ViralSEQ™ Lentivirus Physical Titer Kit

Catalog Numbers A52597 and A52598

Pub. No. MAN0026127 Rev. B.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.

For information about using ViralSEQ™ Lentivirus Physical Titer Kit with AccuSEQ™ Software v2.2 or later, see *ViralSEQ™ Lentivirus Titer Kits User Guide* (Pub. No. MAN0026126).

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°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 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

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 2)

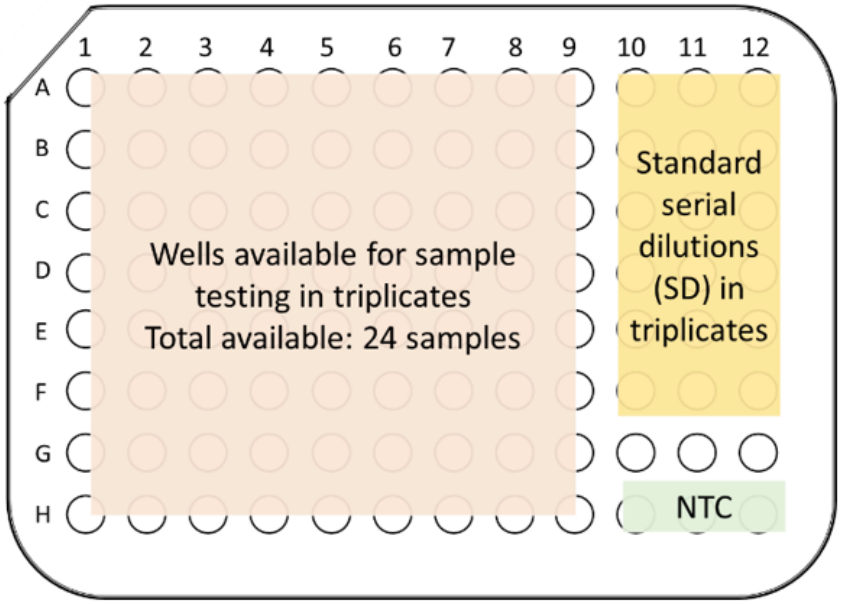


Figure 1 Recommended plate layout

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 recentrifuge.

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

Note: The **ViralSEQ Lentivirus Physical Titer Kit** template is available with AccuSEQ™ Software v3.2 or later.

For information about creating a lentivirus physical titer template using AccuSEQ™ Software v3.1, see *ViralSEQ™ Lentivirus Titer Kits User Guide* (Pub. No. MAN0026126).

1. In the **Home** screen, click the **Factory default/Admin Defined Template** tab, then select a **ViralSEQ Lentivirus Physical Titer Kit** template.
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: Names and comments are not editable post analysis.

   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.

4. Click **Next**.

5. In the **Samples** table in the **Setup** screen, confirm or edit the predefined sample names and control names.
   a. (*Optional*) Click the field in the **Name** column to edit the name of each sample or control.
   b. (*Optional*) Click **Add** to add more samples or controls.

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

Table 3 Example sample names (rename as appropriate)

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. In the **Samples** table, enter the sample volume.

7. In the **Samples** table, scroll to the right, then enter the 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.

8. Define the sample and control wells in the plate layout. For more information, see *AccuSEQ™ Real-Time PCR Software v3.2 User Guide* (Pub. No. MAN0029199).

   Note: The template includes a predefined example plate layout that can be modified.
   a. With **(Grid View)** selected, click or drag to select the plate wells for a particular sample or control.
   b. In the **Targets** table, select the checkboxes for all four targets to add them to the selected well or wells.

      Note: Each assigned well must include all four targets. Do not change the target names, reporter dyes, or quenchers in the table.
   c. Under the **Task** column in the **Targets** table, select the task for each target based on the well type.
      - **U**—Unknown sample
      - **N**—Negative template control
      - **P**—Positive template control
      - **NEC**—Negative extraction control
      - **PEC**—Positive extraction control
      - **S-PEC**—Positive extraction control (in sample)
      - **S-NEC**—Negative extraction control (in sample)
   d. In the **Samples** table, select the checkbox next to a sample or control to add it to the selected well or wells.
   e. Repeat for all wells in the experiment.

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

10. Click **Next** to open the **Run** screen.

11. To save the experiment, exit the experiment, then click **Yes** when prompted to save changes.

    Note: To create a copy of the experiment, click **Save As**.

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

13. Prepare the PCR plate using the plate layout as defined in the experiment, then immediately load the plate in the instrument.

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.

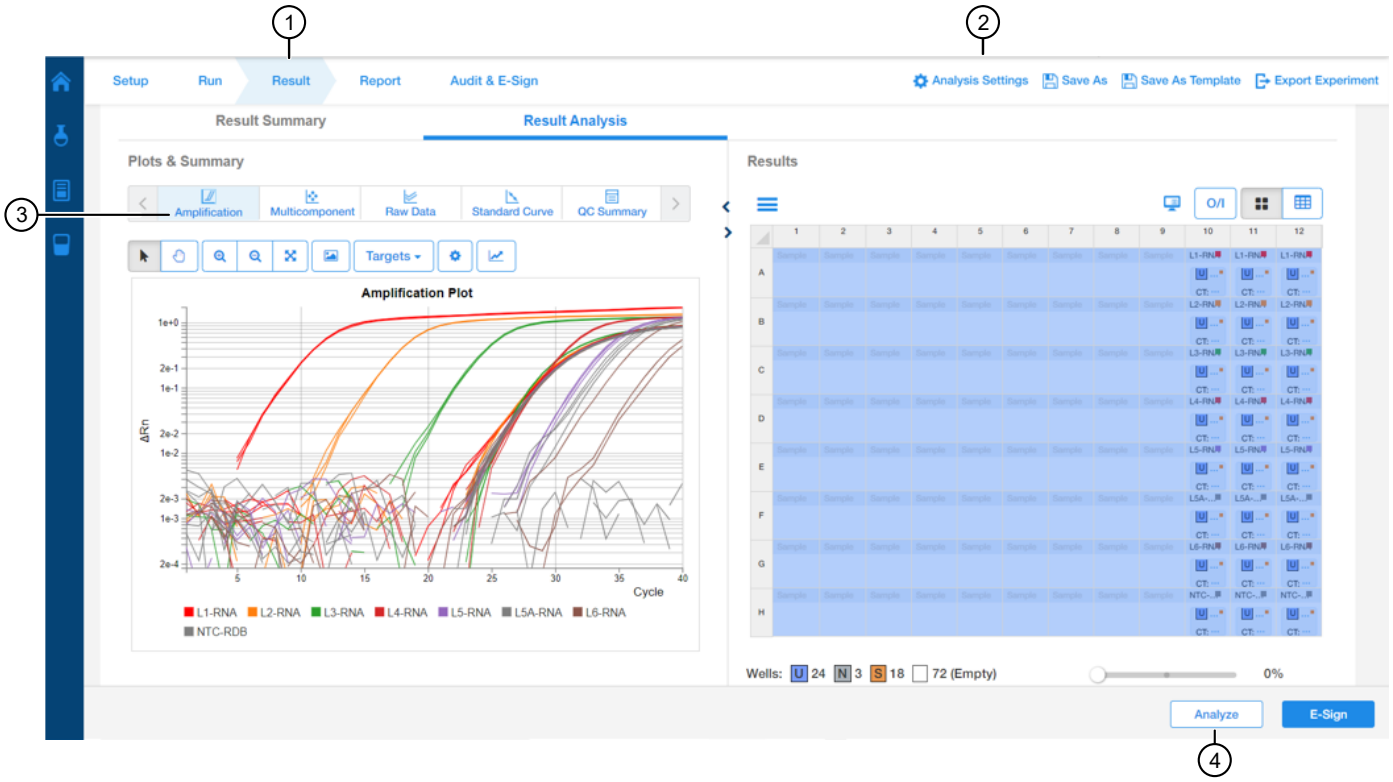
Option	Description
If the experiment is open	Click **Start Run**.
If the experiment is closed	a. Open the experiment. b. Click the **Run** tab. c. Click **Start Run**.

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 v3.2 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.

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 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.

Calculate lentivirus titers from qPCR data

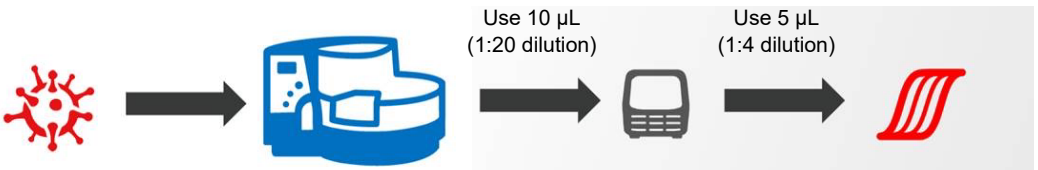
To determine the number of lentivirus RNA copies per mL in the original sample, the copy numbers obtained from the qPCR must be multiplied by the dilution factor of the sample during extraction and DNase I treatment. Since there are 2 copies of RNA/target per lentivirus particle, the number of viral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

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Revision history: Pub. No. MAN0026127 B.0

Revision	Date	Description
B.0	3 January 2024	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.
A.0	23 August 2022	New document for the ViralSEQ™ Lentivirus Physical Titer Kit (Cat. No. A52597).

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

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