Global UltraRapid Lentiviral Titer Kit

Cat. # LV961A-1

(for Titering in Human and Mouse cells)

User Manual

Store kit at -20°C on receipt

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

(ver. 4-051611)

Contents

I. Introduction and Background

A. Purpose of this Manual

This manual describes a real time PCR-based protocol to measure the copy numbers of integrated lentiviral constructs directly from lysates of the cells infected with SBI's lentiviral packaged constructs or libraries. The protocol is based on amplification of a small fragment from the lentivector-specific WPRE (Woodchuck hepatitis virus Post-transcriptional Regulation Element) that is integrated into the genome of transduced cells. The manual does not include information on packaging lentivector constructs into pseudotyped viral particles or transducing your target cells of choice with the particles. This information is available in the user manual *Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells*, which is available on SBI's website (www.systembio.com). Before using the reagents and material supplied with this product, please read the entire user manual.

B. Determining Percentage of Cells Infected with Lentiviral Constructs

Pantropic VSV-G pseudotyped viral particles containing the lentivector expression construct can be used to efficiently deliver and stably express effector and reporter sequences in a wide range of mammalian target cells, but transduction efficiency can vary significantly depending on the transduction conditions and nature of target cells. Therefore, it is a standard procedure to determine the titer of the pseudovirus-containing supernatant in control HT1080 (human) or NIH-3T3 (mouse) cells before proceeding with transduction experiments in your target cells. After transduction of the lentiviral constructs into your target cells of interest, it is also necessary to confirm the transduction efficiency of your experiments. If a lentivector expression construct contains a GFP or RFP reporter, the percentage of infected cells can be easily determined as the percentage of GFP-or RFP-positive cells by fluorescence-activated cell sorting (FACS). However, the procedure requires a FACS machine, and it cannot be used if the vector does not contain a GFP or RFP marker. Additionally, the

percentage of GFP- or RFP-positive cells does not always correlate with the number of infection-competent viral particles present in your viral preparations. This is because multiple viral particles can infect one single cell, especially when infection is conducted at high MOIs.

The relative titer (concentration) of a viral preparation is generally expressed as infection units/ml (IFU/ml) of infection-competent pseudoviral particles. The Global UltraRapid Lentiviral Titer Kit is designed to measure the titers of pseudoviral particles packaged with GeneNet™ siRNA libraries or any SBI lentiviral constructs by amplifying a fragment of WPRE directly from the lysates of infected cells. It can be used to determine the copy number in cells transduced with any lentivector that contains the WPRE element, regardless of the type of selection markers. WPRE in SBI's lentiviral expression vectors and libraries enhances stability and translation of the internal promoter-driven lentiviral transcripts. It is integrated together with the lentiviral expression construct (*e.g.* shRNA or cDNA) into the genomic DNA of transduced cells. Therefore, the copy number of WPRE corresponds to that of the lentiviral expression constructs integrated into cells.

The kit contains calibration standards to measure titer, which can be used to calculate MOI. The calibration standards that are produced from WPRE-containing genomic DNA have been extensively calibrated with cells infected with a copGFP reporter construct at different MOIs. By calculating the amounts of WPRE and the internal UCR1 control amplified from your samples and the calibration standards, you can accurately determine the titer of the virus.

Some key terms used in the protocol:
MOI (multiplicity of infection): The ratio of infectious pseudoviral particles (ifu) to the number of cells being infected. IFU/ # cells = MOI.

IFU/ml (infectious units per ml): The relative concentration of infection-competent pseudoviral particles.

Transduction Efficiency: The average copy number of expression constructs per genome of target cell in the infected population.

C. Product Description and List of Components

The UltraRapid Lentiviral Titer Kit provides sufficient 2X SYBRTaq mix for 100 25-µl PCR reactions, enough for a maximum of 42 individual and singleplex titers. It also contains a cell lysis buffer that allows you to apply the cell lysates directly in the PCR reactions without the need for isolation and concentration measurement of genomic DNA. The set of WPRE PCR primers is universal for any of SBI's HIV or FIV-based lentivectors. The Global Kit also includes the universal UCR1 primers as an internal reference for MOI calculation for both human and mouse lysates. The calibration standards are genomic DNA samples isolated from cells transduced with a broad range of MOI using a copGFP packaged control construct.

Kit Components:

50 µl	**25X Forward and Reverse WPRE Primer Mix**
50 µl	**25X Forward and Reverse UCR1 Primer Mix**
20 µl	**12.5X Calibration Standard 0 - Negative Control**
20 µl	**12.5X Calibration Standard 1**
20 µl	**12.5X Calibration Standard 2**
20 µl	**12.5X Calibration Standard 3**
20 µl	**12.5X Calibration Standard 4**
20 µl	**12.5X Calibration Standard 5**
1.5 ml	**2X SYBRTaq Mix**
5 ml	**Cell Lysis Buffer**

D. Additional Required Materials

For PCR Amplification
Real time PCR System (Recommended: Applied Biosystems 7300 Real time PCR System, Cat# 4351101)

II. Handling the Reagents in the Titering kit

The reagents included in the titering kit are extremely sensitive to changes in temperature. Please note the following storage and handling conditions.

The SYBR Taq mixture should be aliquoted immediately upon receipt, and stored at -20ºC. The SYBR is light-sensitive, so please make sure to keep it in the dark for optimal performance.

The titering kit can be stored at -20 ºC for up to 1 year, however once thawed, the standards and primers should be kept at 4 ºC and should be used within one month.

The Standards, primers, and SYBR Taq are QCed and are lot specific for each lot of titering kit. Please do not substitute other brands of SYBR Taq, or mix and match standards and primers from other SBI kits and products.

III. Protocol

A. Calculate a Standard Curve

Standards should be run in duplicate or triplicate so that an average of the ΔCt can be calculated. The standard curve should be run at the same time as the samples that are being titered. Please review all of the steps in the protocol before proceeding.

1. For each reaction, you will need 9.5 µL of PCR grade water, 12.5 µL of 2X SYBRTaq Mix, and 1 µL of 25X Primer Mix for either UCR1 or WPRE. Prepare **two** PCR master mixtures (**one for UCR1 and the other for WPRE**) enough for all reactions by doubling the volume of each ingredient with 2 plus the number of reactions. Combine the required volumes of PCR Grade Water, 2X SYBRTaq Mix, and the Primer Mix in order.

2. Mix contents by inverting the tubes a few times, and spin the tubes briefly in a microcentrifuge.

3. Aliquot 23 µL of the PCR Master Mix into each test tube or well (if you are using a 96-well plate).

4. Add 2 µL of each of the six control DNA calibration standards or the cell lysates from Step A into the test tubes/ wells from Step 3.

5. Seal the tubes or plate, and place them in the real time PCR system.

6. Commence thermal cycling using the following program:

   50 °C for 2 min
   95 °C for 10 min
   (95 °C for 15 sec; 60 °C for 1 min) for 40 cycles
   Add Dissociation step

7. When the program is complete, check the dissociation curve to make sure there is no significant contamination for WPRE amplification in the negative controls. Then export Ct to an Excel file and calculate the average Ct of UCR1 and WPRE for each standard and sample.
   - Calculate $2^{-\Delta Ct}$, where ΔCt = Average Ct of WPRE – Average Ct of UCR1 of the same standard or sample.
   - Use the Excel software to plot the MOIs* of the standards against the values of $2^{-\Delta Ct}$
   - Use the “add trendline” option of the software to draw the trendline of the standard curve. Set intercept at 0, check the

boxes for Display Equation on chart" and "Display R-squared value on chart".

- Calculate MOI for each of your samples using the equation. For example, if the equation you obtain from your experiment is y = 1.192x, and $2^{-\Delta Ct}$ of one of your samples is 5.1, the MOI of the sample should be 6.08 (i.e. 1.192 multiplied by 5.1).

***IMPORTANT: Please be aware that MOIs for each standard provided may vary from lot to lot. Refer to the tube of each standard for MOIs of the particular lot.**

B. Titering Samples

IMPORTANT: The Global UltraRapid Lentiviral Titer Kit is compatible with both human and mouse cells.

- Plate 50k cells/well in a 24 well-plate. Use 3 wells in duplicate, i.e. 6 wells.
- Add 2 µl of concentrated virus in each of 2 wells.
- Add 0.2 µl (dilute concentrate 1:10 then pipette 2 µl) into each of 2 wells.
- Add 0.02 µl (diluted concentrate 1:100 the pipette 2 µl) into each of the 2 wells.
- Transduce cells as indicated in the TransDux protocol.
- Lyse cells after 72 hours.

C. Lyse the Cells Transduced with Lentiviral Constructs

- Transduce HT1080 (human), NIH-3T3 (mouse) or your target cells of interest in a 24-well plate with packaged lentiviral construct or library using SBI's user manual **"Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells"**. You need to determine the number of cells in a well of the plate upon infection. *For HT1080 cells, the number of cells is around*

75,000 per well if you plate 50,000 cells in each well of a 24-well plate 24 hours before infection.

- Three days after infection, remove the medium, and carefully wash the cells in each well with 1 mL of PBS.
- Remove, as much as possible, all of the PBS from the wells. Add 100 µL of lysis buffer to each well. At this point, you can either store the plate at -80 °C until ready to proceed or quick freeze the plate in dry ice and then thaw the plate at RT.
- Detach the cells in each well by flushing with the lysis buffer and pipetting up and down the cell suspension a few times.
- Transfer as much as possible of the lysed cells into a PCR tube.
- Gently pipette up and down a few times to break down any visible cell clumps.
- Heat the lysate at 95 °C for 2 minutes on a PCR machine.
- Centrifuge the heated lysate at 14,000 RPM for 2 minutes and either put the tubes on ice or store at -20 °C until ready to be used.
- Run the qPCR as described for the Standard Curve calculations.

IMPORTANT: If the cells have been transduced with unpurified pseudoviral stock (directly using viral supernatant from 293 cells), we recommend that after removing the medium containing the transfection reagent, you wash the transduced cells 3 times with fresh media and 1 time with PBS to remove lentiviral plasmid DNA impurities. These may be present in your cells due to residual transfer vector DNA from the 293 cell packaging step.

- The number of viral particles in your viral suspension (IFU/ml) can then be calculated with the following equation: (MOI of the sample) X (The number of cells in the well upon infection) X 1000 / (µl of viral suspension added to the well for infection).

D. Pilot Experiment on Target Cells: What is the Best MOI?

- Plate target cells approximately 50K per well in 6 wells of a 24-well plate.
- Transduce with high MOIs of 0, 1, 2, 5, 10, and 20.
- The MOI with the highest reporter gene expression and healthiest cells is the appropriate amount to use.

UltaRapid qPCR titer setup

	Reaction vol	samples X	<- input # of samples here
	25.0	50.0	
Template	2.0		
MQ Water	9.5	475.0	
2X SYBR mix	12.5	625.0	
Primer Mix (25X)	1.0	50.0	UCR 1 or WPRE primers

to each well add 23.0 **Then add 2 µl of either the standard or cell lysate to the wells as outlined below**

Samples	1	2	3	4	5	6	7	8	9	10	11	12	
A	CS 0 (neg)	CS 1	CS 2	CS 3	CS 4	CS 5	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	UCR 1 Primer
B	CS 0 (neg)	CS 1	CS 2	CS 3	CS 4	CS 5	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	UCR 1 Primer
C	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	UCR 1 Primer
D	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	UCR 1 Primer
E	CS 0 (neg)	CS 1	CS 2	CS 3	CS 4	CS 5	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	WPRE Primer
F	CS 0 (neg)	CS 1	CS 2	CS 3	CS 4	CS 5	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	WPRE Primer
G	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	WPRE Primer
H	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	WPRE Primer

Figure 1. Example qPCR Setup

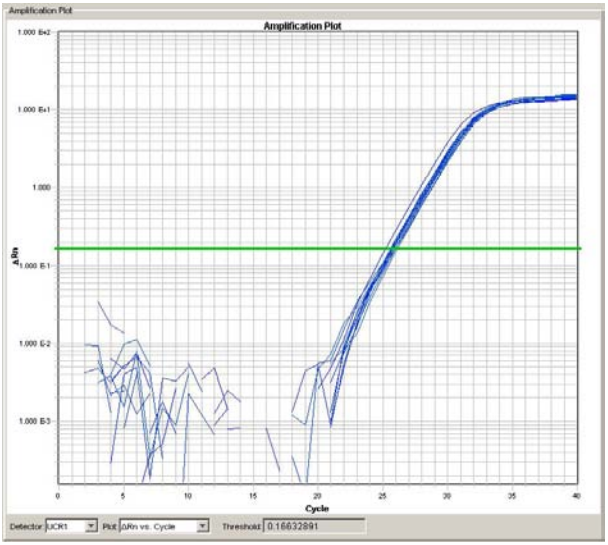

Figure 2. Example UCR Amplification Plot

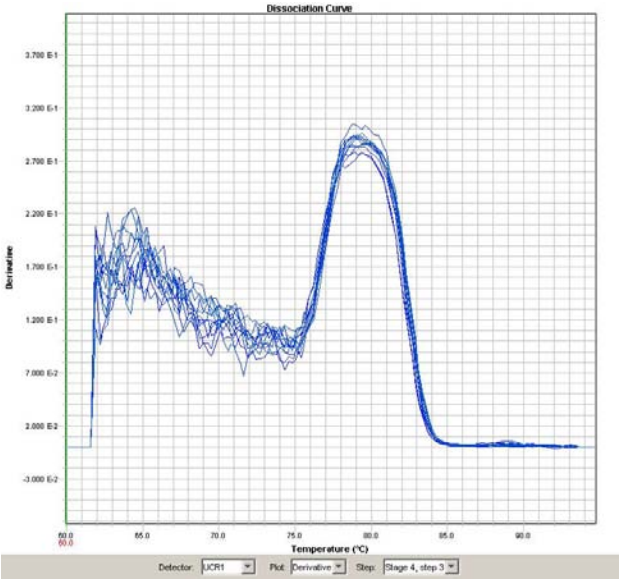

Figure 3. Example UCR Dissociation Curve

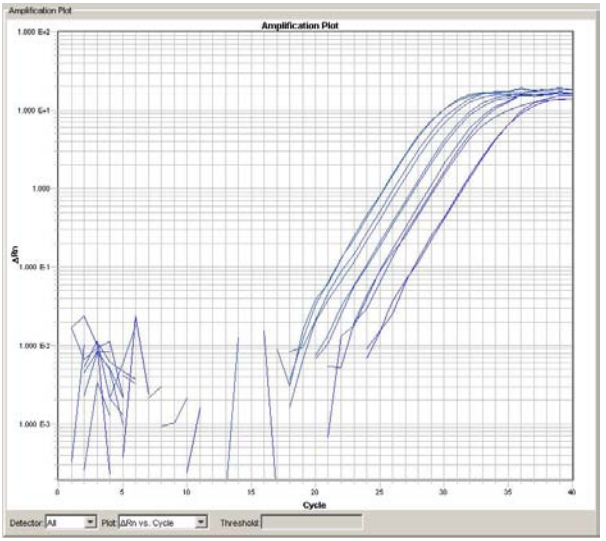

Figure 4. Example WPRE Amplification Plot

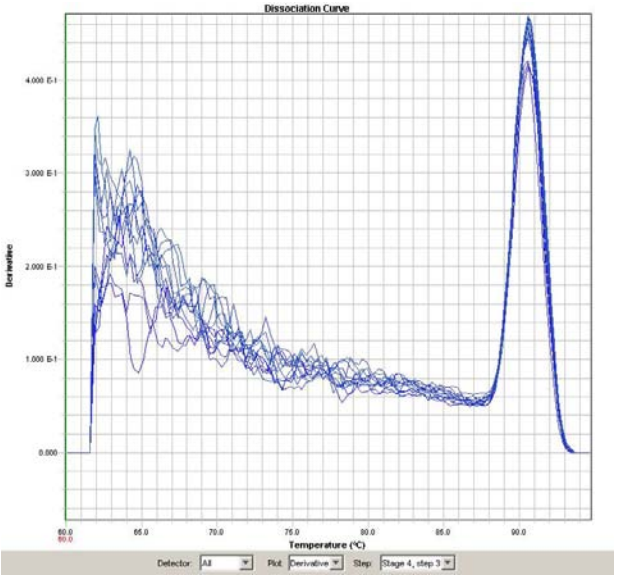

Figure 5. Example WPRE Dissociation Curve

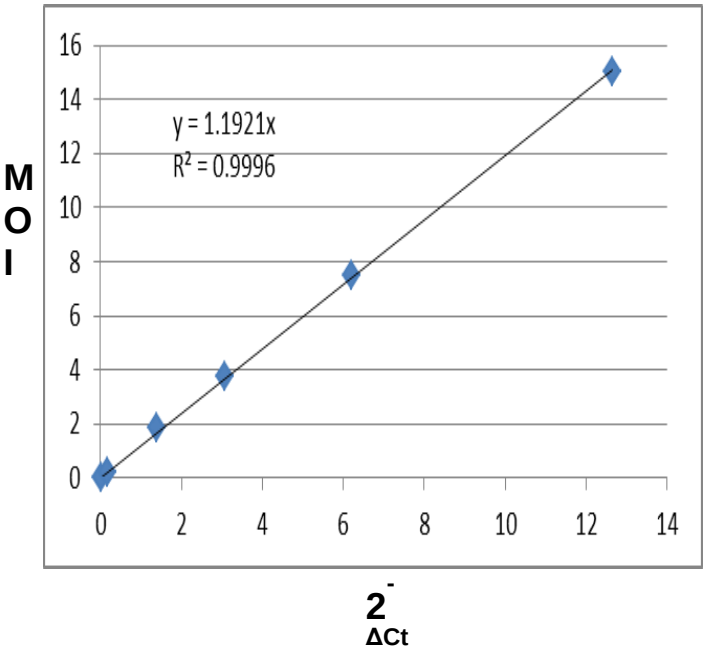

Figure 6. Example Standard Curve

IV. Troubleshooting

A. No PCR product Amplified

No amplification from both calibration standard and sample DNA

Repeat PCR and make sure you have added all the components in the master mix.

No amplification from sample DNA only

The cells are not properly lysed. Make sure the cells are washed carefully with PBS and all residue amount of PBS is removed from the wells.

B. Dissociation Curves of Negative Controls are the Same as Those of Samples.

The negative controls are contaminated with a plasmid or sample containing WPRE in the lab. Make sure you apply all the cautions of PCR set-up to avoid contaminations. Especially, do not touch the inner lid of tubes, always use filtered tips, and avoid generate bubbles during pipetting.

C. MOI

Using an MOI that is too high

This can result in an interferon response in the cells, causing the cells to look ailing.

Using an MOI that is too low

This can result in an insufficient amount of construct to be expressed.

V. Appendix

A. Related Products

Lentivector Packaging Kits

For FIV-based Vectors: pPACKF1™ (Cat. # LV100A-1)

For HIV-based Vectors: pPACKH1™ (Cat. # LV500A-1)

Unique plasmid mixes that produce all the necessary viral proteins and the VSV-G envelope glycoprotein from vesicular stomatitis virus required to make active pseudoviral particles. Producer Cell Line 293TN (SBI Cat. # LV900A-1) transiently transfected with the packaging plasmids and an HIV-based lentiviral construct produce packaged viral particles containing the lentiviral construct of interest.

293TN Human Kidney Producer Cell Line (SBI, Cat. # LV900A-1)

For packaging of plasmid lentivector constructs.

Packaged Positive Transduction Controls

FIV-based: pSIF1-H1-siLuc-copGFP (Cat. # LV201B-1)

HIV-based: pSIH1-copGFP (Cat. # LV600A-1)

Packaged Positive control lentivectors allow you to measure transduction efficiency in target cells based on percent of GFP-positive cells. The H1-siLuc lentivector expresses an siRNA targeting Luciferase.

Transduction Reagent - TransDux^{Tm}(200x) (Cat# LV850A-1)

Transdux^{TM} is an optimized mix of cationic polymers used for efficient transduction of cells. Each tube of Transdux^{TM} provides enough material to transduce 80 wells in a 24 well plate format

PureFection Reagent – (Cat# LV750A-1)

Pure-Fection Transfection Reagent is a new versatile and powerful polymer based gene delivery tool that ensures effective delivery of DNA into mammalian cells with low toxicity.

B. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

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Technical Support: tech@systembio.com

Ordering Information: orders@systembio.com

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Limited Use License

Use of the Lentivector Rapid Titer Kit (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

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