Cat. # 6196

For Research Use

TaKaRa

LVpro™ Provirus qPCR Quantitation Kit

Product Manual

v202511w

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I. Description

The LVpro Provirus qPCR Quantitation Kit uses real-time PCR to measure the vector copy number integrated into the genome when genes are introduced into human cells using lentiviral (LV) vectors. This product can measure the provirus copy number in normal human cells transfected with commonly used lentiviral vectors.

1 Principles of real-time PCR (probe method)

This product detects targets using probe detection (5′-nuclease method). This product contains a probe modified with fluorescent substance (FAM/HLX) on the 5′ end and a quencher substance (BHQ-1) on the 3′ end. During PCR amplification, the probes hybridize specifically to their respective target genes, but their fluorescence is suppressed by the quencher. During the extension reaction, the hybridized probe is degraded by the 5′ → 3′ exonuclease activity of the thermostable DNA polymerase, disconnecting the quencher and allowing fluorescence to be emitted and detected.

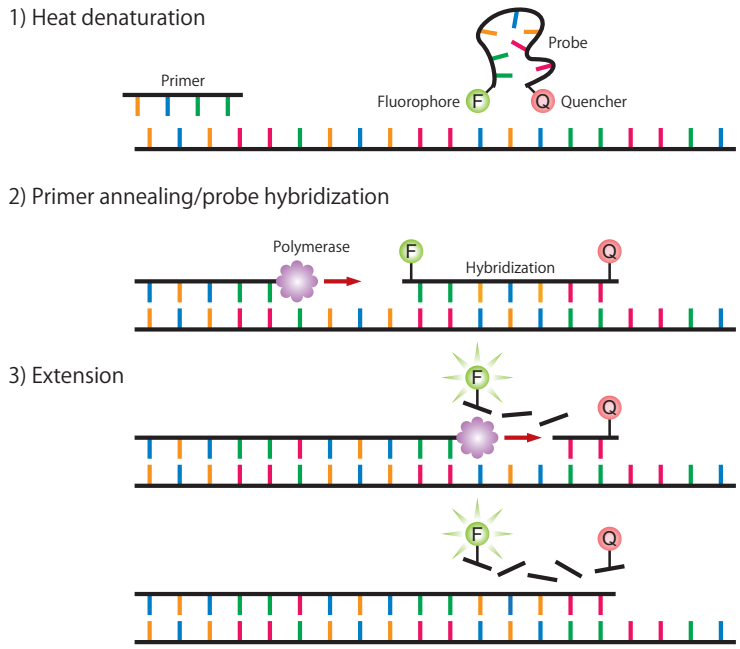
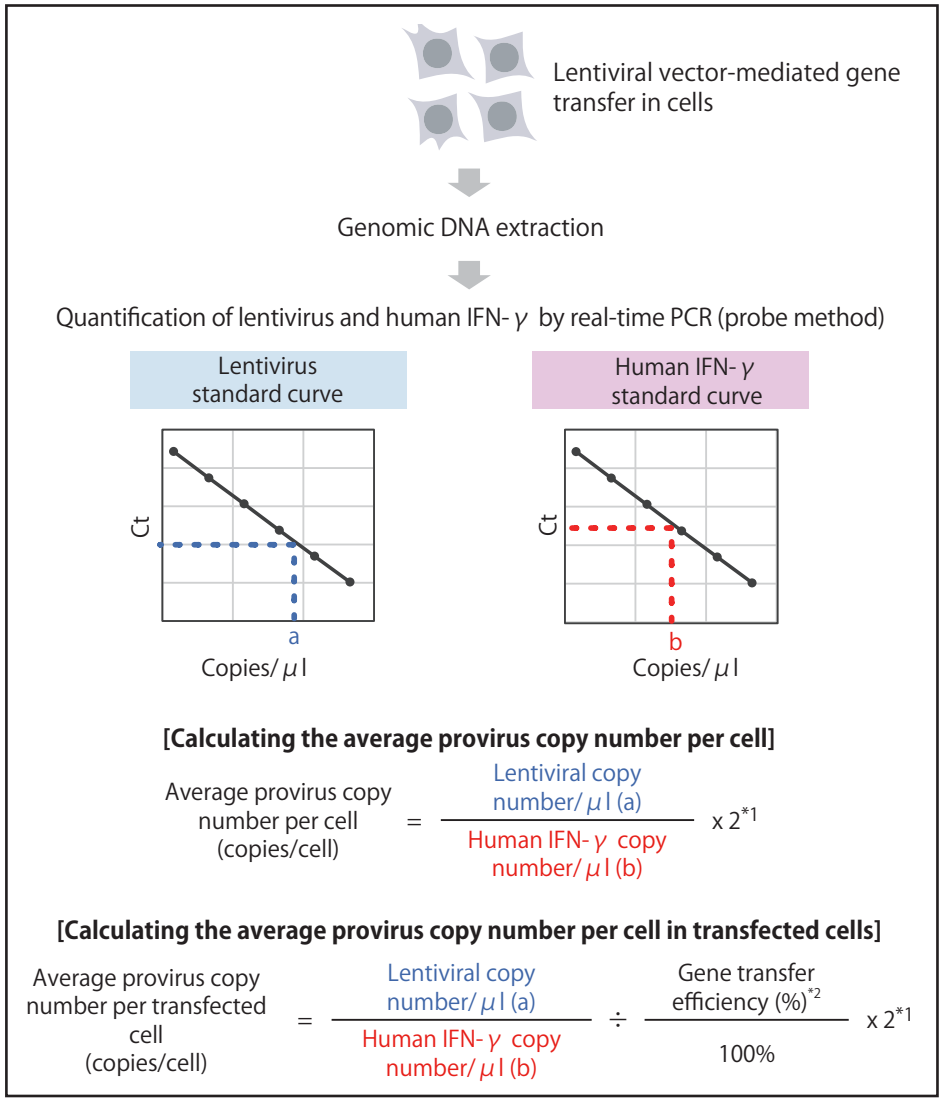


Figure 1. Principle of the probe method

2. Provirus copy number measurement



[Calculating the average provirus copy number per cell]

$$\text{Average provirus copy number per cell (copies/cell)} = \frac{\text{Lentiviral copy number/}\mu\text{l (a)}}{\text{Human IFN-}\gamma\text{ copy number/}\mu\text{l (b)}} \times 2^{*1}$$

[Calculating the average provirus copy number per cell in transfected cells]

$$\text{Average provirus copy number per transfected cell (copies/cell)} = \frac{\text{Lentiviral copy number/}\mu\text{l (a)}}{\text{Human IFN-}\gamma\text{ copy number/}\mu\text{l (b)}} \div \frac{\text{Gene transfer efficiency (\%)}^{*2}}{100\%} \times 2^{*1}$$

Figure 2. Principle of provirus copy number measurement used in this product

Using the Provirus Control Template, which contains one copy each of portions of the lentivirus sequence and the human IFN-γ sequence, standard curves for lentivirus and human IFN-γ are prepared. These curves are then used to quantify the copy numbers (copies/μl) of lentivirus (a) and human IFN-γ (b) in the genomic DNA of the target sample. Since one copy of human IFN-γ exists in the genome, the ratio of lentivirus to human IFN-γ is a/b. Since human autosomes are diploid, the average provirus copy number per cell can be calculated by multiplying the ratio of lentiviral vector copies to human IFN-γ (a/b) by 2*1.

*1 If the host cell is diploid, the number will be "2". If using cell lines, the ploidy may vary, so set the value as needed.
*2 Please measure the gene transfer efficiency separately and calculate based on that value.

II. Components (100 reactions, 25 μl volume per reaction)

○ Probe qPCR Mix, with UNG (2X conc.)*1	625 μl x 2
Lentivirus Primer/Probe Mix for Provirus (10X conc.)	250 μl
hIFNg Primer/Probe Mix for Provirus (10X conc.)	250 μl
H_2O H_2O	1 ml
ROX Reference Dye (50X conc.)*2	200 μl
ROX Reference Dye II (50X conc.)*2	200 μl
Provirus Control Template (4.13 x 10^7 copies/μl)*3	25 μl
EASY Dilution (for Real Time PCR)	1 ml x 2

*1 Includes dNTP mixture, Mg^{2+}, enzymes, etc. required for the reaction. Adding uracil-*N*-glycosylase (UNG) to the reaction system prevents false positives due to carryover of PCR amplification products.

*2 Used when analyzing with a device that compensates for fluorescent signals between wells, such as an Applied Biosystems real-time PCR device.

◆ Models that add ROX Reference Dye
- Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)

◆ Models that add ROX Reference Dye II
- Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)
- QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific)

◆ Models that do not require additives
- Thermal Cycler Dice™ Real Time System IV with PC (Cat. #TP1010)
- Thermal Cycler Dice Real Time System III with PC (Cat. #TP970)
- CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad)
- LightCycler 96 System (Roche Diagnostics) etc.

*3 Provirus control template containing the lentivirus sequence and the amplified region of the human IFN-γ sequence. Be sure to avoid contaminating other reagents.

III. Storage -20℃

IV. Materials Required but not Provided

【Reagents】

- DNA extraction kit
  For extracting genomic DNA from cells, we recommend NucleoSpin Tissue (Cat. #740952.10/.50/.250)*.
- Recombinant DNase I (RNase-free) (Cat. #2270A)
  If lentiviral DNA contamination is suspected, we strongly recommend that you perform DNase I treatment before extracting genomic DNA from cells.
- Dulbecco's phosphate buffered saline (D-PBS) to wash cells.

【Materials】

- 200 μl, 20 μl, and 10 μl micropipettes
- Micropipette tips (with hydrophobic filter)
- Dedicated reaction tube or plate

【Equipment】

- High speed microcentrifuge
- Heat block (used for DNase I treatment and genomic DNA extraction)
- Real-time PCR machine
  - Thermal Cycler Dice Real Time System IV with PC (Cat. #TP1010)*
  - Thermal Cycler Dice Real Time System III with PC (Cat. #TP970)*

    Note: For TP970, a HEX/VIC filter must be purchased separately:
    Filter Unit Premium (HEX/VIC) for LED (Cat. #TP704)*
  - QuantStudio 5 Real-Time PCR System (96-well, 0.2 mL block) (Thermo Fisher Scientific)
  - CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad)
  - LightCycler 96 System (Roche Diagnostics) etc.

* Not available in all geographic locations. Check for availability in your area.

V. Precautions for Use

Read these precautions before use and follow them when using this product.

1. Before using Probe qPCR Mix with UNG, mix by gently inverting the tube, taking care not to create bubbles, to ensure that the reagent is evenly mixed. Sufficient reactivity may not be obtained if the reagent composition is uneven. Do not mix with a vortex mixer. If sediment forms during storage, mix by gently inverting the tube after lightly warming it with your hand or briefly leaving it at room temperature to completely dissolve. Before use, be sure to mix gently until uniform.

2. Immediately place the thawed reagent on ice.

3. When dispensing the reagent, be sure to use a new disposable tip and take every precaution to prevent sample-to-sample contamination.

4. Accurate detection will not be possible if the sample or primer is degraded by contamination with nucleolytic enzymes (i.e., nucleases). Since nuclease contamination from the operator's sweat or saliva can occur, pay close attention to operational precautions such as wearing disposable gloves and masks.

5. The DNA polymerase used in this product is a hot-start PCR enzyme that utilizes an anti-Taq antibody to inhibit polymerase activity. Do not perform the 5 - 15 min activation step at 95℃ that is required with other companies' chemically modified hot-start PCR enzymes. Excessive heat treatment will decrease enzyme activity, which tends to affect amplification efficiency and quantitative accuracy. Even if initial template denaturation is required before PCR, 30 seconds at 95℃ is usually sufficient.

6. With this product, there is no need to analyze the amplified products by electrophoresis or other methods after the reaction is complete. Do not remove the amplification products from the tube, as this could contaminate the laboratory with nucleic acid.

7. Operate the real-time PCR system according to the manufacturer's instructions. If the correction functions of the analysis software are not appropriate, it may cause inaccurate results. If necessary, manually adjust the analysis parameters according to the instruction manual for the real-time PCR system.

VI. Protocol

VI-1. Sample preparation

Genomic DNA is extracted from cells.

* We recommend using NucleoSpin Tissue (Cat. #740952.10/.50/.250) for genomic DNA extraction.
* If lentiviral DNA contamination is suspected, perform DNase I treatment before extracting genomic DNA.

A-1. When treating with DNase I

The recommended cell number is 1 x 10^6 to 5 x 10^6 cells per sample. The following describes how to prepare a sample using Recombinant DNase I (RNase-free) (Cat. #2270A).

1. Prepare the following DNase I mixture.
   After preparation, mix by pipetting and spin down briefly.

   [DNase I Mixture for 1 reaction]

Reagent	Volume
10X DNase I Buffer	2 μl
Recombinant DNase I (RNase-free) (5 U/μl)	2 μl
D-PBS	16 μl
Total	20 μl

2. Aliquot 1 x 10^6 to 5 x 10^6 cells into a 1.5 ml microfuge tube, centrifuge at 1,000*g* for 2 min, and carefully remove the supernatant.
3. Add 1,000 μl of D-PBS, centrifuge at 1,000*g* for 2 min, and carefully remove the supernatant.
4. Add 20 μl of DNase I Mixture to step 3, mix, and incubate at 37℃ for 30 min.
   Note: Reducing the reaction time may result in residual lentiviral DNA.
5. Add 1,000 μl of D-PBS, centrifuge at 1,000*g* for 2 min, and carefully remove the supernatant.
6. Repeat step 5 (washing with D-PBS) again.
7. Remove the supernatant and extract genomic DNA from the cell pellet (proceed to Section B).

A-2. When not treating with DNase I

For cells that are not likely to be contaminated with lentiviral DNA, DNase I treatment can be skipped.

1. Aliquot 1 x 10^6 to 5 x 10^6 cells into a 1.5 ml microfuge tube, centrifuge at 1,000*g* for 2 min, and carefully remove the supernatant.
2. Add 1,000 μl of D-PBS, centrifuge at 1,000*g* for 2 min, and carefully remove the supernatant.
3. Remove the supernatant and extract genomic DNA from the cell pellet (proceed to Section B).

B. Genomic DNA extraction using NucleoSpin Tissue

Extract genomic DNA from cells using a DNA extraction kit such as NucleoSpin Tissue according to the manufacturer's instructions.

When using NucleoSpin Tissue, please note the following:

- After adding Lysis Buffer T1, thoroughly pipette the mixture to break up the cell pellet. If the number of cells is large, the solution may become very viscous, so be careful when handling.
- When Lysis Buffer B3 is added, a white hazy precipitate may temporarily appear. This is a normal reaction; continue pipetting until the haze disappears and the solution becomes homogeneous.
- Even after cell lysis by heat treatment, precipitates such as cell clumps may remain. If this is the case, centrifuge the sample at 11,000*g* for 5 min and transfer the resulting supernatant to a new microfuge tube.
- Store the eluted DNA solution at 4℃, or at -20℃ for long-term storage.

VI-2. Real-time PCR reaction solution preparation and reaction initiation

1. Dilute the sample.

   Use sterile purified water to dilute the extracted sample to a concentration of 10 to 100 ng/μl (20 ng/μl recommended).

2. Heat denature the sample at 95℃ for 5 min, then rapidly cool it at 4℃ for at least 5 min.*1

3. Prepare standard samples for the calibration curve.
   1) Dispense 45 μl of EASY Dilution (for Real Time PCR) into each tube according to the following procedure.

[1]	4.13 x 10^6 copies/μl(Provirus Control Template stock solution 5 μl + EASY Dilution 45 μl)
[2]	4.13 x 10^5 copies/μl(5 μl of 4.13 x 10^6 copies/μl solution of [1] + 45 μl of EASY Dilution)
[3]	4.13 x 10^4 copies/μl(5 μl of 4.13 x 10^5 copies/μl solution of [2] + 45 μl of EASY Dilution)
[4]	4.13 x 10^3 copies/μl(5 μl of 4.13 x 10^4 copies/μl solution of [3] + 45 μl of EASY Dilution)
[5]	4.13 x 10^2 copies/μl(5 μl of 4.13 x 10^3 copies/μl solution of [4] + 45 μl of EASY Dilution)
[6]	4.13 x 10 copies/μl (5 μl of 4.13 x 10^2 copies/μl solution of [5] + 45 μl of EASY Dilution)

   2) Take 5 μl of the Provirus Control Template (4.13 x 10^7 copies/μl) and add it to the tube in [1]. Mix thoroughly by vortexing five times for 1 sec each, then spin down.
   3) Repeat step 2) to create a dilution series.
   4) Heat denature the diluted standard at 95℃ for 5 min, then rapidly cool at 4℃ for at least 5 min.*1

*1 The standard may also be cooled on ice. Be sure to perform this heat denaturation step, as skipping it may negatively impact results.

4. Prepare the real-time PCR reaction mixture.

   Prepare for the required number of reactions plus a few more, and dispense 20 μl into real-time PCR tubes or plates.

【When ROX Reference Dye is not used*2】

[Reaction solution for 1 reaction]

Reagent	Volume
○ Probe qPCR Mix, with UNG	12.5 μl
● Lentivirus Primer/Probe Mix for Provirus (FAM)	2.5 μl
● hIFNg Primer/Probe Mix for Provirus (HEX)	2.5 μl
(H2O) H_2O	2.5 μl
Total	20.0 μl

*2 Applicable models
- Thermal Cycler Dice Real Time System IV with PC (Cat. #TP1010)
- Thermal Cycler Dice Real Time System III with PC (Cat. #TP970)
- CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad)
- LightCycler 96 System (Roche Diagnostics) etc.

【When using ROX Reference Dye*3】

When analyzing with a device that compensates for fluorescent signals between wells, such as an Applied Biosystems real-time PCR device, use ROX Reference Dye.

[Reaction solution for 1 reaction]

Reagent	Volume
○ Probe qPCR Mix, with UNG	12.5 μl
● Lentivirus Primer/Probe Mix for Provirus (FAM)	2.5 μl
● hIFNg Primer/Probe Mix for Provirus (HEX)	2.5 μl
● ROX Reference Dye or ● Dye II	0.5 μl
(H2O) H_2O	2.0 μl
Total	20.0 μl

*3 Applicable models

◆ Models that add ROX Reference Dye
- Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)

◆ Models that add ROX Reference Dye II
- Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)
- QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific)

5. Add the template.

   Add 5 μl of the test sample or standard sample prepared in steps 1 to 3 to the PCR reaction mixture dispensed in step 4 and close the lid tightly. For the negative control, add 5 μl of (H2O) H_2O and close the lid tightly. Then, spin down the reaction tube and set it in a real-time PCR device.

6. Perform the reaction using the following conditions:
   * See the operating instructions provided by the manufacturer for details about using your real-time PCR device.
   * If using Thermal Cycler Dice Real Time System III/IV, QuantStudio 5 Real-Time PCR System, set Run mode/Ramp speed to "Fast."

<Hold>
(25℃ 10 min)*4
95℃ 30 sec

<PCR: 40 cycles>
95℃ 5 sec
60℃ 30 sec (fluorescence detection: FAM/HEX (VIC))

*4 If PCR contamination is suspected, perform the (25℃ for 10 min) step. UNG action degrades the PCR products.

【Detection target and fluorescence detection filter】

Detection target	fluorescence detection filter
Lentivirus	FAM
Human IFN-γ	HEX (VIC)

VI-3. Analysis

After the PCR reaction is complete, refer to the manufacturer's instructions for your real-time PCR instrument to obtain the Ct (or Cq) value. There are two main methods for calculating Ct values: the Crossing Point method (CP method) and the Second Derivative Maximum method (SDM method). The CP method is a method for determining the Ct value from the intersection of the amplification curve and the threshold. The SDM method uses the maximum second derivative (a curve differentiated twice) of the amplification curve as the Ct value. The latter method allows for highly accurate analysis because the Ct value does not fluctuate depending on the threshold setting and is not affected by detection errors in the instrument. For the reasons explained above, we recommend calculating Ct values using the SDM method for this product. However, some devices do not allow you to choose between the Crossing Point method and 2nd Derivative Maximum method.

To calculate the average provirus copy number per cell, perform the following analysis:

1. Create a standard curve using the Ct values measured from the standard samples using the lentivirus detection system and human IFN-γ detection system and the common logarithm ($\log_{10}$) of copies/μl.

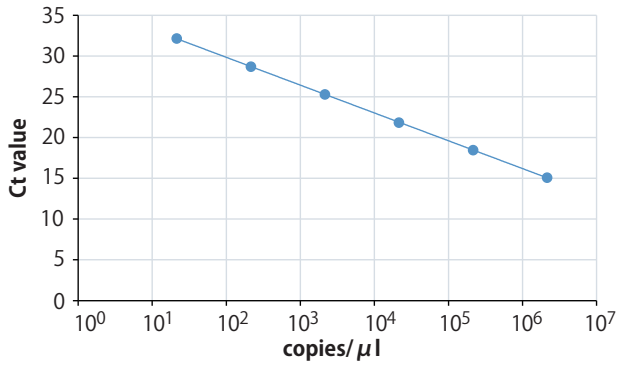


Figure 3. Example of a standard curve using the Provirus Control Template

2. Using the standard curve in step 1, calculate the copies/μl of lentivirus and human IFN-γ from the Ct value of each sample.

3. Calculate the average provirus copy number per cell using the following formula:

【Calculating the average provirus copy number per cell】

$$\text{Average provirus copy number per cell (copies/cell)} = \frac{\text{Lentiviral copy number/}\mu\text{l}}{\text{Human IFN-}\gamma\text{ copy number/}\mu\text{l}} \times 2^{*5}$$

【Calculating the average provirus copy number per cell in transfected cells】

$$\text{Average provirus copy number per transfected cell (copies/cell)} = \frac{\text{Lentiviral copy number/}\mu\text{l}}{\text{Human IFN-}\gamma\text{ copy number/}\mu\text{l}} \div \frac{\text{Gene transfer efficiency (\%)}^{*6}}{100\%} \times 2^{*5}$$

*5 If the host cell is diploid, the number will be "2". If using cell lines, the ploidy may vary, so set the value as needed.
*6 Please measure the gene transfer efficiency separately and calculate based on that value.

VII. Related Products

[Lentiviral vector plasmid]
pLVpro2-Promoterless-Km Vector (Cat. #6977)
pLVpro2-MND-Km Vector (Cat. #6978)
pLVpro2-EF1α-Km Vector (Cat. #6979)
pLVpro2-EFS-Km Vector (Cat. #6980)
pLVpro2-MSCV-Km Vector (Cat. #6981)

[Packaging Mix]
LVpro™ Packaging Mix (Cat. #6195)
LVpro™ Packaging Mix (pLVpro-MSCV Vector) (Cat. #6962)
LVpro™ Packaging Mix (pLVpro-MSCV-EI Vector) (Cat. #6963)
LVpro™ Packaging Mix (pLVpro-EF1α Vector) (Cat. #6964)
LVpro™ Packaging Mix (pLVpro-MSCV-ZsGreen1 Vector) (Cat. #6965)
LVpro™ Packaging Mix (pLVpro-MSCV-EI-ZsGreen1 Vector) (Cat. #6966)
LVpro™ Packaging Mix (pLVpro-EF1α-ZsGreen1 Vector) (Cat. #6967)

[Titer measurement]
Lenti-X™ qRT-PCR Titration Kit (Cat. #631235)
Lenti-X™ p24 Rapid Titer Kit (Cat. #632200)
Lenti-X™ GoStix™ Plus (Cat. #631280/631281)

VIII. Note

- Use of this product requires basic skills in genetic engineering and cell culture.
- This product does not contain lentiviral vectors, and use of this product does not result in the production of lentiviral vectors. However, for your safety and the safety of others around you, it is imperative to fully understand the potential hazards of working with recombinant lentiviruses and the necessary precautions for their use in the laboratory. The National Institutes of Health and Center for Disease Control have designated recombinant lentiviruses as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it.
  For more information on Biosafety Level 2 agents and practices, download the following reference: CDC & NIH. Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition | CDC Laboratory Portal | CDC. U.S. Dep. Heal. Hum. Serv. (2020). at https://www.cdc.gov/labs/bmbl/?CDC_AAref_Val
- Viral supernatants produced by lentiviral vector systems may contain dangerous viruses depending on the insert, so appropriate measures must be taken when producing and handling recombinant viruses. Always use a safety cabinet to prevent inhalation and contact.
- When using this product, follow the ministerial ordinance and the instructions of your organization's safety committee for recombinant DNA experiments, and take sufficient care to ensure safety.
- Please note that we cannot be held responsible for any accidents or damages that may occur as a result of your use of this product.