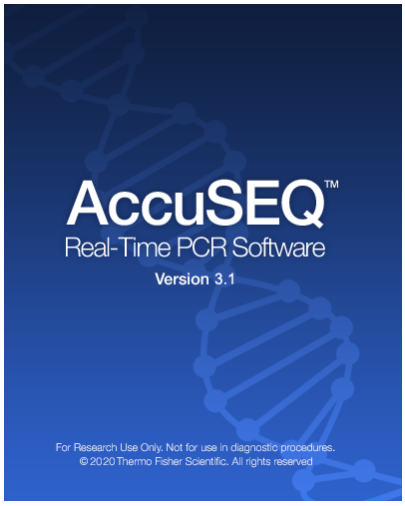
Set up, run, and analyze samples with AccuSEQ™ Software v3.1 or later on the QuantStudio™ 5 Instrument

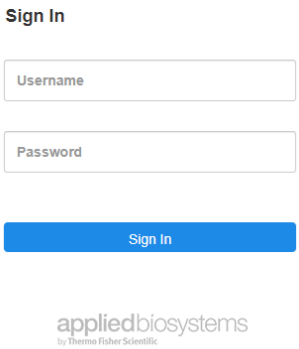
Sign in to the AccuSEQ™ Real-Time PCR Software

Thermo Fisher Scientific recommends configuring the Windows™ 10 screen save feature to require sign in when the screensaver is activated. This prevents other users from accessing the AccuSEQ™ Real-Time PCR Software and making changes.

1. Launch the AccuSEQ™ Real-Time PCR Software by double-clicking the AccuSEQ icon .
2. Enter the **Username**, then **Password**.

   (First login only) The default username is **Administrator** and the default password is **Administrator**.
3. Click **Sign in**.





The following restrictions may be seen in the software:

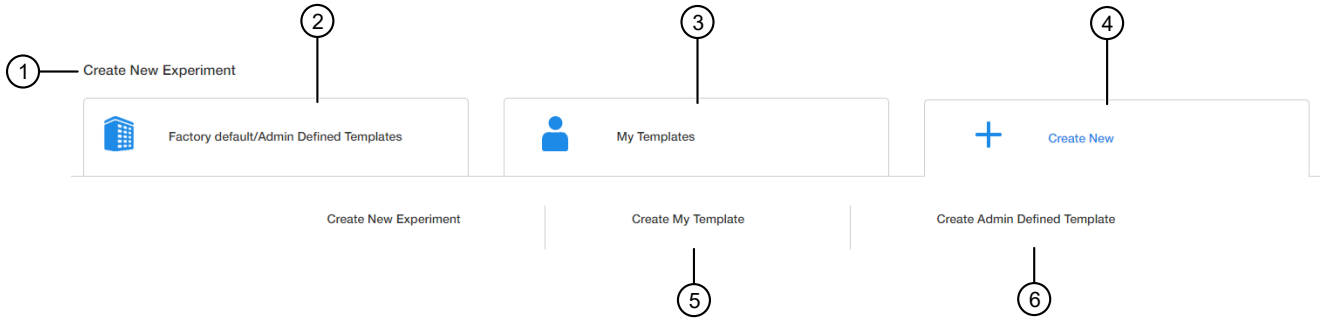
- Access to functions in the software is based on the permissions associated with the individual user account.
- If a user account does not have permission to perform a function, the function is grayed out in the software.
- If the system is configured for password expiration, you will be periodically prompted to change your password. If the 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.

Create a ViralSEQ™ template in AccuSEQ™ Real-Time PCR Software v3.1

Create a new template in the **(Home)** screen of the AccuSEQ™ Real-Time PCR Software v3.1.

Note: AccuSEQ™ Real-Time PCR Software v3.2 or later has a **Factory default/Admin Defined Template** for the ViralSEQ™ Lentivirus Proviral DNA Titer Kit. See "Create a ViralSEQ™ Lentivirus Proviral DNA Titer Kit experiment in AccuSEQ™ Real-Time PCR Software v3.2 or later" on page 64.

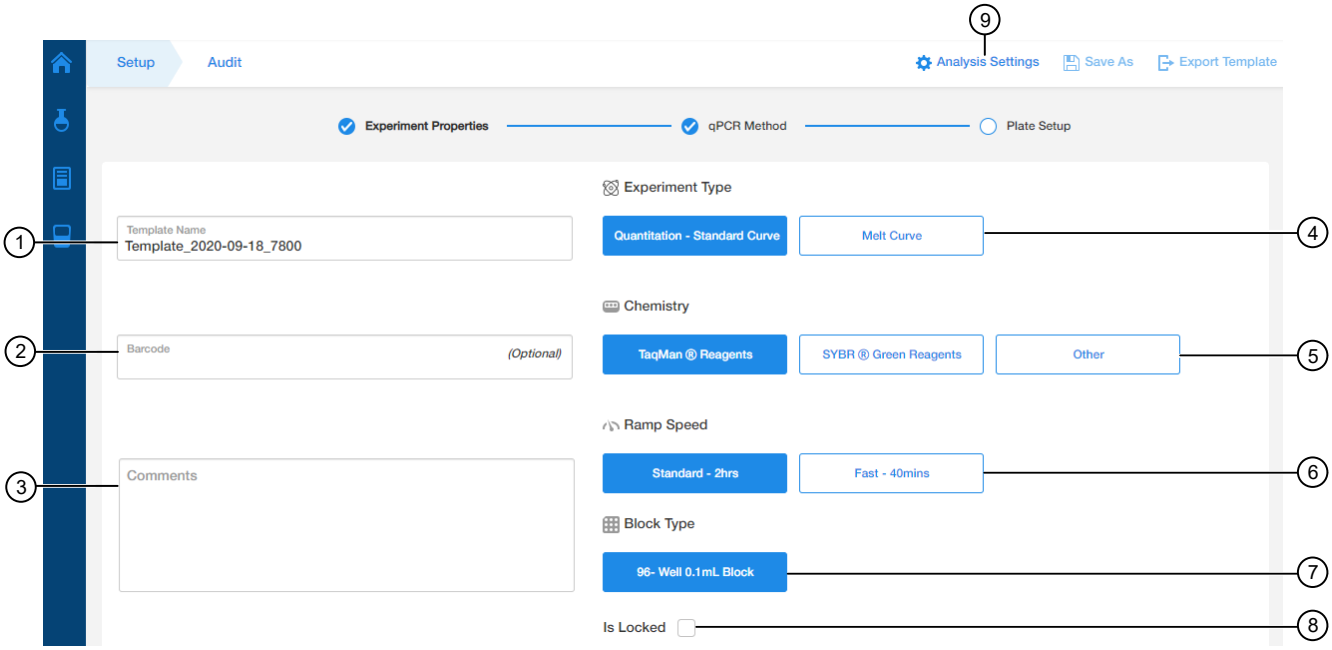
1. Click **Create New** on the home screen.



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).

2. Select **Create My Template** or **Create Admin Defined Template**.

3. Edit the **Experiment Properties** as required.



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**

a. In the **Template Name** field, modify the template name. For example, LV Titer template.

b. (*Optional*) Enter information in the **Comments** field.

c. In the **Setup** tab, select:
   - **Experiment Type—Quantitation-Standard Curve**
   - **Chemistry—TaqMan Reagents**
   - **Ramp Speed—Standard-2hrs**
   - **Block Type—96-Well 0.1mL Block**

d. (*Optional*) Select **Is Locked** to lock the template. If locked, users are unable to edit the template.

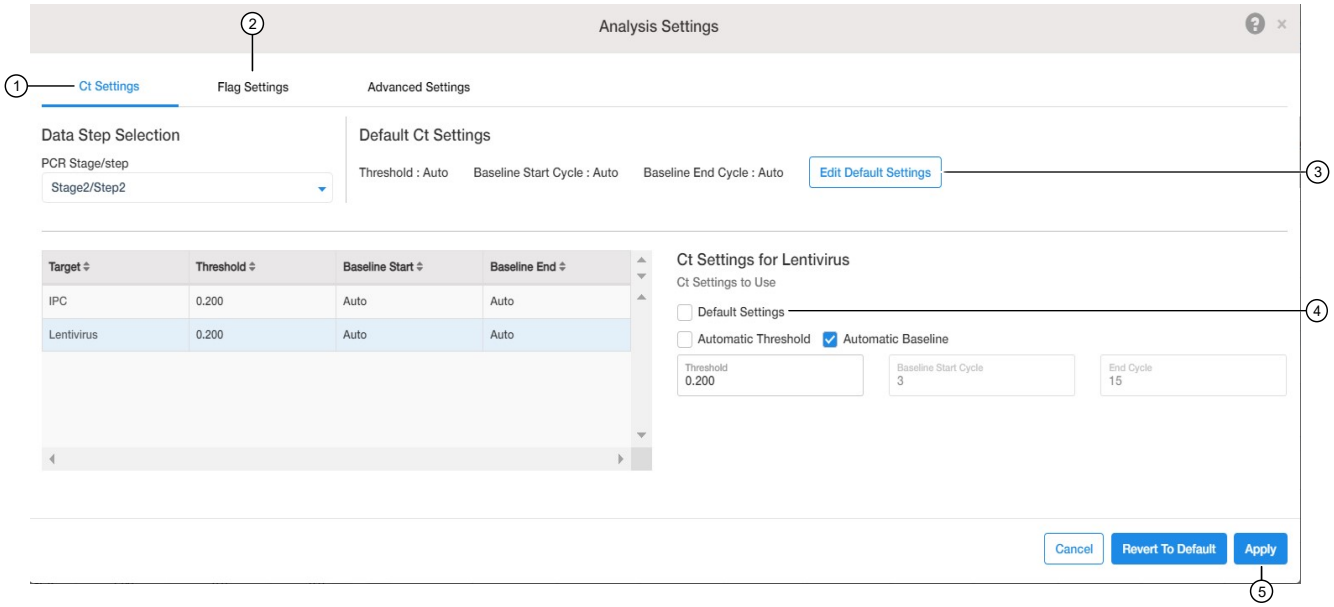
4. Click **Analysis settings** to change the default C_t Settings and Flag Settings.
   a. In the **C_t Settings** tab, click **Edit Default Settings**.

   b. Deselect **Automatic Threshold**, then enter **0.200**.

   c. Ensure that **Automatic Baseline** is selected.

   d. Click **Save Changes**.

e. Deselect **Default Settings**, then click **Apply** to save any changes before closing the window.



① **C_t Settings**
② **Flag Settings**
③ **Edit Default Settings** button
④ **Default Settings** checkbox
⑤ **Apply** button

f. In the **Flag Settings** tab, deselect the following flags.

- **CQCONF**—Low Cq confidence
- **EXPFAIL**—Exponential algorithm failed
- **NOAMP**—No amplification
- **NOSIGNAL**—No signal in well

Note: Use the scrollbar on the right to scroll down the list of flags.

g. Click **Apply** to save any changes before closing the window.

5. Click **Next**.

Template name cannot be changed after this step.

The qPCR Method screen is displayed.

Edit the run method and optical filter selection

This section provides general procedures to edit the run method and optical filter selection in the qPCR Method. To edit the default run method, see the *AccuSEQ™ Real-Time PCR Software v3.2 User Guide* (Pub. No. MAN0029199).

1. Set the reaction volume to **30 µL**.
2. Set **Step 1** of the **Hold Stage** to 95°C for 10 minutes.
3. Set **Step 1** of the **PCR Stage** to 95°C for 15 seconds.
4. Edit **Step 2** of the **PCR Stage** to 60°C for 1 minute.
5. Set the cycle number to **40**.
6. Ensure that **Data Collection** occurs after **Step 2**.

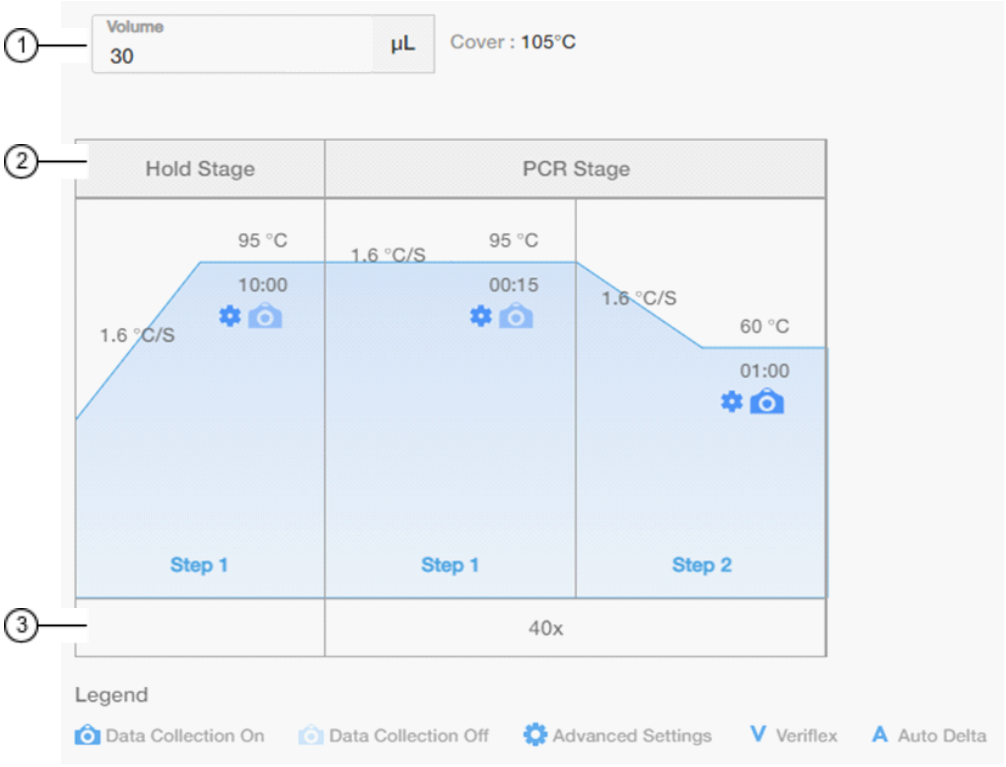


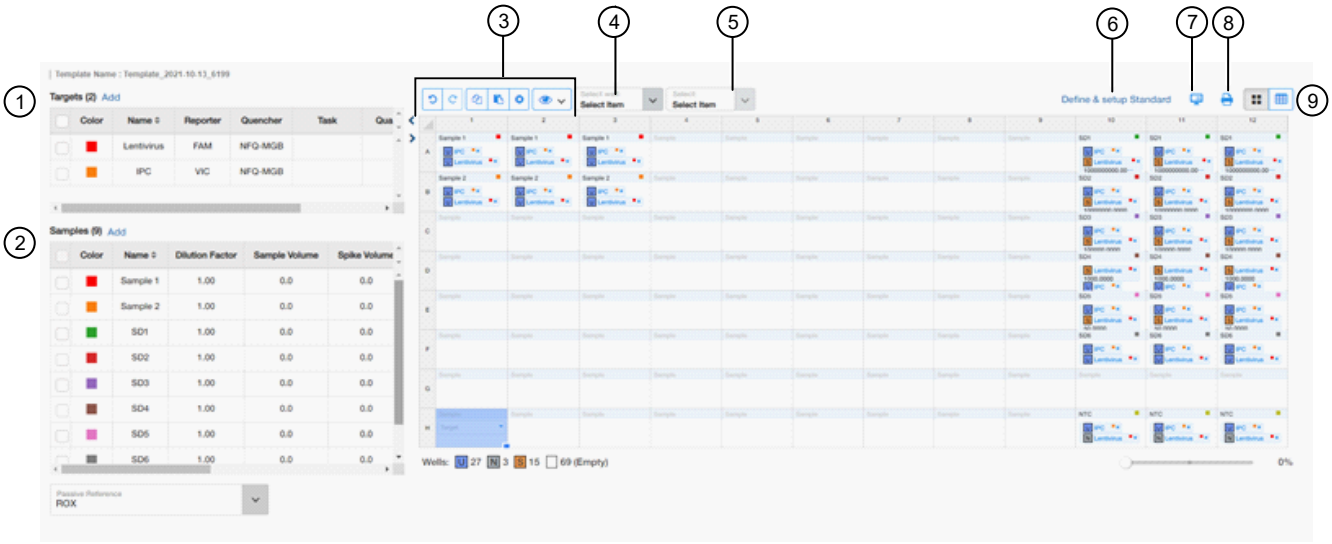
Figure 6 Lentivirus Proviral Titer Run Method

① Reaction volume- set to 30µL
② Stage
③ Cycle number- set to 40 cycles

7. (*Optional*) Click **(Optical Filter Settings)** to view the default filter settings.
   - The default optical filter selection is suitable for the ViralSEQ™ Lentivirus Proviral DNA Titer Kit.
   - The ViralSEQ™ Lentivirus Proviral DNA Titer Kit requires the QuantStudio™ 5 System to be calibrated for FAM™, VIC™, and ROX™.
   - 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).
8. Click **Next**.

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 *ViralSEQ™ Lentivirus Titer Kits User Guide* (Pub. No. MAN0026126). Do not change **Targets** for default assay templates.



1. Targets
2. Samples
3. Plate setup toolbar
4. Select Item to highlight (Sample, Target, or Task).
5. Select Item. For example, Sample 1. Sample 1 replicates are highlighted.
6. Define & setup Standard
7. (View Legend)
8. (Print Preview)
9. 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. Each well should have a Sample Name under **Samples**, as well as the appropriate Targets under **Targets**. Reporters should be **FAM™** dye for Lentivirus Proviral Titer, and **VIC™** dye for internal positive control (IPC).

   a. 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 if needed. The new sample or target is then selectable within the wells of the plate.

   b. For each sample (for example DNase-treated lentivirus sample, standard curve dilution, or NTC), two targets should be included.
      - Select the **FAM™** dye reporter for Lentivirus Proviral Titer detection.
      - Select the **VIC™** dye for IPC detection.

   c. Select **NFQ-MGB** as the quencher for both targets.

   d. For standard curve dilution samples (SD1 to SD5), the **Task** for Lentivirus Proviral Titer target should be indicated as **S** for Standard, with the appropriate copy number written under **Quantity**. For instance, the quantity for SD1 is 1E7 copies. Change the **Task** by clicking on the field and using the dropdown menu. Copy numbers can be indicated using scientific notation (for example “1E7”) and the program converts it to numerical format.

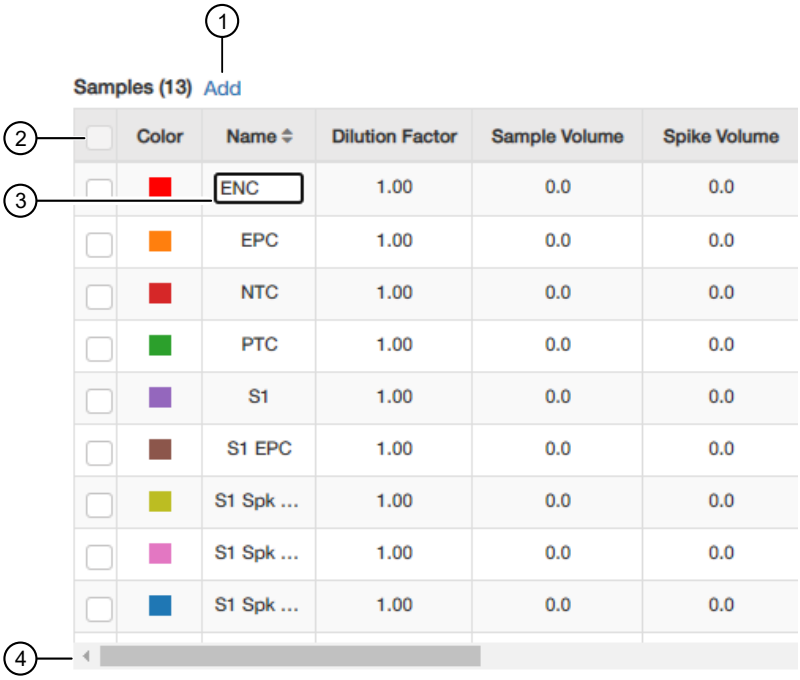
   e. For unknown or test samples and SD6, set the **Task** for Lentivirus Proviral Titer target to **U** for Unknown.

   f. For NTC wells, set the **Task** for Lentivirus Proviral Titer target to **N** for NTC.

   g. For IPC wells, set the **Task** to **U** for all samples.

h. 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.

When a **Sample** or **Target** name are edited, two entries are added to the Audit trail (one for Delete, and another for Create).



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 more 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.

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

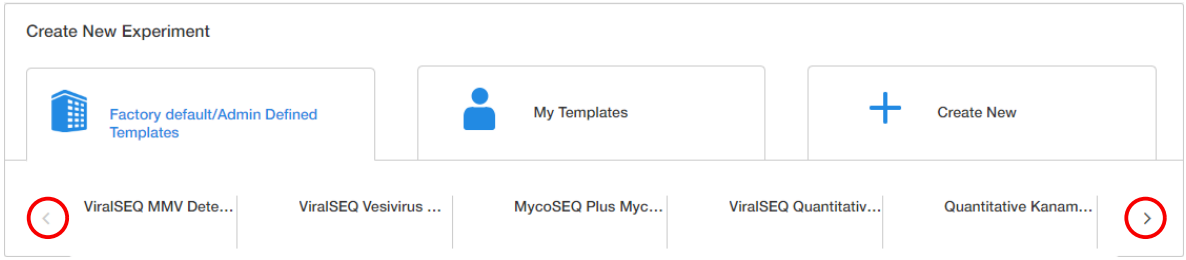
4. Select ROX™ dye from the **Passive Reference** dropdown list (lower-left of screen).

5. Click **Save** to save the template.
   This template can then be used to create experiments.

Create a ViralSEQ™ Lentivirus Proviral DNA Titer Kit experiment in AccuSEQ™ Real-Time PCR Software v3.2 or later

1. In the **Home** screen, click the **Factory default/Admin Defined Template** tab, then select a **ViralSEQ Lentivirus Proviral DNA Titer Kit** template.

   To navigate to through the available templates click the arrows to either side of the available templates.



To create an experiment from an existing ViralSEQ™ Lentivirus Proviral DNA Titer Kit experiment, see *AccuSEQ™ Real-Time PCR Software v3.2 User Guide* (Pub. No. MAN0029199).

2. In the **Experiment Properties** pane of the **Setup** screen:
   a. (*Optional*) Change the system-generated name of the experiment.

   Note: Names must be unique. Deleted experiment names can not be reused.

   b. (*Optional*) Enter the plate barcode in the **Barcode** field, then add comments in the **Comments** field.

   Note: Comments are not editable post analysis.

   Default ViralSEQ™ Lentivirus Proviral DNA Titer Kit settings (cannot be changed)
   - **Experiment Type—Quantitation-Standard Curve**
   - **Chemistry—TaqMan™ Reagents**
   - **Ramp Speed—Standard**

   c. Click **Next**.

   Note: Experiment names cannot be changed after this step.

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

Note: This assay is run with a **Standard** ramp speed.

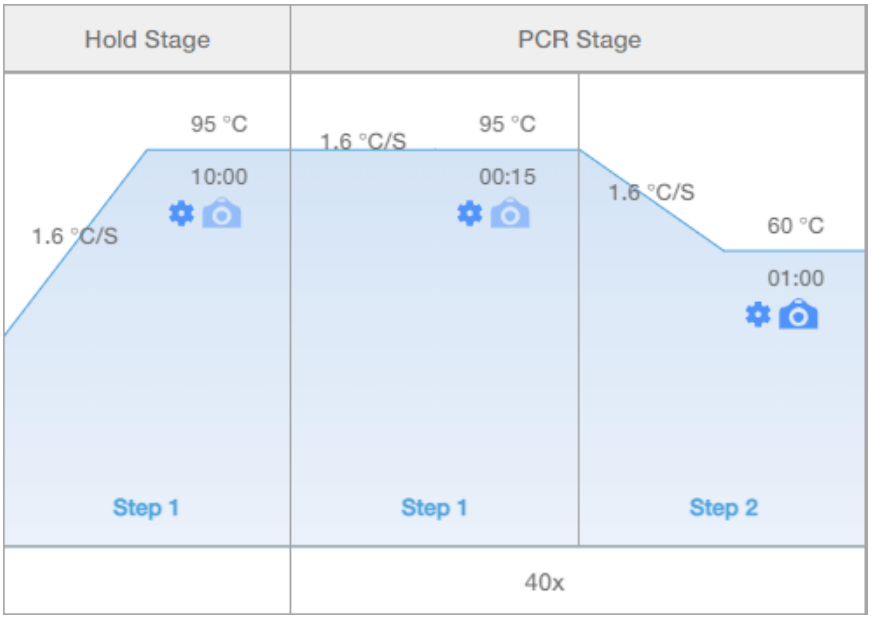


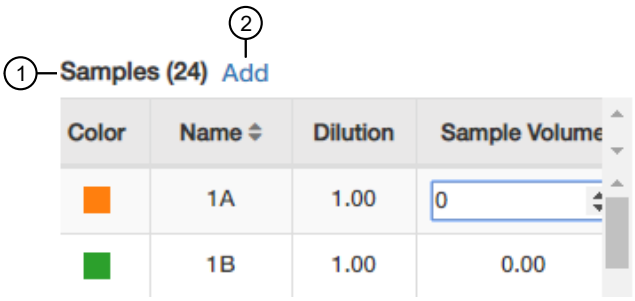
Figure 7 ViralSEQ™ Lentivirus Proviral DNA Titer Kit template default cycling conditions

4. Click **Next**.

5. In the **Samples** table of the **Plate Setup** pane, enter the sample name. Sample volume is not needed. Click **Add** to add more samples if needed.

Note: The software calculates provirus copies per cell in the results if the number of cells extracted is entered.

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



① **Samples** table
② **Add**—adds more samples

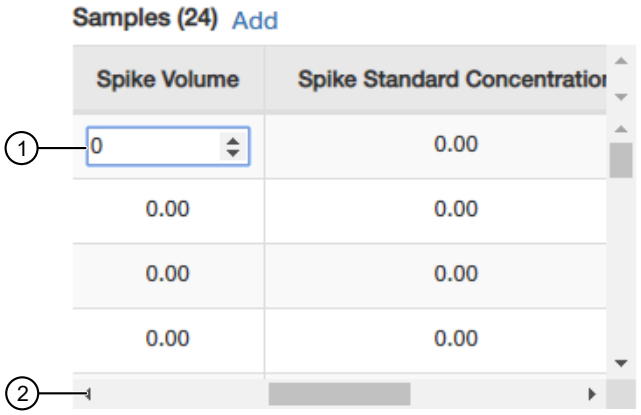
For more information about plate setup, see “Assign plate and well attributes” on page 62.

- **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, 10 copies).
- **Reference**—the non-spiked sample; the mean quantity of reference is subtracted during % recovery calculation.

- **Spike Input**—calculated (double check if correct).

Note: If not correct, ensure **Spike Volume** is set to 10 and **Spike Standard Concentration** is the expected copies spike per PCR reaction.

- (Optional) **Comments**
- **Number of Cells Extracted**—needed to calculate provirus copies per cell in the results.



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

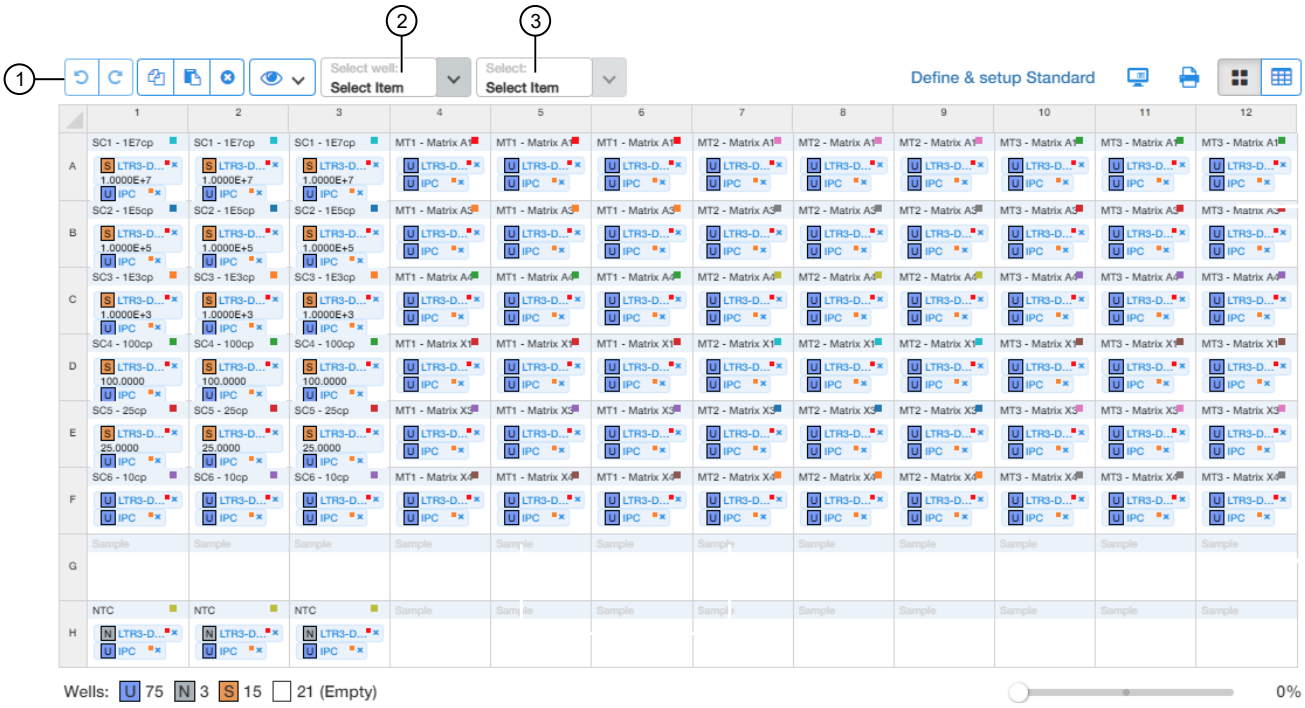


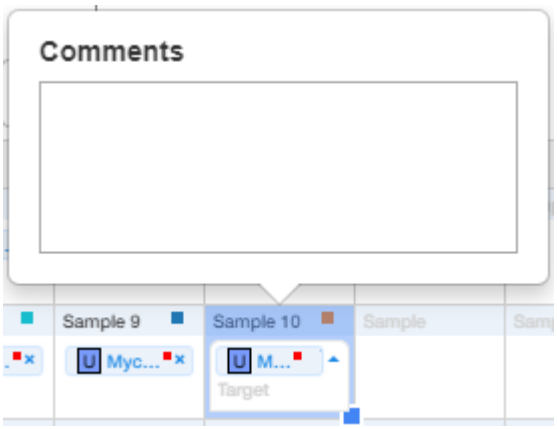
Figure 8 ViralSEQ™ Lentivirus Proviral Titer Kit template default sample plate layout

① Toolbar (in order: Undo, Redo, Copy, Paste, Delete, View)
② Select type (Sample, Target, or Task) to highlight.
③ Select specific items of that type. For example, Sample 1. Sample 1 replicates are highlighted.

Table 19 Plate well descriptions

Name	Description
SC1 to SC 6	Standard curve dilutions in triplicate
MT1 to MT3	18 samples in triplicate
NTC	No template control in triplicate

6. (*Optional*) Double-click wells to add **Comments**. Comments can also be added post-analysis.



7. Click **Next**.
   The **Run** screen 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** creates a copy of the experiment.

9. (*Optional*) Click **Print** to print the plate layout for use in preparing the reactions.

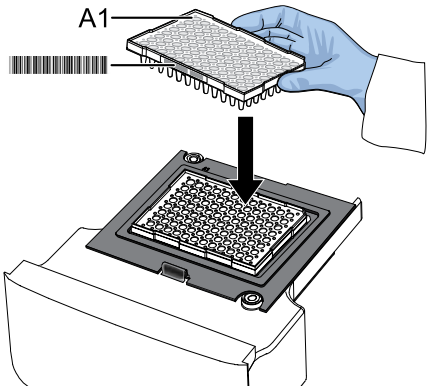
10. Assemble the PCR reactions following the manufacturer's instructions for the reagents and following the plate layout set up in the template.

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.

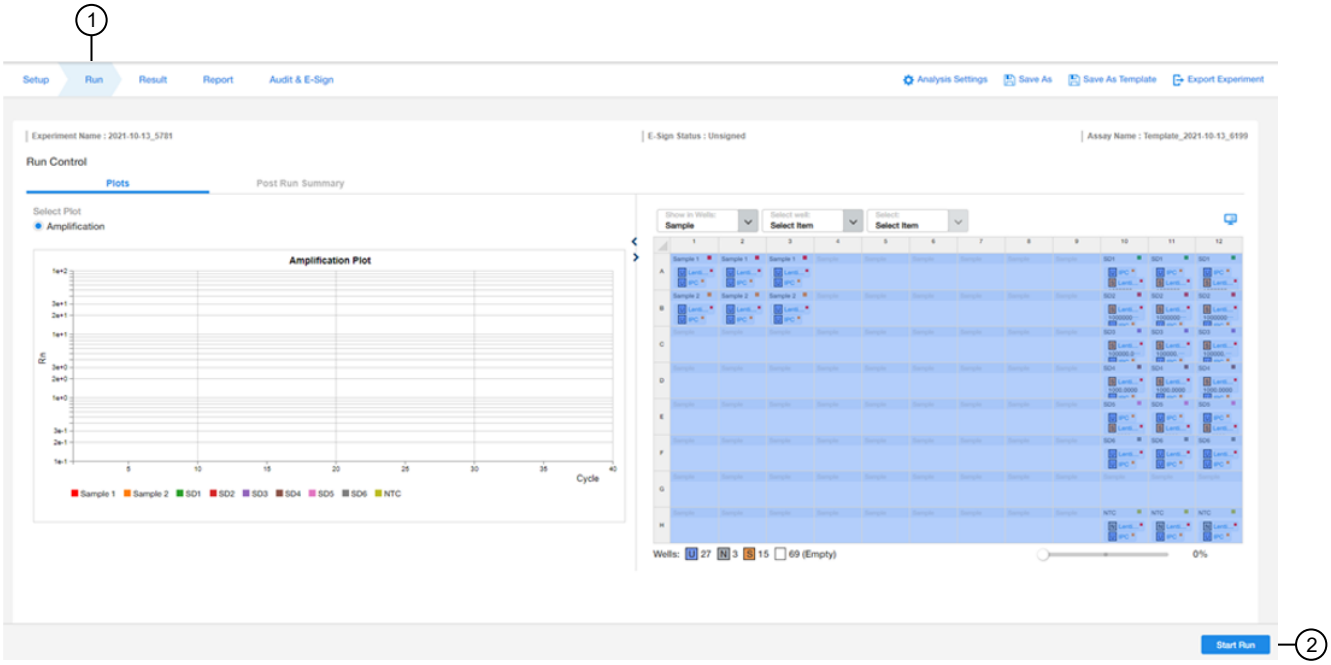
3. Touch ⏏ to close the instrument drawer.

Start the run (QuantStudio™ 5 Real-Time PCR Instrument)

Ensure that the plate is loaded in the QuantStudio™ 5 Real-Time PCR Instrument.

Start the run in the AccuSEQ™ Software v3.1 or later.

Option	Description
If the experiment is open	Click **Start Run**.
If the experiment is closed	a. Open the experiment. b. Click the **Run** tab. c. 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:

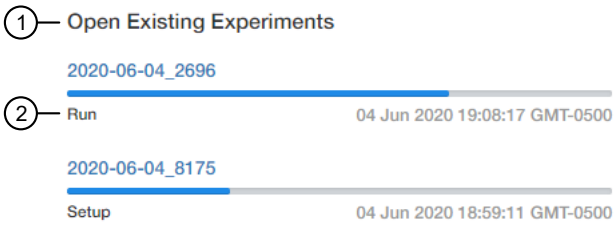
- On the instrument touchscreen.
- In the **Monitor the Run** pane of the AccuSEQ™ Software **(Home)** screen.

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: You cannot start another run while the instrument status is **Running**.

When the run is complete, the status changes to **Analysis** and the status bar displays as complete.



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.

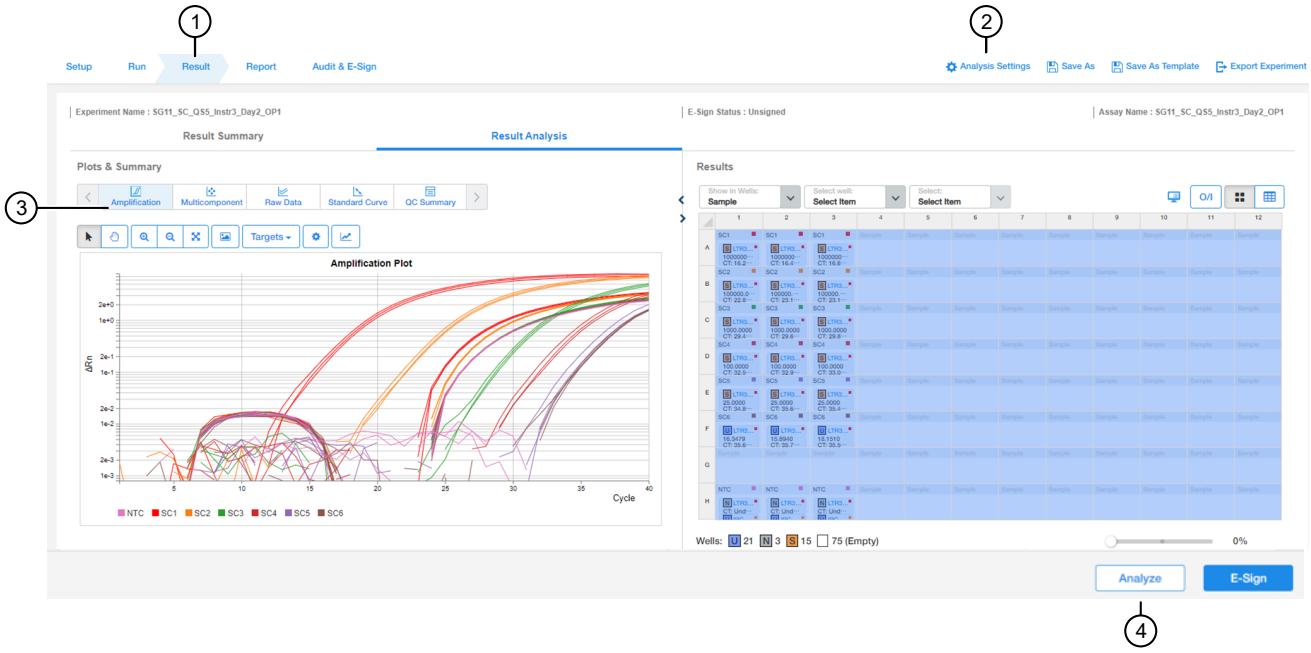
On run completion, the **Post Run Summary** displays the run length, user and instrument information, and a list of any errors that occurred.

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

Review 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 v3.2 User Guide* (Pub. No. MAN0029199).

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



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

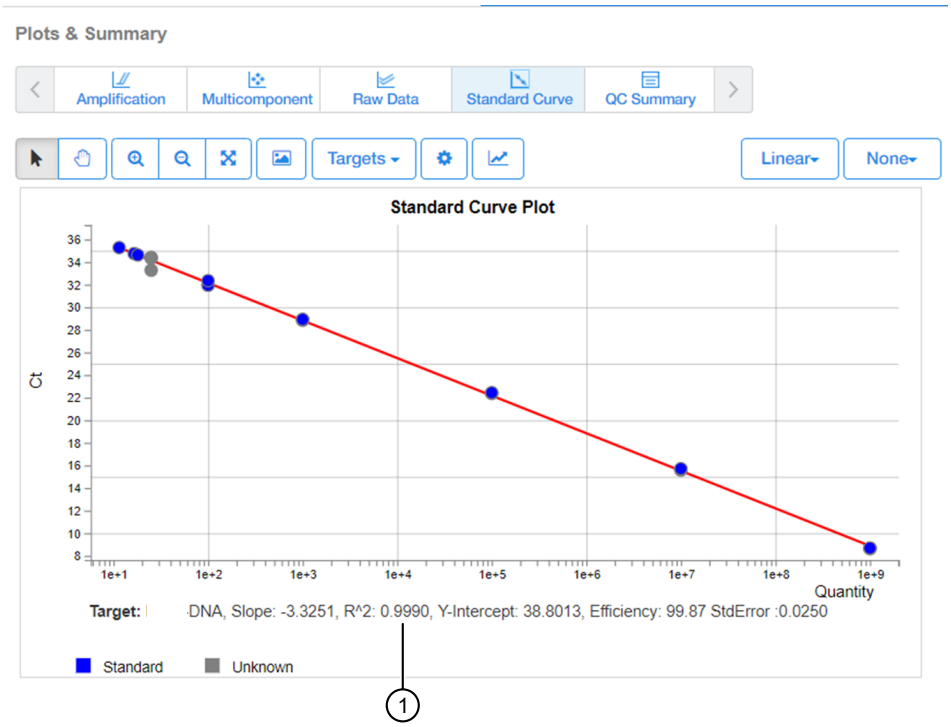
2. In the **Result Analysis** tab, select individual targets, then review the Amplification Curve plots for amplification profiles in the controls, samples, and the standard curve. Ensure that threshold is set to 0.200 with an automatic baseline.

Note: If any settings are changed, click the **Analyze** button to apply the new settings to the results. Target names and Sample names (under the **Setup** tab) can also be changed after the run is completed.

---

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, R^2, and Efficiency are within acceptable limits.



① Slope, R^2, and Efficiency values

Note: The **Standard Curve** efficiency should be between 90–110% and the R^2>0.99. If these criteria are not met, up to two points, not in the same triplicate, can be removed from the standard curve data, and the analysis repeated.

5. In Table View, ensure that C_t values are within the standard curve range.
   - Samples with C_t values that exceed the upper limit of quantitation (10^7 copies) of the standard curve should be diluted and re-run.
   - Samples with C_t values that exceed the lower limit of detection (LoD of 10 copies) and IPC shows no signs of PCR inhibition, suggests the absence of provirus.

6. (*Optional*) Outliers can be excluded from the results. To exclude, select the well, then click **Omit/Include**, then reanalyze by clicking **Analyze**.

7. (*Optional*) Select **File ▸ Print Report** to generate a hard copy of the experiment, or click **Print Preview** to view and save the report as a PDF or HTML file.

8. Export the results.
   a. Navigate to the **Report** screen.
   b. Check all boxes under **Contents**.
   c. Select **Export Data in One File**.

d. Select the **XLS** format, then click `Export`.

Calculate the infectious titer

Note: After analysis, AccuSEQ™ Software v3.2 or later provides provirus copies per cell in the table view of the **Results** screen. Use of the **Proviral Titer Calculation Tool** is needed to calculate the infectious titer.

1. Download the **Proviral Titer Calculation Tool**.
   a. Go to **thermofisher.com**.
   b. Search for the ViralSEQ™ Lentivirus Proviral DNA Titer Kit.
   c. Download the tool from the **Documents** section.
2. Open the tool, then follow the instructions in the tool to calculate the titer.

Analyze the data without AccuSEQ™ Real-Time PCR Software

1. Set the threshold to 0.2 and use automatic baseline.
2. Export the C_t values to a Microsoft™ Excel™ spreadsheet.
3. For the standard curve, plot $\log_{10}$ of the standard quantity versus the mean C_t.
4. Determine the equation of the standard curves by adding a trendline and displaying the equation.
5. Determine the quantity of the unknown samples using the C_t values and the equation generated from the trendline.

Calculate provirus copies and lentivirus titers from qPCR data

Each provirus has 2 copies of the assay target (LTR).

1. To determine the number of provirus copies in the extracted sample, the copy numbers obtained from the qPCR must be multiplied by 0.5 and then by the dilution factor of the sample during extraction. The concentration of provirus per cell can be calculated based on the number of cells used in the extraction.

   $$\text{Provirus copies per cell} = \frac{\text{qPCR copies x sample dilution factor x 0.5}}{\text{Number of harvested cells used for extraction}}$$

   For help in determining the qPCR copy numbers, see the *QuantStudio™ Design and Analysis Desktop Software User Guide* (Pub. No. MAN0010408).

   For example, if the following parameters were used,

   - 10,000 cells were extracted with the KingFisher™ Flex Purification System with 96 Deep-Well Head and eluted in 200 µL.
   - 5 µL of this eluate (40x dilution) was used for the qPCR reaction.

   then, the calculation would be:

   $$\text{Provirus copies per cell} = \frac{\text{qPCR copies x 40 x 0.5}}{\text{10,000}}$$

   Note: The number of cells extracted can be determined by the counting of cells or nucleic acid quantity equivalence (1 cell = 6.6 pg for diploid cells). Users should establish the nucleic acid quantity equivalence for cells that are non-diploid.

2. To estimate the infectious viral titer (or transduction unit TU/mL), use the volume of lentivirus (mL) that was used to infect the cells seeded for lentivirus transduction and the number of cells seeded for transduction [No. of cells (Day 1)].

   $$\text{Infectious viral titer per mL} = \frac{\text{Provirus copies per cell x No. of cells (Day 1)}}{\text{Volume of lentivirus (mL)}}$$

A

Troubleshooting

PrepSEQ™ sample preparation

Observation	Possible cause	Recommended action
Poor extraction efficiency (low yields)	Overdrying the sample.	Start the 5 minute timer before removing ~300 µL from the first 6–8 samples. Then continue removing wash buffer from the remaining samples.
	Magnetic Particles are difficult to resuspend during the elution.	Incubate the pellets at 70°C for >7 minutes. Vigorously vortex the tubes three times during incubation to help resuspension. Do not overdry. If necessary, repeat the incubation and vortexing steps.
	Formation of precipitate in Magnetic Particles.	Incubate the Magnetic Particle suspension at 37°C with intermittent vortexing at 900 rpm until the particles are completely suspended.
	PK Buffer was used instead of PK Buffer II.	Use PK Buffer II.
Particles no longer produce consistent results (fine brown sandy particles and brown color are observed in the supernatant)	Samples have low pH.	Measure the pH of the sample and adjust the pH to between 6 and 8 using NaOH or HCl as needed.
	Magnetic Particles were stored at –20°C.	Order new materials and store them at room temperature.

ViralSEQ™ assays

Observation	Possible cause	Recommended action
Δ Rn and Ct values are inconsistent with replicates	Evaporation of reaction mixture from some wells occurred because the optical adhesive cover was not correctly sealed to the reaction plate or due to over-drying the eluates in the PrepSEQ™ workflow.	1. Select the **Component** tab. Confirm that affected wells generated significantly less fluorescence than unaffected replicates. 2. Check the amount of solution in each well of the reaction plate. Confirm that the wells affected by evaporation contained less solution than unaffected wells, and corresponded with the inconsistent results. 3. For subsequent runs, ensure that the optical adhesive cover is correctly sealed to the reaction plate.

Observation	Possible cause	Recommended action
Δ Rn and Ct values are inconsistent with replicates *(continued)*	Incorrect volume of PCR reaction mix was added to some reactions.	1. Select the **Component** tab. Confirm that affected wells generated significantly less fluorescence than unaffected replicates. 2. Select the **Spectra** tab. Confirm that the wells with the incorrect volume of PCR reaction mix generated significantly different amounts of fluorescence than the unaffected wells. 3. For subsequent runs, ensure the correct volume of PCR reaction mix.
Jagged amplification plots	Weak lamp or incorrect replacement.	Replace the lamp or make sure that the existing replacement is correct.
No defined amplification plots	An incorrect detector was selected on the amplification plot. *or* An incorrect detector was applied to the reactions when setting up the plate document.	1. Confirm that the correct detector was selected on the amplification plot. 2. If the correct detector was not selected, then in the plate document, double-click a well to view the Well Inspector, verify that the detector settings are correct, and reanalyze.
Slope for the standard curve is outside the typical range, or R^2 value is significantly less than 0.99.	When applying detectors for standards, the Task and Quantity were applied to the wrong detector. *or* The incorrect Quantity was entered. *or* Adjust baseline settings. *or* Poor standard curve preparation technique (forgot to mix, inaccurate pipetting).	1. In the SDS software, from the plate document, double-click a well containing a DNA standard to view the Well Inspector. 2. Ensure that the correct Task and Quantity are applied to the correct detector, then reanalyze. 3. Compare std curve statistics using autobaseline or manual baseline. The upper limit of the manual baseline setting must be 2 cycles before uptick in amplification. Verify in Rn vs C_t linear view. 4. Increase final standard volumes (SD1 to SD6) up to 500 µL if required. Larger volumes will have less inaccuracies when pipetting.
No target-specific signal (FAM dye) is detected in PCR positive control and/or extraction positive control wells	FAM™ dye detector not selected.	Make sure the FAM™ dye detector is selected in the analysis software. Repeat the analysis with the FAM™ dye detector selected.
IPC signal is low	Inhibition of PCR or insufficient amount of assay mix added.	Repeat the sample preparation, then repeat the assay. If PCR inhibition continues, contact your FAS, or dilute the sample (for example, 1:5 or 1:10) to dilute inhibitors.

Observation	Possible cause	Recommended action
No IPC signal is detected, but target-specific signal is detected	High copy number of target cDNA/DNA resulting in preferential amplification of the target-specific cDNA/DNA.	Dilute sample (to within standard curve range) and repeat the assay.
Abnormal ΔRn values or negative ΔRn values	Incorrect passive reference was selected when setting up the plate document.	1. From the plate document, double-click a well to view the Well Inspector. 2. Ensure that ROX™ dye was selected as the Passive Reference.
No IPC signal is detected	The VIC™ dye detector was not selected.	Make sure the correct detector is selected in the analysis software. Repeat the analysis with the correct detector selected.
Amplification plot crosses the threshold but no target-specific signal is amplified	Probe hydrolysis or non-optimal baseline setting.	View amplification plots for the affected wells using Rn vs. C_t to confirm no up-tick in amplification. Change baseline settings. Check manual or autobaseline. Contact Technical Support.
No dye signal in test sample well	Error in preparation.	Repeat the sample preparation and perform a new PCR reaction, using freshly prepared sample and reagents.
NTC signals are < SD6 (LOD)	Contamination of master mix, assay mix, pipettes, or work area from improper handling of samples or control template.	Decontaminate the work area and use clean, dedicated pipettes when preparing reaction mixes. Where possible, carry out experiment in a biosafety cabinet or on dedicated work benches.
Multiple bands (3 or more) observed when PCR product is run on an agarose gel.	Secondary or nonspecific PCR products are produced in later cycles of PCR.	The probe does not bind to the nonspecific PCR products and thus does not affect the accuracy of the results. The user does not need to troubleshoot.

B

Use the ViralSEQ™ Lentivirus Physical Titer Kit with the 7500 Fast Real-Time PCR Instrument and AccuSEQ™ software v2.x

Required materials not supplied

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
Instrument	
7500 Fast Real-Time PCR System with AccuSEQ™ software v2.x	Contact your local sales representative
Generic consumables	
Disposable gloves	MLS
Aerosol-resistant pipette tips	MLS
Pipettors: • Positive-displacement • Air-displacement • Multichannel	MLS
Consumables for the 7500 Fast Real-Time PCR System	
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL, 20 plates; for use with 7500 Fast Real-Time PCR System	4346906

(continued)

Item	Source
MicroAmp™ Optical 96-Well Reaction Plate with Barcode & Optical Adhesive Films, 100 plates with covers	4314320
MicroAmp™ Optical 8-Cap Strips, 300 strips	4323032
MicroAmp™ Optical Adhesive Film Kit, 20 covers, 1 applicator, 1 optical cover compression pad	4313663
MicroAmp™ Optical Adhesive Film	4360954

Create a ViralSEQ™ Lentivirus Physical Titer Kit experiment in AccuSEQ™ Software v2.0 or v2.1

Note: AccuSEQ™ Software v2.2 or later has a factory default **SEQ Experiment** for the Lentivirus Physical Titer Kit. For information about the factory default template, see "Create a ViralSEQ Lentivirus Physical Titer Kit experiment in AccuSEQ™ Software v2.2 or later" on page 84.

Use this workflow for the Lentivirus Physical Titer Kit in AccuSEQ™ Software v2.0 or v2.1.

Plate document: settings

If you have run the assay frequently, you can use the table below to enter settings in Plate Document fields. If you are a new user, follow the detailed instructions in the following sections.

Summary of settings for the Plate Document		
In this field…		**Use these settings**
Detector	Lentivirus	FAM™ dye (Select **NFQ_MGB** dye for quencher)
	IPC	VIC™ dye (Select **NFQ_MGB** dye for quencher)
	Passive reference	ROX™
RT-PCR	Ramp rate	100%[1]
	RT	Temp: **45**°C Time: **30:00**
	Hold	Temp: **95**°C Time: **10:00**

(continued)

Summary of settings for the Plate Document		
In this field…		**Use these settings**
RT-PCR	Cycling (Standard Mode)	Cycles: **40** Temp: **95**°C Time: **0:15** Temp: **60**°C Time: **0:45**
Analysis	Lentivirus	**Automatic Baseline** Threshold: **0.2**

[1] 1.6°C/second for other systems.

Create a plate document in AccuSEQ™ Software v2.0 or 2.1

1. In the home screen, select **Create Custom Experiment**.
2. In the **Experiment name** field, enter a unique name for the experiment.
3. Specify experiment information.
   a. Select experiment type **Quantitation – Standard Curve**.
   b. Select reagents **TaqMan™ Reagents**.
   c. Select ramp speed **Standard**.
4. In the **Plate Setup** screen, select the **Define Targets and Samples** tab.
5. Specify target information.
   a. Click **Add New Target**.
   b. Enter a host cell name in the target name field.
   c. Select reporter **FAM™** dye and quencher **NFQ_MGB** dye.
   d. Select a color for this target.
6. Specify IPC target information.
   a. Click **Add New Target**.
   b. Enter **IPC** in the target name field.
   c. Select reporter **VIC™** dye and quencher **NFQ_MGB** dye.
   d. Select a color for this target.
7. Define new samples.
   a. Click **Add New Sample**.

b. In **Sample Name**, add the names of the samples you want to define.

c. Click **Next**, or select the **Assign Targets and Samples** tab.

8. In the **Assign Targets and Samples** tab, define new targets.
   a. Follow the instructions in the top of the tab to set up the standards, unknowns, and negative controls.

   b. Click **Define and Set Up Standards** to open the **Define and Set Up Standards** dialog box to enter the appropriate settings and define the standard curve. When defined, click **Apply** and **Close**. The new standard curve is applied to the plate layout screen.

9. Assign the IPC to the standard curve wells.

10. In the **Run Method** screen, in the **Graphical View** tab.
    a. In **Reaction Volume Per Well**, enter **25 µL**

11. Click the **Analysis** button in the left panel. In the **Analysis Settings** window on the right, set the default settings.
    a. On the **Ct Settings** tab, click **Edit Default Settings**. Then set **Threshold** to 0.2, set to **Automatic Baseline**, and then click **Save Changes**.

    b. Select (highlight) both targets.

    c. In the right-hand window, select **Use Default Settings**.

    d. Click **Apply Analysis Settings**.

12. Select **File ▸ Save as**, confirm that the file is named **LV TiterTemplate** or similar, then select **Save as a template file** in the drop-down list and close the template plate document.

Note: You can reuse the plate template document to run an assay by opening the template file and choosing **Save As** to save the file with the experiment name.

Run the plate in AccuSEQ™ Software v2.0 and v2.1

1. In the toolbar, select **File ▸ Open**, navigate to the **LV Titer Template** file, then click **Open**.

2. In the **Experiment Name** field, enter the appropriate experiment name, then click **Finish**.

3. Make any necessary changes to the test sample labels.
   - **Sample Volume**—not applicable; leave as default (0).
   - **Spike Volume**—volume of DNA added to the PCR (set to 3).
   - **Spike Standard Concentration**—expected spike amount per reaction (for example, 5,000 copies).
   - **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 copies per PCR reaction.

4. Select **Save As** to save the new experiment as an EDS experiment file with the same name as entered in the **Experiment Name** field.
5. Load the plate into the instrument.
6. Click **Start Run**.
7. Select a run screen (**Amplification** plot, **Temperature** plot, or **Run method**) to monitor the progress of the run.

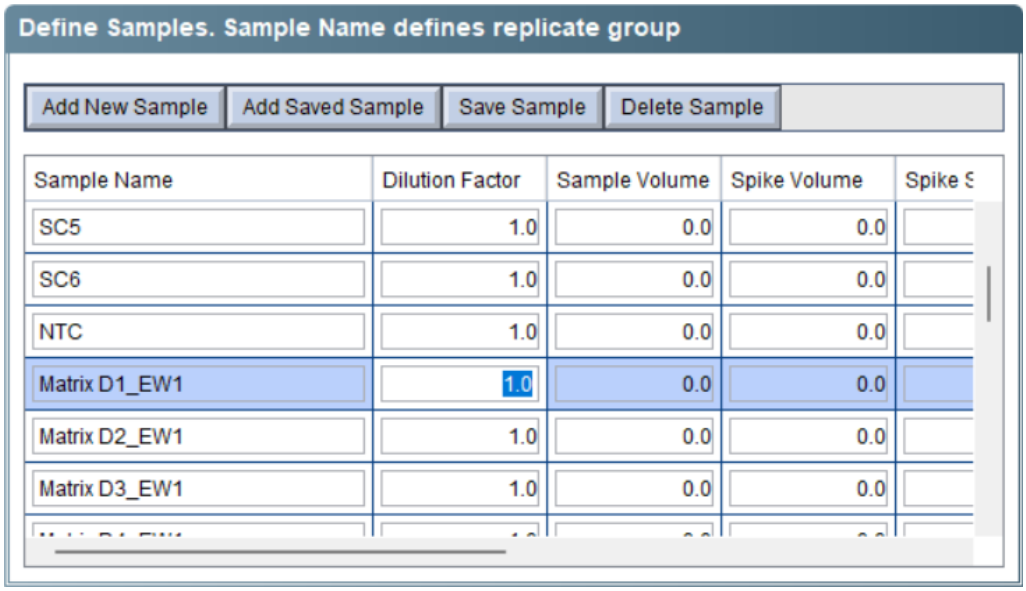
Create a ViralSEQ Lentivirus Physical Titer Kit experiment in AccuSEQ™ Software v2.2 or later

Note: The **ViralSEQ Lentivirus Physical Titer Kit** SEQ experiment is available with AccuSEQ™ Software v2.2 or later.

1. Click **Create SEQ Experiment** in the home screen.
2. Select **ViralSEQ Lentivirus Physical Titer Kit** from the assay list, then click **Next** .
   The **Plate Setup** screen opens.
3. In the **Experiment Menu** navigation bar, click **Experiment Properties**.
4. Enter the experiment name.

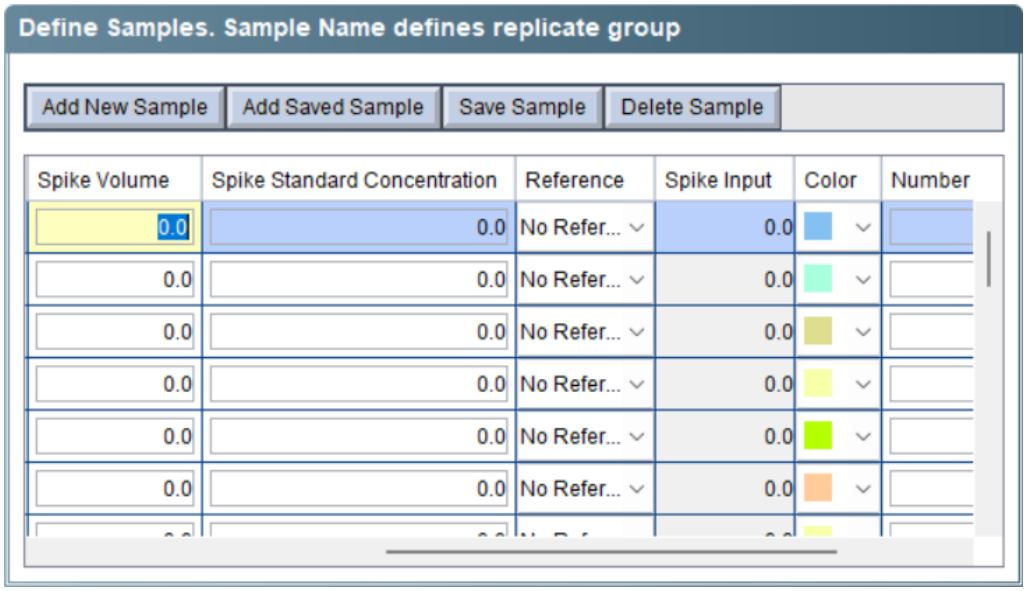
   Note: The experiment name can be up to 100 letters and numbers. Spaces are not allowed.
5. *(Optional)* Enter a plate barcode and comments.
6. In the **Experiment Menu** navigation bar, click **Plate Setup**.
7. In the **Define Samples** pane of the **Define Targets and Samples** tab of the **Plate Setup** pane, enter the sample name, then enter the sample dilution. Click **Add New Sample** to add more samples if needed.

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



For information about adding and defining samples, see *AccuSEQ™ Real-Time PCR Software v2.2 User Guide* (Pub. No. MAN0029201).

8. In the **Define Samples** pane in the **Define Targets and Samples** tab, scroll to the right, then enter the sample volume and spike information.



- **Sample Volume**—add sample volume to calculate viral particles per mL in the results.
- **Spike Volume**—volume of DNA added to the PCR (set to 10).
- **Spike Standard Concentration**—expected spike amount per reaction (for example, 10 copies).
- **Reference**—the non-spiked sample; the mean quantity of reference is subtracted during % recovery calculation.

- **Spike Input**—calculated (double check if correct).

  Note: If not correct, ensure **Spike Volume** is set to 10 and **Spike Standard Concentration** is the expected copies spike per PCR reaction.

- **Protein Concentration**—Drug substance protein concentration (if total DNA in copies RNA/mg protein is needed).
- (Optional) **Comments**

Note: The software calculates viral particles per mL if the sample volume was entered.

9. Click the **Assign Targets and Samples** tab.
The default plate layout is shown.

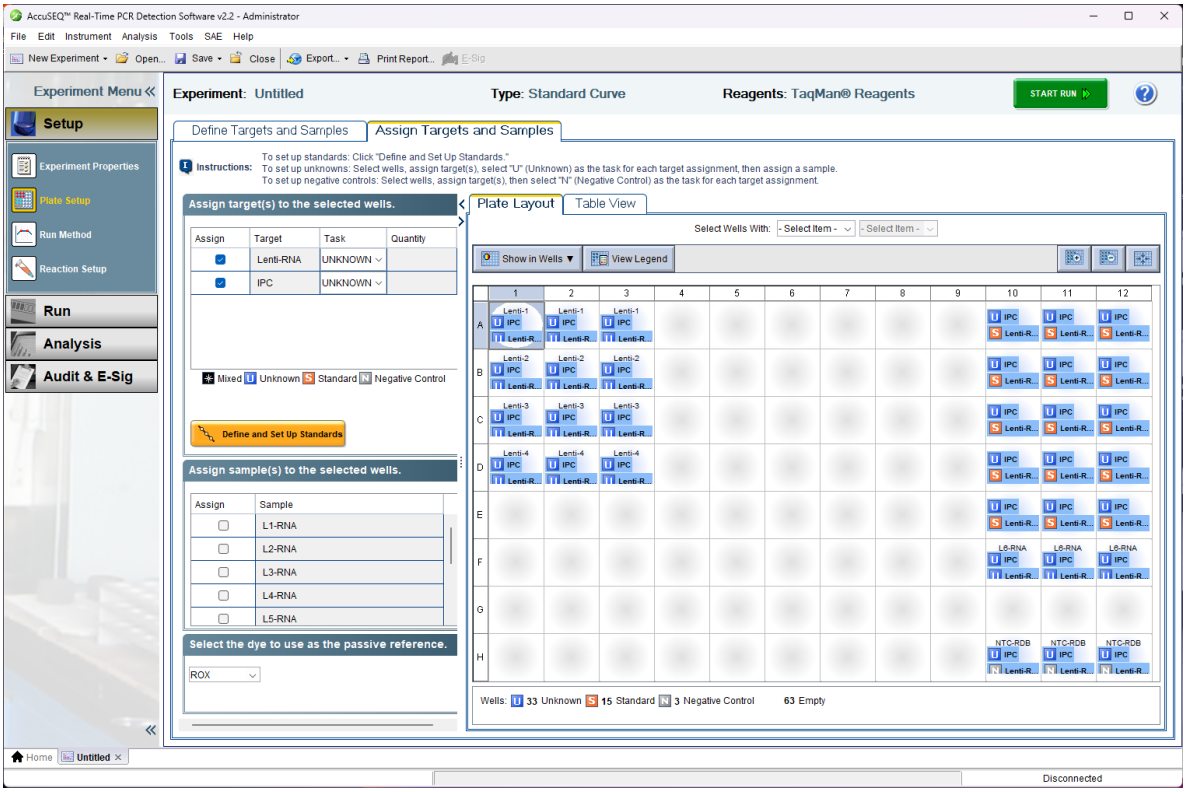


Table 20 Plate well descriptions

Name	Description
Lenti-1 to Lenti-4	4 samples in triplicate
L1-RNA to L6-RNA	Standard curve dilutions in triplicate
NTC-RDB	No template control in triplicate

10. *(Optional)* Edit the plate.

To change the plate layout display, see *AccuSEQ™ Real-Time PCR Software v2.2 User Guide* (Pub. No. MAN0029201).

a. Assign the targets to wells in the plate layout.

b. Assign samples to wells in the plate layout.

11. Confirm that ROX™ dye is selected as the passive reference.

12. *(Optional)* Save the plate layout as an image.
    a. Right-click the plate layout.
    b. Select **Save As**.
    c. Specify a file name and location for the JPEG file, then click **Save**.

    Note: Electronic signature information is not included in an image file. Use Print Report to include Electronic signature information with the plate layout.

13. *(Optional)* Print the plate layout
    a. Right-click the plate layout.
    b. Click **Print Preview** or **Print**.

14. In the **Experiment Menu** navigation bar, click **Run Method**.

15. In the **Run Method** screen, view the default volume and cycling conditions.

    IMPORTANT! Do not modify the run method for factory default SEQ experiments.

16. In the **Experiment Menu** navigation bar, click **Reaction Setup**.

17. In the **Reaction Setup** screen, complete the reaction setup. For more information about the reaction setup, see *AccuSEQ™ Real-Time PCR Software v2.2 User Guide* (Pub. No. MAN0029201).

18. Assemble the PCR reactions following the instructions of the manufacturer for the reagents and following the plate layout set up in the template.

Analyze the results

After the qPCR run is finished, use the following general procedure to analyze the results:

1. In the toolbar, select **Analysis ▸ Analysis Settings**.
2. Click Analyze (**Analyze**).
3. Select **Analysis ▸ QC Summary** in the left panel of the screen. Review the flag summary.
4. In the left panel, select **Analysis ▸ Standard Curve**. Verify the values for the Slope, Y-Intercept, R2, and Efficiency.
5. Select **File ▸ Export**. In the **Export Data** menu, select file type ***.xls**. Click **Start Export**.

6. Calculate titers using the **Physical Titer Calculation Tool**.

IMPORTANT! In AccuSEQ™ software v2.x, **Quantity** values above 2.1474836×10^7 copies per reaction do not display correctly in the software, which also results in incorrect display of the **Mean Quantity** and **Quantity Standard Deviation**. Disregard values in the table view of the software with a **Quantity** of 2.1474836×10^7, and instead use data in the Excel™ spreadsheet exported from the software. This error does not impact plotting of data on the standard curve or in the Excel™ report.

Calculate lentivirus titers from qPCR data

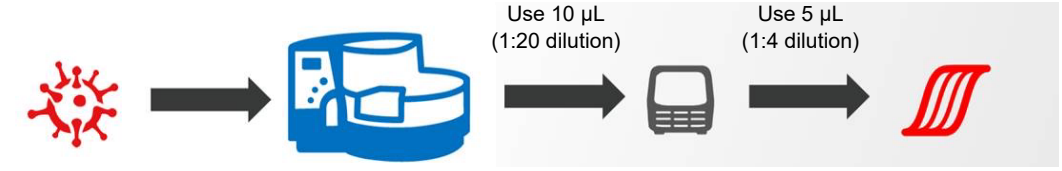
To determine the number of lentivirus RNA copies per mL in the original sample, the copy numbers obtained from the qPCR must be multiplied by the dilution factor of the sample during extraction and DNase I treatment. Since there are 2 copies of RNA/target per lentivirus particle, the number of viral particles per mL (VP/mL) is 0.5x the number of lentivirus RNA copies.

$$\text{Viral particles per mL} = \frac{\text{qPCR copies x sample dilution factor x 0.5}}{\text{Volume of sample used (mL)}}$$

For help in determining the qPCR copy numbers, see the *QuantStudio™ Design and Analysis Desktop Software User Guide* (Pub. No. MAN0010408).

For example, if the following parameters were used,

- 10 µL of lentivirus culture was extracted with the KingFisher™ Flex Purification System with 96 Deep-Well Head and eluted in 200 µL
- 10 µL of this eluate (20x dilution) was treated with DNase I, RNase-free (1 U/µL) in a total volume of 20 µL.
- 5 µL of the DNase-treated sample (4x dilution) was used for the qPCR reaction.



then, the calculation would be:

$$\text{Viral particles per mL} = \frac{\text{qPCR copies x (20x4) x 0.5}}{\text{0.01 (mL)}}$$

Note: qPCR can only determine the number of physical particles in a virus culture. To determine the numbers of infectious units, cell-based transduction experiments must be carried out. The titers of physical particles are often higher than infectious titers by 10-1000 fold, depending on the purity of the lentivirus preparation and the levels of infectious particles within the culture.

C

Use the ViralSEQ™ Lentivirus Proviral DNA Titer Kit with the 7500 Fast Real-Time PCR Instrument and AccuSEQ™ software v2.x

Required materials not supplied

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
Instrument	
7500 Fast Real-Time PCR System with AccuSEQ™ software v2.x	Contact your local sales representative
Generic consumables	
Disposable gloves	MLS
Aerosol-resistant pipette tips	MLS
Pipettors: • Positive-displacement • Air-displacement • Multichannel	MLS
Consumables for the 7500 Fast Real-Time PCR System	
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL, 20 plates; for use with 7500 Fast Real-Time PCR System	4346906

(continued)

Item	Source
MicroAmp™ Optical 96-Well Reaction Plate with Barcode & Optical Adhesive Films, 100 plates with covers	4314320
MicroAmp™ Optical 8-Cap Strips, 300 strips	4323032
MicroAmp™ Optical Adhesive Film Kit, 20 covers, 1 applicator, 1 optical cover compression pad	4313663
MicroAmp™ Optical Adhesive Film	4360954

Create a ViralSEQ™ Lentivirus Proviral DNA Titer Kit experiment in AccuSEQ™ Software v2.0 or v2.1

Note: AccuSEQ™ Software v2.2 or later has a factory default **SEQ Experiment** for the Lentivirus Proviral DNA Titer Kit. For information about the factory default template, see "Create a ViralSEQ Lentivirus Proviral DNA Titer Kit experiment in AccuSEQ™ Software v2.2 or later" on page 93.

Use this workflow for the Lentivirus Proviral DNA Titer Kit in AccuSEQ™ Software v2.0 or v2.1.

Plate document: settings

If you have run the assay frequently, you can use the table below to enter settings in Plate Document fields. If you are a new user, follow the detailed instructions in the following sections.

Summary of settings for the Plate Document		
In this field…		**Use these settings**
Detector	Provirus	FAM™ dye (Select **NFQ_MGB** dye for quencher)
	IPC	VIC™ dye (Select **NFQ_MGB** dye for quencher)
	Passive reference	ROX™
qPCR	Ramp rate	100%[1]
	Hold	Temp: **95**°C Time: **10:00**
	Cycling (Standard Mode)	Cycles: **40** Temp: **95**°C Time: **0:15** Temp: **60**°C Time: **1:00**
Analysis	Provirus	**Automatic Baseline** Threshold: **0.2**

[1] 1.6°C/second for other systems.

Create a plate document in AccuSEQ™ Software v2.0 and v2.1

1. In the home screen, select **Create Custom Experiment**.
2. In the **Experiment name** field, enter a unique name for the experiment.
3. Specify experiment information.
   a. Select experiment type **Quantitation – Standard Curve**.
   b. Select reagents **TaqMan™ Reagents**.
   c. Select ramp speed **Standard**.
4. In the **Plate Setup** screen, select the **Define Targets and Samples** tab.
5. Specify target information.
   a. Click **Add New Target**.
   b. Enter a host cell name in the target name field.
   c. Select reporter **FAM™** dye and quencher **NFQ_MGB** dye.
   d. Select a color for this target.
6. Specify IPC target information.
   a. Click **Add New Target**.
   b. Enter **IPC** in the target name field.
   c. Select reporter **VIC™** dye and quencher **NFQ_MGB** dye.
   d. Select a color for this target.
7. Define new samples.
   a. Click **Add New Sample**.
   b. In **Sample Name**, add the names of the samples you want to define.
   c. Click **Next**, or select the **Assign Targets and Samples** tab.
8. In the **Assign Targets and Samples** tab, define new targets.
   a. Follow the instructions in the top of the tab to set up the standards, unknowns, and negative controls.
   b. Click **Define and Set Up Standards** to open the **Define and Set Up Standards** dialog box to enter the appropriate settings and define the standard curve. When defined, click **Apply** and **Close**. The new standard curve is applied to the plate layout screen.
9. Assign the IPC to the standard curve wells.

10. In the **Run Method** screen, in the **Graphical View** tab.
    a. In **Reaction Volume Per Well**, enter **30 µL**

11. Click the **Analysis** button in the left panel. In the **Analysis Settings** window on the right, set the default settings.
    a. On the **Ct Settings** tab, click **Edit Default Settings**. Then set **Threshold** to 0.2, set to **Automatic Baseline**, and then click **Save Changes**.
    b. Select (highlight) both targets.
    c. In the right-hand window, select **Use Default Settings**.
    d. Click **Apply Analysis Settings**.

12. Select **File ▸ Save as**, confirm that the file is named **LV Proviral Template** or similar, then select **Save as a template file** in the drop-down list and close the template plate document.

Note: You can reuse the plate template document to run an assay by opening the template file and choosing **Save As** to save the file with the experiment name.

Run the plate in AccuSEQ™ Software v2.0 and v2.1

1. In the toolbar, select **File ▸ Open**, navigate to the **LV Proviral Template** file, then click **Open**.
2. In the **Experiment Name** field, enter the appropriate experiment name, then click **Finish**.
3. Make any necessary changes to the test sample labels.
   - **Sample Volume**—not applicable; leave as default (0).
   - **Spike Volume**—volume of DNA added to the PCR.
   - **Spike Standard Concentration**—expected spike amount per reaction (for example, 5,000 copies).
   - **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 correctly and **Spike Standard Concentration** is the expected copies per PCR reaction.
4. Select **Save As** to save the new experiment as an EDS experiment file with the same name as entered in the **Experiment Name** field.
5. Load the plate into the instrument.
6. Click **Start Run**.
7. Select a run screen (**Amplification** plot, **Temperature** plot, or **Run method**) to monitor the progress of the run.

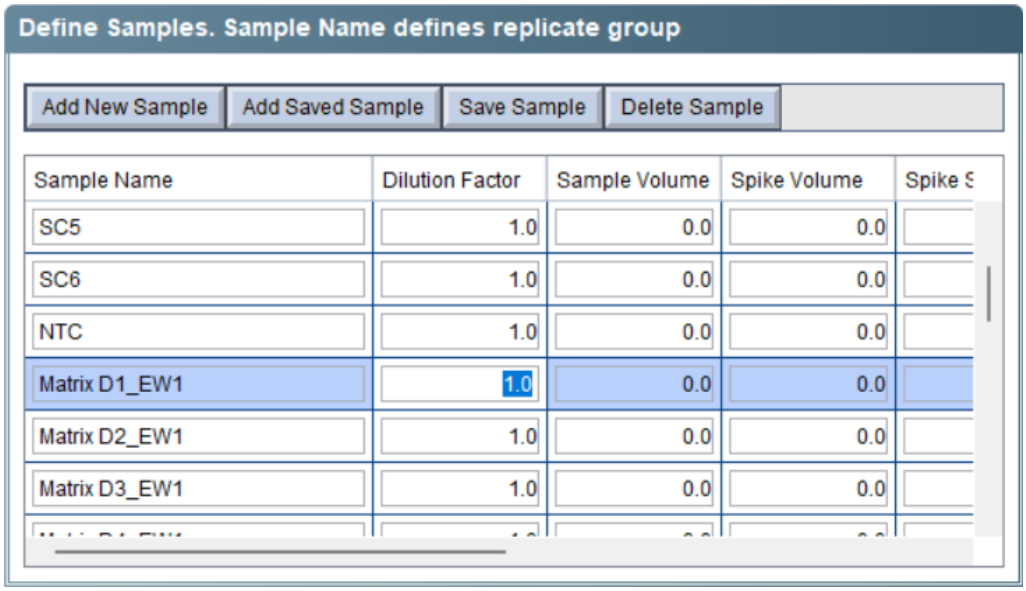
Create a ViralSEQ Lentivirus Proviral DNA Titer Kit experiment in AccuSEQ™ Software v2.2 or later

Note: The **ViralSEQ Lentivirus Proviral DNA Titer Kit** SEQ experiment is available with AccuSEQ™ Software v2.2 or later.

1. Click **Create SEQ Experiment** in the home screen.
2. Select **ViralSEQ Lentivirus Proviral DNA Titer Kit** from the assay list, then click **Next** .
   The **Plate Setup** screen opens.
3. In the **Experiment Menu** navigation bar, click **Experiment Properties**.
4. Enter the experiment name.

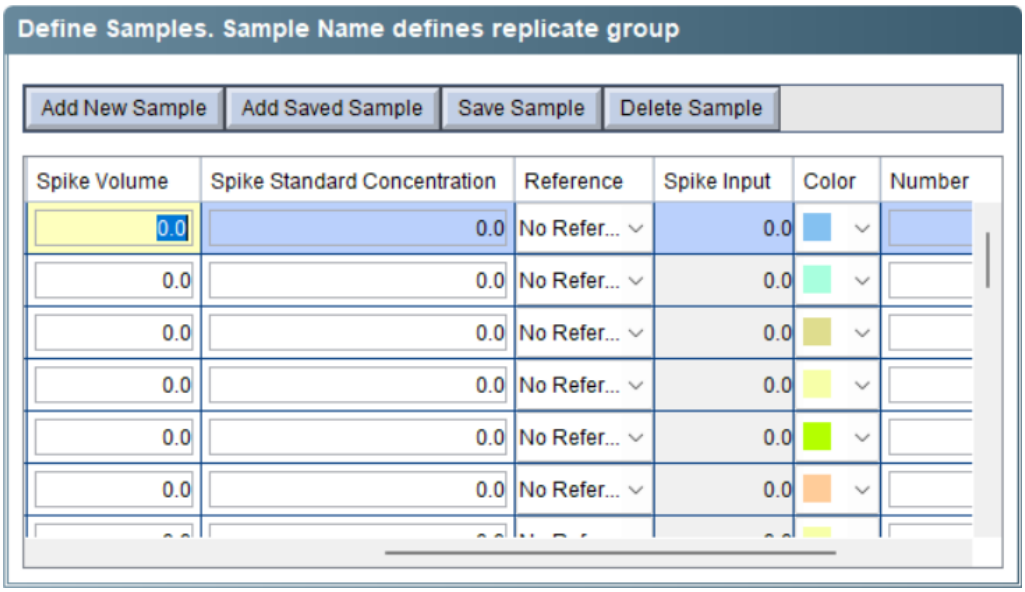
   Note: The experiment name can be up to 100 letters and numbers. Spaces are not allowed.
5. *(Optional)* Enter a plate barcode and comments.
6. In the **Experiment Menu** navigation bar, click **Plate Setup**.
7. In the **Define Samples** pane of the **Define Targets and Samples** tab of the **Plate Setup** pane, enter the sample name, then enter the sample dilution. Sample volume is not needed. Click **Add New Sample** to add more samples if needed.

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



For information about adding and defining samples, see *AccuSEQ™ Real-Time PCR Software v2.2 User Guide* (Pub. No. MAN0029201).

8. In the **Define Samples** pane in the **Define Targets and Samples** tab, scroll to the right, then enter the spike information.

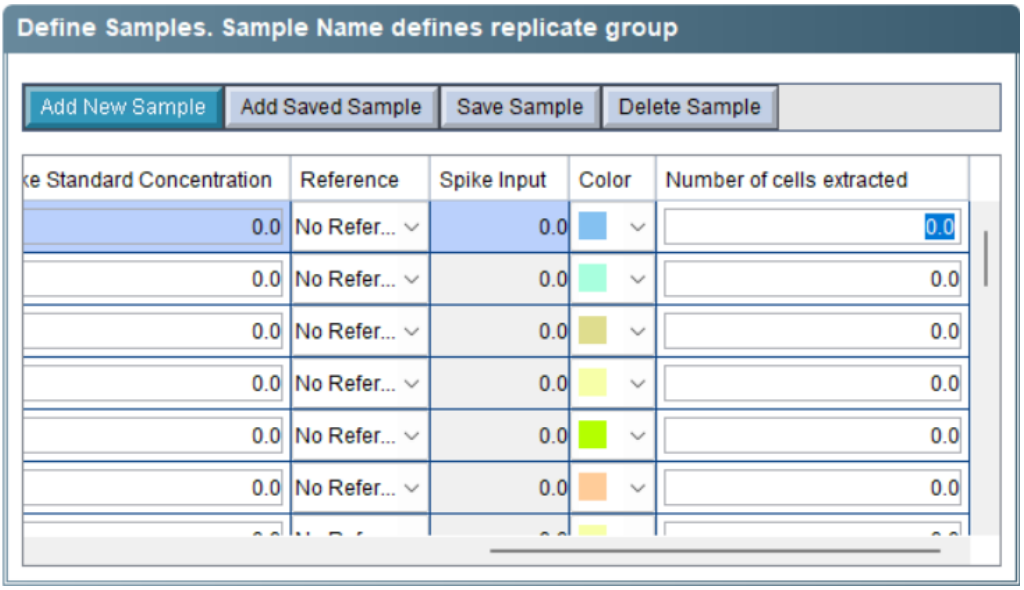


- **Sample Volume**—not needed; leave as default (0).
- **Spike Volume**—volume of DNA added to the PCR (set to 10).
- **Spike Standard Concentration**—expected spike amount per reaction (for example, 10 copies).
- **Reference**—the non-spiked sample; the mean quantity of reference is subtracted during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

  Note: If not correct, ensure **Spike Volume** is set to 10 and **Spike Standard Concentration** is the expected copies spike per PCR reaction.

- (Optional) **Comments**

- **Number of Cells Extracted**—needed to calculate provirus copies per cell in the results.



Note: The software calculates provirus copies per cell if the number of cell extracted was entered.

9. Click the **Assign Targets and Samples** tab.
The default plate layout is shown.

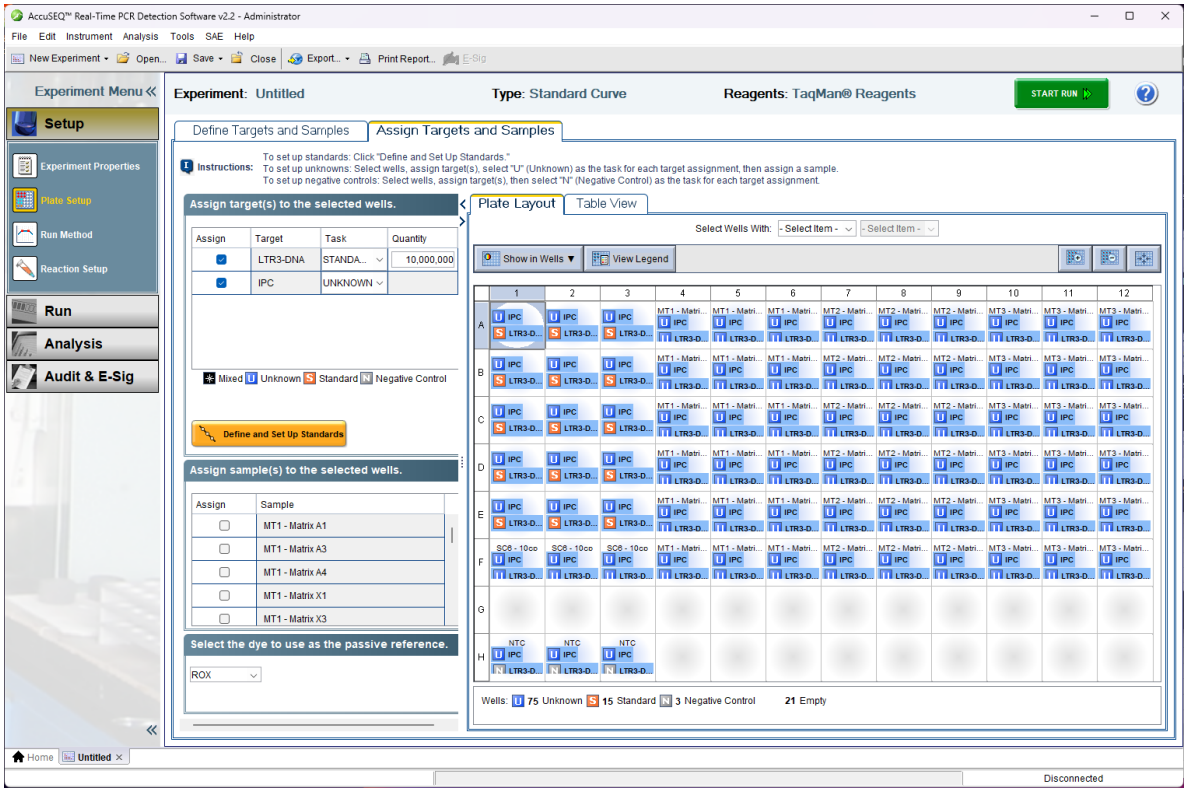


Table 21 Plate well descriptions

Name	Description
SC1 to SC 6	Standard curve dilutions in triplicate
MT1 to MT3	3 samples with 6 matrices
NTC	No template control in triplicate

10. *(Optional)* Edit the plate.

    To change the plate layout display, see *AccuSEQ™ Real-Time PCR Software v2.2 User Guide* (Pub. No. MAN0029201).

    a. Assign the targets to wells in the plate layout.

    b. Assign samples to wells in the plate layout.

11. Confirm that ROX™ dye is selected as the passive reference.

12. *(Optional)* Save the plate layout as an image.

    a. Right-click the plate layout.

    b. Select **Save As**.

    c. Specify a file name and location for the JPEG file, then click **Save**.

    Note: Electronic signature information is not included in an image file. Use Print Report to include Electronic signature information with the plate layout.

13. *(Optional)* Print the plate layout

    a. Right-click the plate layout.

    b. Click **Print Preview** or **Print**.

14. In the **Experiment Menu** navigation bar, click **Run Method**.

15. In the **Run Method** screen, view the default volume and cycling conditions.

    IMPORTANT! Do not modify the run method for factory default SEQ experiments.

16. In the **Experiment Menu** navigation bar, click **Reaction Setup**.

17. In the **Reaction Setup** screen, complete the reaction setup. For more information about the reaction setup, see *AccuSEQ™ Real-Time PCR Software v2.2 User Guide* (Pub. No. MAN0029201).

18. Assemble the PCR reactions following the instructions of the manufacturer for the reagents and following the plate layout.

Analyze the results

After the qPCR run is finished, use the following general procedure to analyze the results:

1. In the toolbar, select **Analysis** ▸ **Analysis Settings**.
2. Click Analyze (**Analyze**).
3. Select **Analysis** ▸ **QC Summary** in the left panel of the screen. Review the flag summary.
4. In the left panel, select **Analysis** ▸ **Standard Curve**. Verify the values for the Slope, Y-Intercept, R2, and Efficiency.
5. Select **File** ▸ **Export**. In the **Export Data** menu, select file type ***.xls**. Click **Start Export**.
6. Calculate titers using the **Proviral Titer Calculation Tool**.

Calculate provirus copies and lentivirus titers from qPCR data

Each provirus has 2 copies of the assay target (LTR).

1. To determine the number of provirus copies in the extracted sample, the copy numbers obtained from the qPCR must be multiplied by 0.5 and then by the dilution factor of the sample during extraction. The concentration of provirus per cell can be calculated based on the number of cells used in the extraction.

   $$\text{Provirus copies per cell} = \frac{\text{qPCR copies x sample dilution factor x } 0.5}{\text{Number of harvested cells used for extraction}}$$

   For help in determining the qPCR copy numbers, see the *QuantStudio™ Design and Analysis Desktop Software User Guide* (Pub. No. MAN0010408).

   For example, if the following parameters were used,

   - 10,000 cells were extracted with the KingFisher™ Flex Purification System with 96 Deep-Well Head and eluted in 200 µL.
   - 5 µL of this eluate (40x dilution) was used for the qPCR reaction.

   then, the calculation would be:

   $$\text{Provirus copies per cell} = \frac{\text{qPCR copies x } 40 \text{ x } 0.5}{10{,}000}$$

   Note: The number of cells extracted can be determined by the counting of cells or nucleic acid quantity equivalence (1 cell = 6.6 pg for diploid cells). Users should establish the nucleic acid quantity equivalence for cells that are non-diploid.

2. To estimate the infectious viral titer (or transduction unit TU/mL), use the volume of lentivirus (mL) that was used to infect the cells seeded for lentivirus transduction and the number of cells seeded for transduction [No. of cells (Day 1)].

$$\text{Infectious viral titer per mL} = \frac{\text{Provirus copies per cell x No. of cells (Day 1)}}{\text{Volume of lentivirus (mL)}}$$

D

Good laboratory practices

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.

Good pipetting practices

- Use calibrated pipettes that are appropriate for volumes recommended by the manufacturer. Use pipette tips compatible with the pipettes.
- Immerse the pipette tip 2-3 mm below the meniscus during aspiration. Avoid air aspiration or pressing the tip on the container/tube bottom.
- Press and release the plunger at a consistent speed and pressure.
- Check the pipette tip before dispensing to ensure that there is appropriate volume drawn and that there is no additional droplet on the outside of the tip.
- When dispensing, press and hold the plunger to the second stop until tip is removed from the liquid. This prevents re-aspiration of the sample. Check the tip after dispensing to ensure that there is no residual liquid left in the tip.

Avoiding false positives due to cross-contamination

To avoid false positives due to cross-contamination:

- Prepare and close all negative control and unknown sample tubes before pipetting the positive control.
- Do not open tubes after amplification.
- Use different sets of pipettors when pipetting negative control, unknown, and positive control samples.

Plate layout suggestions

- For each plate row, dispense in sequence from left to right the: negative controls, unknown samples, and positive controls (at the end of the row or column).
- Place positive controls in one of the outer columns.
- If possible, separate all samples from each other by at least one well; if space is limiting, place at least one well between unknown samples and controls.

E 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, visit **thermofisher.com/support**.

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)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

Documentation and support

Related documentation

Document	Publication number
ViralSEQ™ Lentivirus Physical Titer Kit Quick Reference	MAN0026127
ViralSEQ™ Lentivirus Proviral Titer Kit Quick Reference	MAN0026218
PrepSEQ™ Nucleic Acid Sample Preparation Kit User Guide	MAN0026641
AccuSEQ™ Real-Time PCR Software v3.2 User Guide	MAN0029199
AccuSEQ™ Real-Time PCR Software v2.2 User Guide	MAN0029201
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407
QuantStudio™ Design and Analysis Desktop Software User Guide	MAN0010408
Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Getting Started Guide: Absolute Quantitation using Standard Curve	4347825

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

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

Limited product warranty

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