ViralSEQ™ Lentivirus Physical Titer Kit

Catalog Numbers A52597 and A52598

Pub. No. MAN0026127 Rev. A.0

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *ViralSEQ™ Lentivirus Titer Kits User Guide* (Pub. No. MAN0026126). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Product description

The Applied Biosystems™ ViralSEQ™ Lentivirus Physical Titer Kit is a TaqMan™-based RT-qPCR kit. The kit measures viral count based on highly sensitive viral RNA quantitation from the supernatants of cell-based, bioproduction systems. Viral titers of 10^4 to 10^{11} viral particles (VP) per mL can be quantitated using a standard curve generated from the synthetic RNA control included with the kit. Lentivirus quantitation by RT-qPCR is accurate, sensitive, and reproducible.

The ViralSEQ™ Lentivirus Physical Titer Kit is compatible with the PrepSEQ™ Nucleic Acid Sample Preparation Kit (Cat. A50485), which offers both a manual and automated sample preparation workflow. For real-time PCR, the ViralSEQ™ Lentivirus Physical Titer Kit has 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 and security, audit, and e-signature capabilities to help enable 21 CFR Pt 11 compliance.

For more information about reagent use, see the *ViralSEQ™ Lentivirus Titer Kits User Guide* (Pub. No. MAN0026126).

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℃

   Set reaction volume: 20 µL

Step	Temperature	Time
DNase I inactivation	75℃	10 minutes
Hold	4℃	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 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 1 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.

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 2 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

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 2)

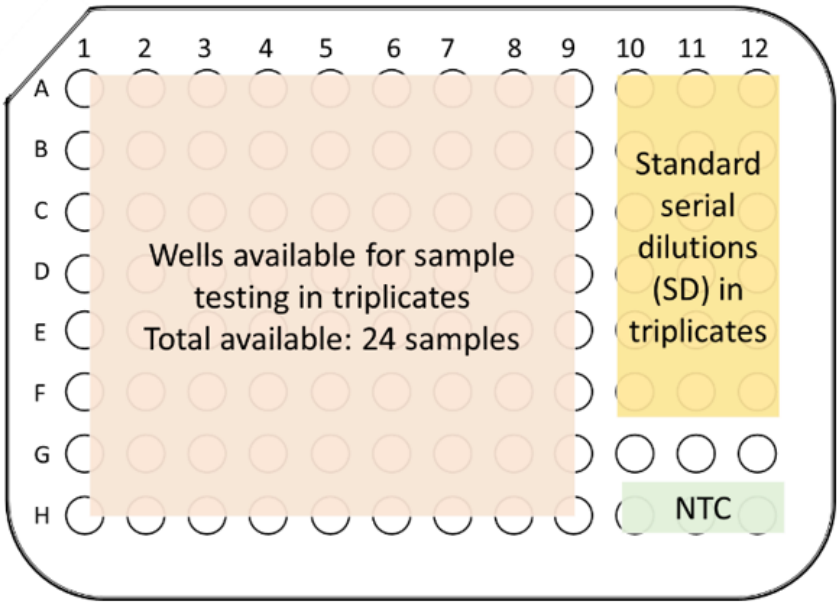


Figure 1 Recommended plate layout

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.

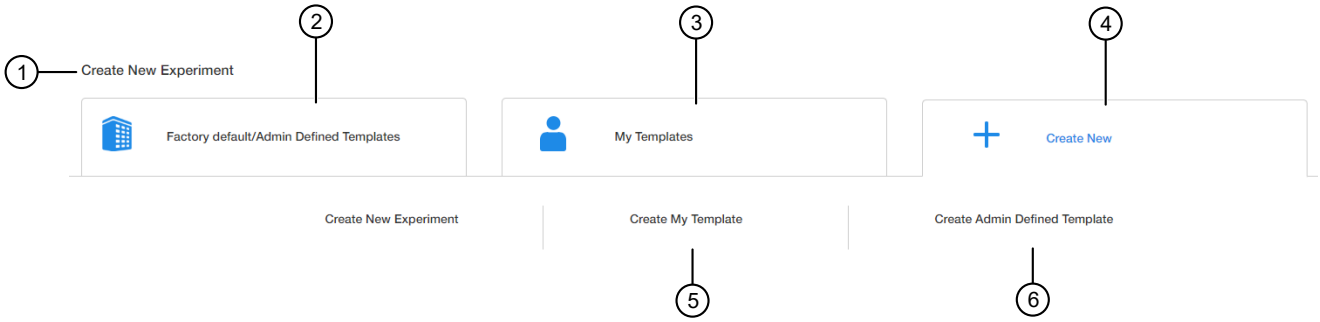
   Note: Ensure there are no bubbles in the reaction wells. If present, tap the well gently to remove bubbles, then re-centrifuge.

Proceed immediately to "Start the run (QuantStudio™ 5 Real-Time PCR Instrument)".

Create a ViralSEQ™ template

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

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**).

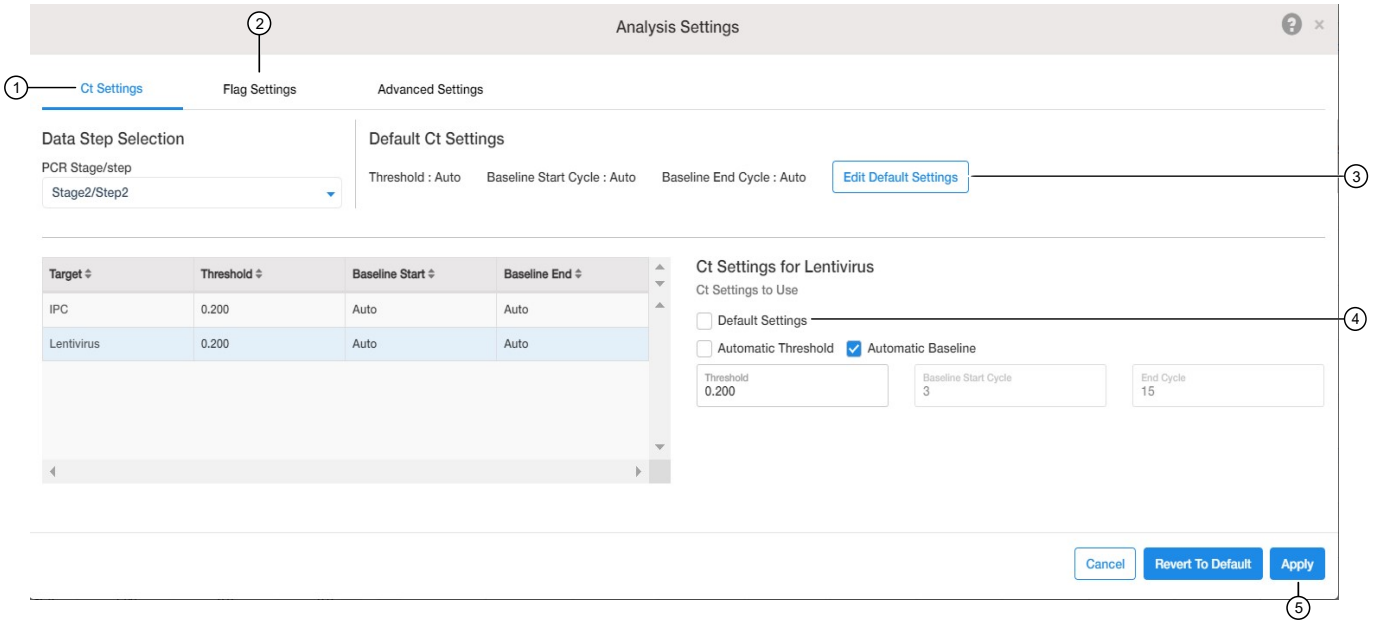
⑥ **Create Admin Defined Template**—Used to create a new template (Administrator only).

2. Select **Create My Template** or **Create Admin Defined Template**.
3. Edit the **Experiment Properties** as required.
   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°C for 30 minutes.
3. Set **Step 2** of the **Hold Stage** to 95°C for 10 minutes.
4. Set **Step 1** of the **PCR Stage** to 95°C for 15 seconds.
5. Edit **Step 2** of the **PCR Stage** to 60°C for 45 seconds.
6. Set the cycle number to **40**.
7. Ensure that **Data Collection** occurs after **Step 2**.

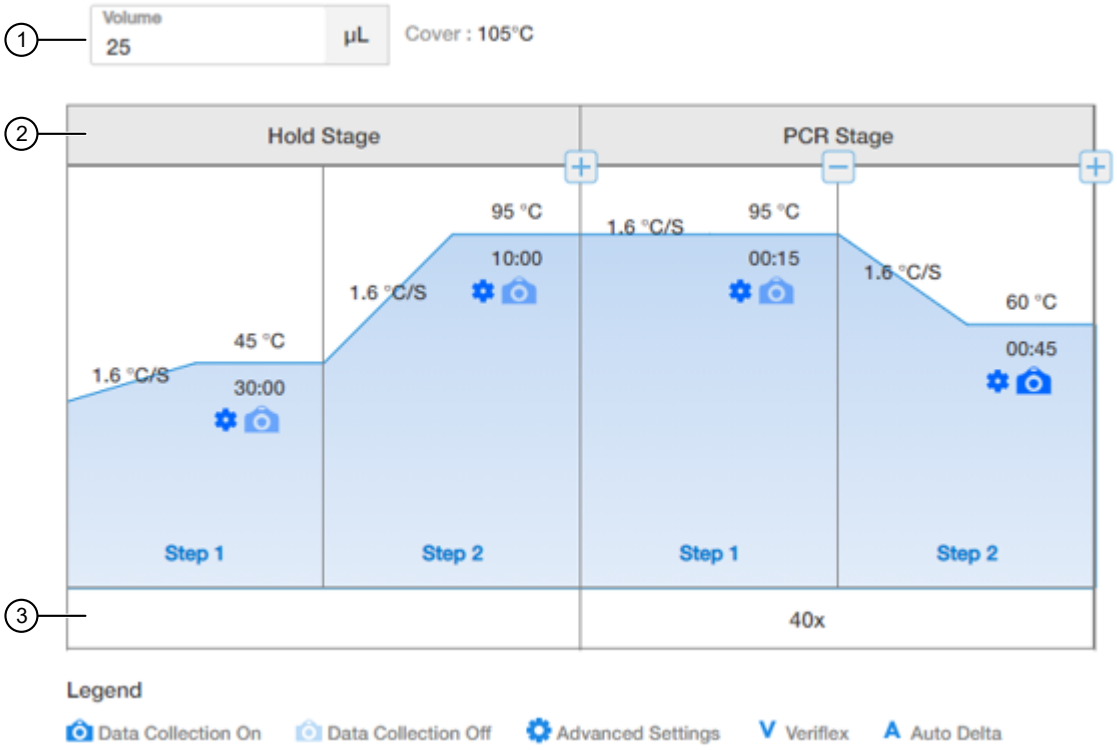


Figure 2 Lentivirus Physical Titer Run Method

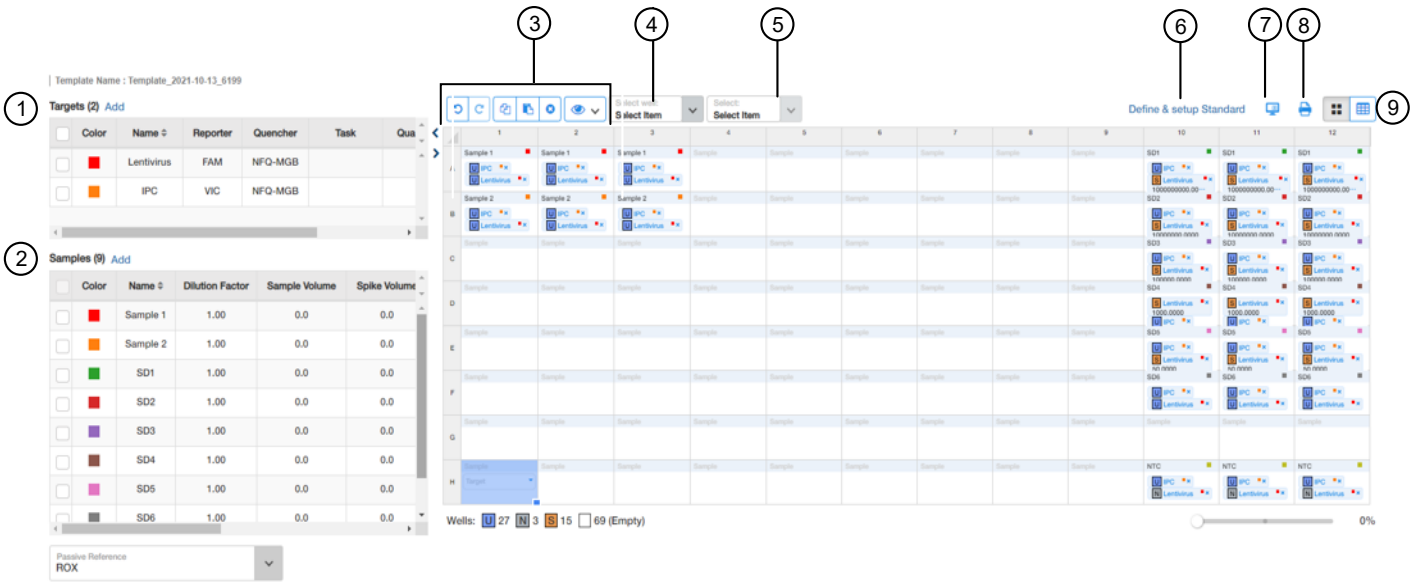
① Reaction volume- set to 25µL
② Stage
③ Cycle number- set to 40 cycles

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.



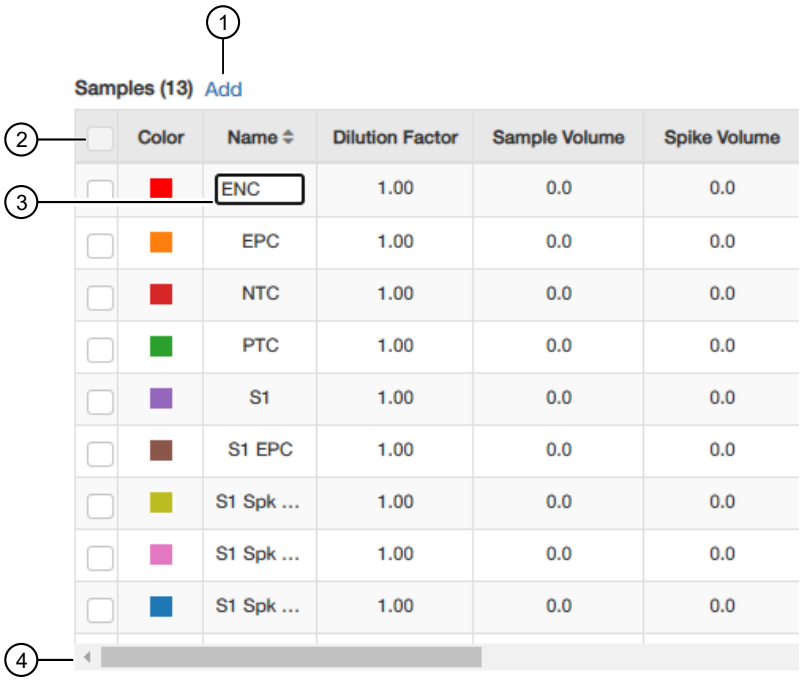
① Targets
② Samples
③ Plate setup toolbar
④ Select Item to highlight (Sample, Target, or Task).
⑤ Select Item. For example, Sample 1. Sample 1 replicates are highlighted.
⑥ Define & setup Standard
⑦ (View Legend)
⑧ (Print Preview)
⑨ View (Grid View or Table View)

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.

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.

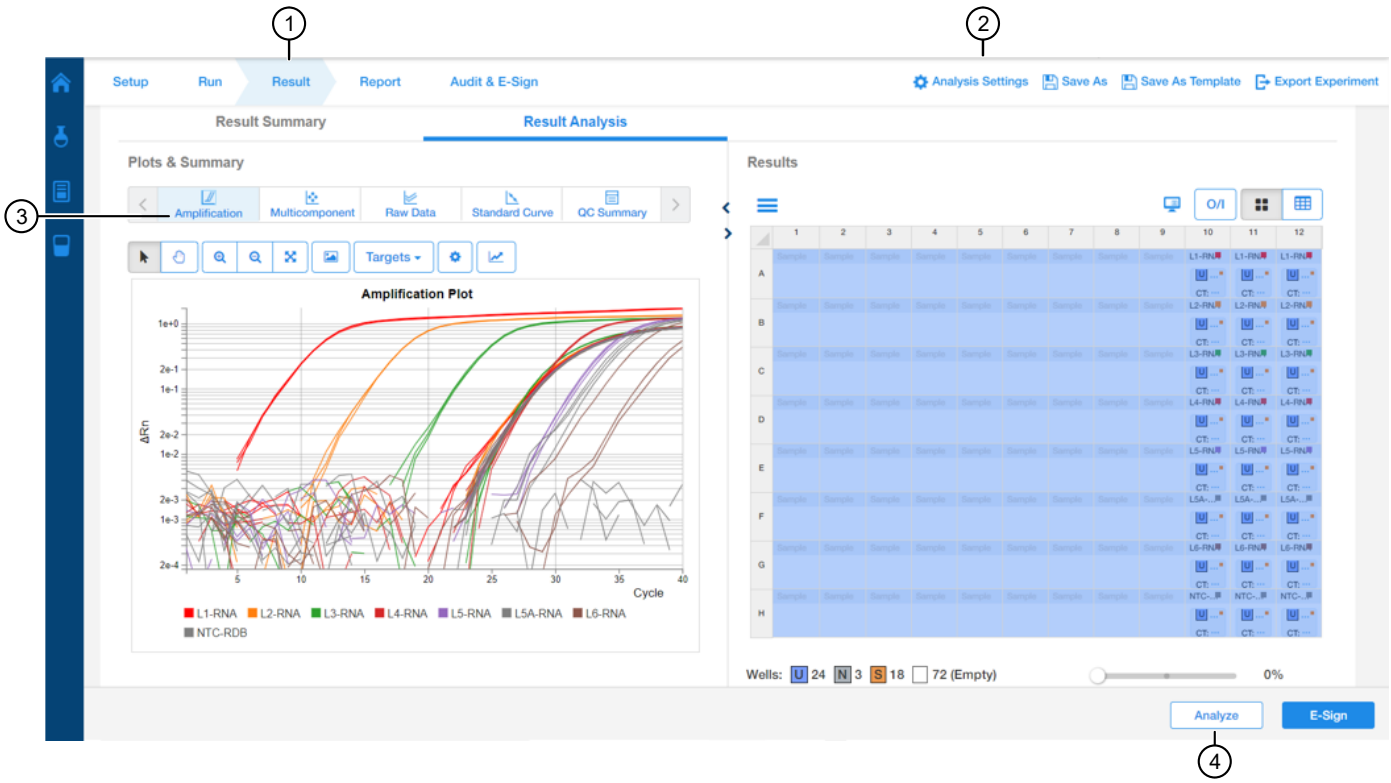
Option	Description
If the experiment is open	Click **Start Run**.
If the experiment is closed	1. Open the experiment. 2. Click the **Run** tab. 3. Click **Start Run**.

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

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 v3.1 User Guide* (Pub. No. 100094287).

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



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

2. In the **Result Analysis** tab, 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.

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.

   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 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 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** tab.
   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)

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.

Calculate lentivirus titers from qPCR data

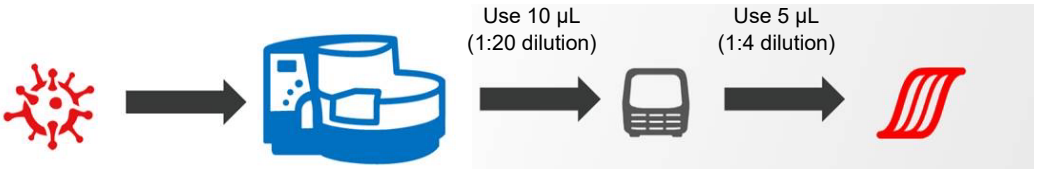
To determine the number of lentivirus RNA copies per mL in the original sample, the copy numbers obtained from the qPCR must be multiplied by the dilution factor of the sample during extraction and DNase I treatment. Since there are 2 copies of RNA/target per lentivirus particle, the number of viral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

Limited product warranty

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

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Revision history: Pub. No. MAN0026127

Revision	Date	Description
A.0	23 August 2022	New document for the ViralSEQ™ Lentivirus Physical Titer Kit (Cat. No. A52597).