Cat. # 6195, 6962 - 6967

For Research Use

TaKaRa

LVpro Packaging Mix with pLVpro Series

Product Manual

v202202Da

Table of Contents

I. Introduction

A. Gene transduction and expression using recombinant lentivirus

Recombinant lentiviral vector is a viral vector that can transduce genes into almost all mammalian cells types, including primary culture cells, stem cells, nerve cells, and non-dividing cells. This system is designed to produce safe, high-titer, non-proliferating lentiviral vectors that can transduce a broad range of cell types with high transgene expression.

This product uses an expression system with SIN (Self INactivating)-type pLVpro Vectors (pLVpro-MSCV Vector, pLVpro-MSCV-EI Vector, pLVpro-EF1 α Vector) in combination with LVpro Packaging Mix (Cat. #6195). Simply co-transfect Lenti-X™ 293T cells (Lenti-X 293T Cell Line, Cat. #632180) with pLVpro Lentivirus Vector Plasmid carrying your gene of interest, and LVpro Packaging Mix for easy preparation of a non-replicating recombinant virions (virus particles).

B. LVpro Packaging Mix

LVpro Packaging Mix is designed with an optimal plasmid mixture that expresses the components necessary for lentiviral vector preparation and virus packaging for high-titer recombinant lentivirus production. Co-transfection of Lenti-X 293 cells with pLVpro lentivirus vector and the Packaging Mix induces transient expression of Gag, Pol, Rev, and VSV-G envelope proteins, which facilitates the recombinant viral RNA (transcribed from the pLVpro lentiviral vector) to be incorporated into complete virus particles (Fig. 1). Using this optimized Packaging Mix and Lenti-X 293T cells with highly efficient *Trans*IT-VirusGEN (Mirus Bio, No. MIR6700) or *Trans*IT-293 (Mirus Bio, No. MIR2700) Transfection Reagents, makes it possible to obtain a lentiviral vector with a high viral titer, which, in many cases, can be used to infect the target cells directly, without concentration.

C. pLVpro Lentivirus Vector Plasmid

pLVpro Lentivirus Vector Plasmid is a SIN-type lentiviral vector plasmid that contains various sequences that, together with the HIV-1 LTR (CMV-5'LTR and 3'LTR/ΔU3) and the Lentivirus packaging signal (Ψ), can improve the expression of the transgene, the virus titer, and the overall vector function. We offer 3 types of internal promoters — MSCV-U3, MSCV-EI (containing exons and introns derived from human EF1 α genes downstream of the MSCV-U3 promoter), and EF1 α — for use with different cell-types or transgenes. The pLVpro vector is a Tat-independent 3rd-generation lentiviral vector in which the U3 region of 5'LTR is replaced with the CMV promoter, and the HIV-derived sequences near the packaging signal are eliminated without affecting the infectious titer.

- **WPRE** (woodchuck hepatitis virus post-transcriptional regulatory element):
  WPRE promotes processing and maturation of RNA and increases its transport from the nucleus by preventing readthrough at the polyA site (Zufferey, *et al*., 1999; Higashimoto, *et al*., 2007).
  It acts upon the viral genome transcript within the packaging cell to promote vector packaging and increase viral titer. Since WPRE promotes maturation of the mRNA produced by the vector's internal promoter, it promotes expression of the desired gene within the target cell. The potentially carcinogenic sequences within the WPRE have been modified (WPRE2) in the pLVpro Lentivirus Vector Plasmid.
- **cPPT/CTS** (central polypurine tract-central termination sequence):
  The "DNA flap" formed by cPPT and CTS promotes transport of the viral genome into the nucleus during infection of the target cell. For this reason, the cPPT/CTS element improves recombination of the vector into the genome and the transduction efficiency (Zennou, *et al.*, 2000).
- **RRE** (Rev response element)**:**
  Improves viral titer by promoting transport of unspliced viral genome from the nucleus (Cochrane, *et al.*, 1990).

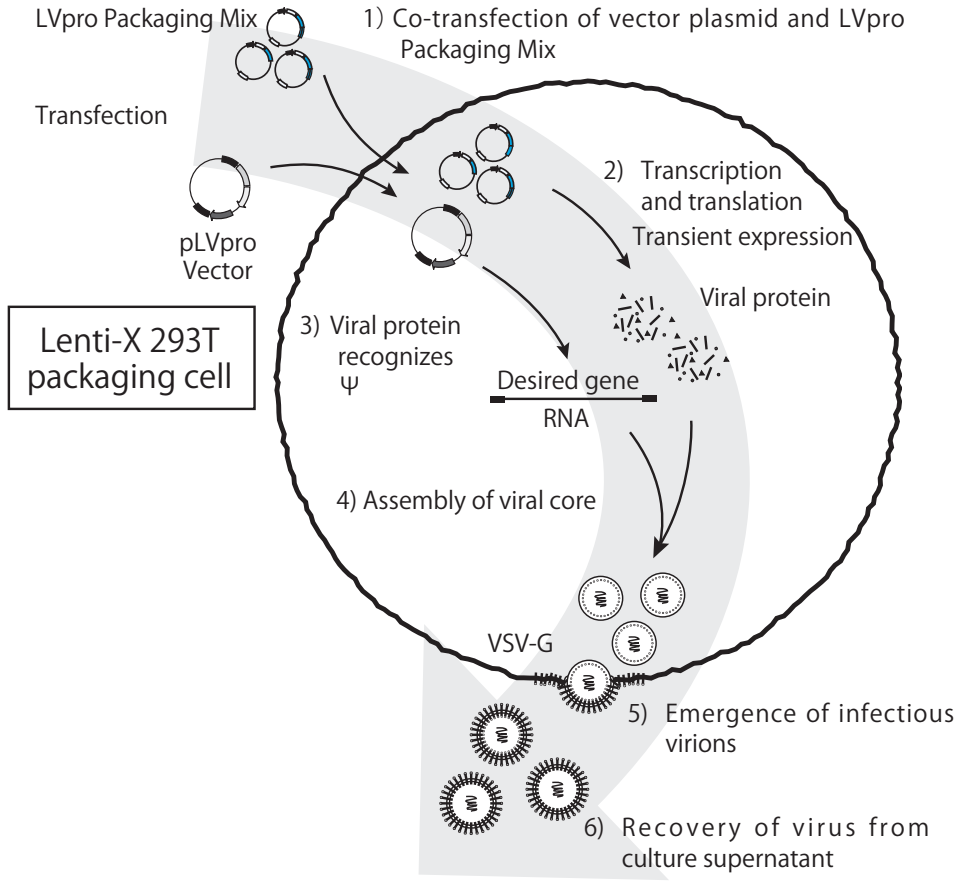


Fig. 1. Production of lentivirus using LVpro Packaging Mix and Lenti-X 293T cells

Co-transfection with the LVpro Packaging Mix and pLVpro Lentivirus Vector Plasmid carrying the desired gene (Step 1), results in the production of corresponding recombinant lentivirus genome RNA transcripts and viral packaging proteins (Step 2). The packaging signal (Ψ) on the recombinant viral RNA genome is recognized by the packaging protein (Step 3), resulting in incorporation of the recombinant viral RNA into the packaging protein, and the formation and transport of the viral core to the cell membrane (Step 4). There, the core is enveloped by the cell membrane that includes the VSV-G envelope protein. Mature infectious virions (viral particles) emerge from the cell (Step 5) and are released into the culture medium. The viral particles are then recovered from the culture medium (Step 6).

Although viral particles produced with this kit possess infectivity, they lack several genes that are necessary for reproduction and multiplication within the target cell. This kit uses a number of different plasmids for viral protein expression, making it hard to produce virus with replication capacity unless low-frequency recombination occurs many times over, thus making the kit extremely safe.

D. Biosafety

The protocols in this User Manual require the production, handling, and storage of infectious lentivirus. It is imperative to fully understand the potential hazards of, and take necessary precautions for the laboratory use of lentiviruses.

Although biosafety regulations and practices vary from country to country, the National Institute 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.

The pseudotyped lentiviruses packaged from the HIV-1-based vectors described here are capable of infecting human cells. The viral supernatants produced by these lentiviral systems could, depending on your insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*.

For more information on Biosafety Level 2 agents and practices, download the following reference: Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition (February 2007) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH. If possible, observe and learn the practices described below from someone who has experience working with lentiviruses.

Summary of Biosafety Level 2: Practices: — Standard microbiological practices — Limited access to work area — Biohazard warning signs posted — Minimize production of aerosols — Decontaminate potentially infectious wastes before disposal — Use precautions with sharps (e.g., syringes, blades) — Biosafety manual defining any needed waste decontamination or medical surveillance policies.

Note： Please be advised that our company cannot be held responsible for any accidents or damage related to the handling and use of this product.

II. Components

LVpro Packaging Mix (Cat. #6195)

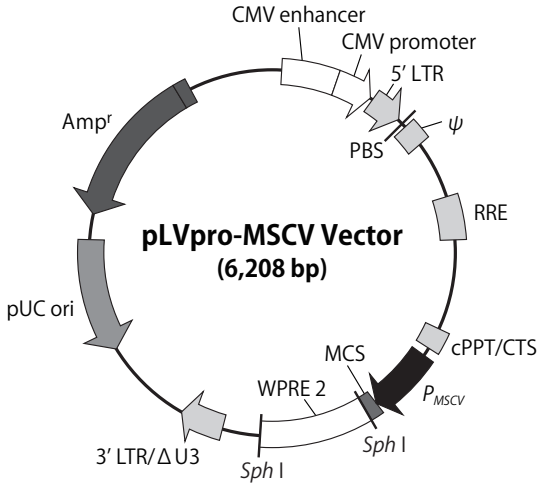
LVpro Packaging Mix, 60 doses*(140 μl × 3)

* When 100-mm dish is used.

LVpro Packaging Mix (pLVpro-MSCV Vector) (Cat. #6962)
LVpro Packaging Mix (pLVpro-MSCV-EI Vector) (Cat. #6963)
LVpro Packaging Mix (pLVpro-EF1 α Vector) (Cat. #6964)
LVpro Packaging Mix (pLVpro-MSCV-ZsGreen1 Vector) (Cat. #6965)
LVpro Packaging Mix (pLVpro-MSCV-EF1-ZsGreen1 Vector) (Cat. #6966)
LVpro Packaging Mix (pLVpro-EF1 α -ZsGreen1 Vector) (Cat. #6967)

Cat. Nos. 6962 to 6967 are sets of LVpro Packaging Mix (Cat. #6195) and each pLVpro Vector (Cat. #6956–6961; not sold separately).
pLVpro Vectors (Cat. #6956–6961; 0.5 μg/μl, 40 μl)

< Vector maps >



Note: Included in Cat. #6962.

MCS :

*Sse*8387 I *Not* I *Xho* I *Cla* I *Sma* I
*Bam*H I *Hpa* I *Mlu* I *Apa* I *Smi* I

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GGATCCTGCAGGTTAACGCGGCCGCACGCGTCTCGAGGGCCCATCGATTTAAATCTAGACCCGGG
CCTAGGACGTCCAATTGCGCCGGCGTGCGCAGAGCTCCCGGGTAGCTAAATTTAGATCTGGGCCC
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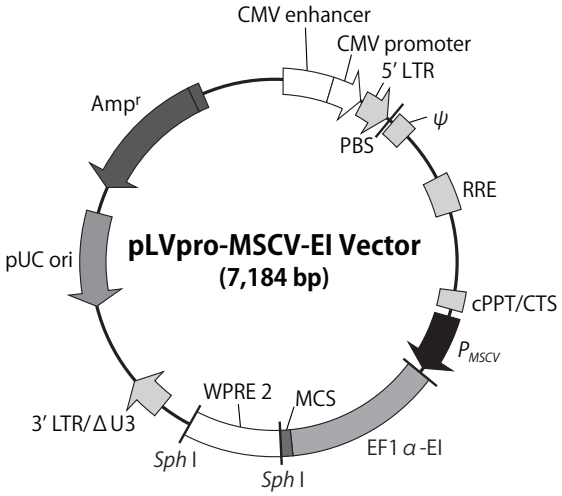

Note: Included in Cat. #6963.

MCS :

Hpa I *Mlu* I *Smi* I
*Sse*8387 I *Not* I *Cla* I *Sma* I

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GGACGTCCAATTGCGCCGGCGTGCGCAGAGCTCCCGGGTAGCTAAATTTAGATCTGGGCCC
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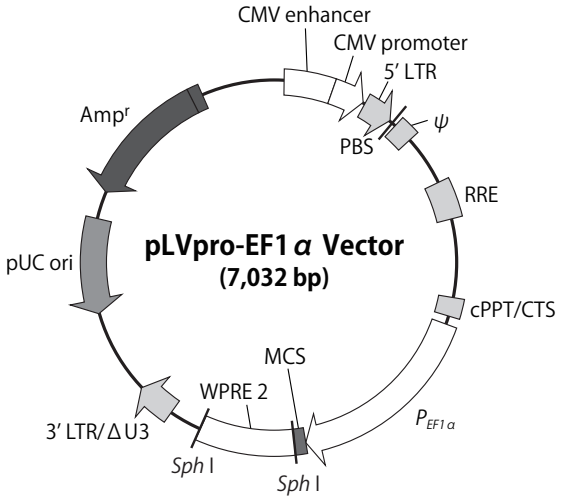

Note: Included in Cat. #6964.

MCS :

*Sse*8387 I, *Not* I, *Cla* I, *Xba* I
*Bam*H I, *Hpa* I, *Mlu* I, *Smi* I, *Sma* I

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GGATCCTGCAGGTTAACGCGGCCGCACGCGTCTCGAGGGCCCATCGATTTAAATCTAGACCCGGG
CCTAGGACGTCCAATTGCGCCGGCGTGCGCAGAGCTCCCGGGTAGCTAAATTTAGATCTGGGCCC
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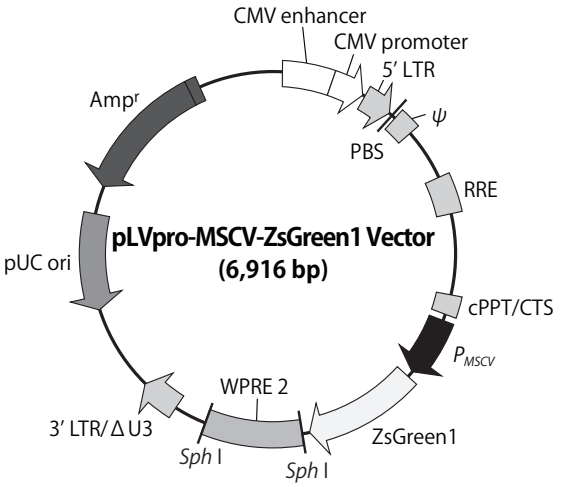
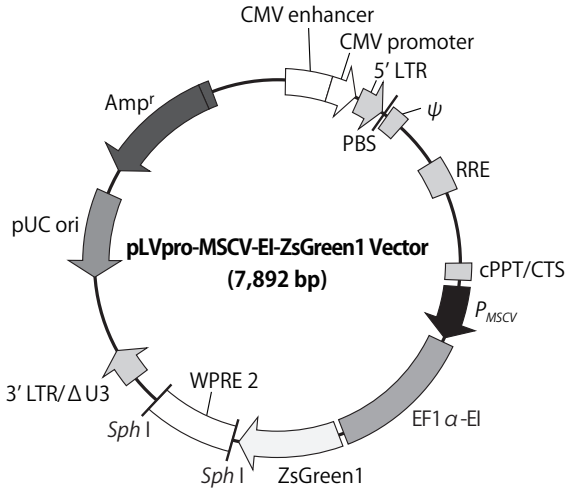


Note: Included in Cat. #6965.



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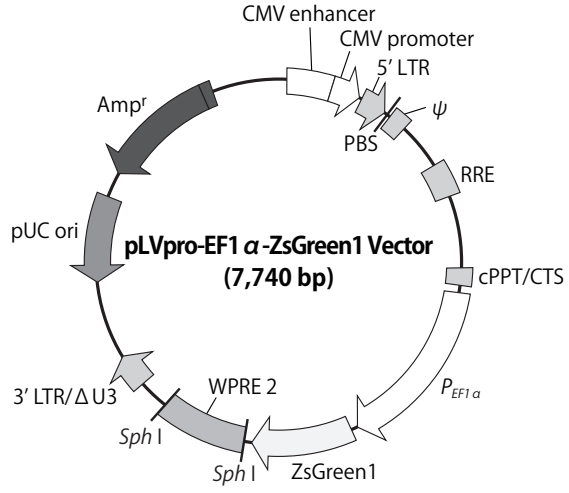

Note: Included in Cat. #6967.

III. Storage -20℃

IV. Additional Materials Required, not Provided

A. Cell lines for lentivirus packaging and titration

- **Lenti-X 293T Cell Line (Cat. #632180)**
  This is a HEK 293T–derived cell line optimized for virus production with a high transfection efficiency. To obtain a high-titer infectious lentivirus, you can use LVpro Packaging Mix and pLVpro Vector Plasmid carrying the desired gene, in combination with *Trans*IT-VirusGEN or *Trans*IT-293 Transfection Reagents, to co-transfect Lenti-X 293T cells. The transfected cells will transiently produce a high-titer recombinant lentivirus. You can also use HEK 293T cell lines, such as HEK 293T/17 from American Type Culture Collection (ATCC No. CRL-11268), instead of these cells. A number of lineages of the HEK 293T strain are commercially available, but we recommend using one with a high transfection efficiency.

- **HT-1080 cell line**
  American Type Culture Collection HT-1080 (ATCC No. CCL-121) (recommended). With this cell line, transduction can easily be performed using recombinant lentiviruses, and it is often used in the measurement of lentivirus titer.

B. Reagents

- One of the following transfection reagents[*1]
  a. *Trans*IT-VirusGEN Transfection Reagent (Mirus Bio, No. MIR6700)
  b. *Trans*IT-293 Transfection Reagent (Mirus Bio, No. MIR2704)
  c. CalPhos™ Mammalian Transfection Kit (Cat. #631312)
- Vectors as positive control[*2]
  d. pLVpro-MSCV-ZsGreen1 Vector (Cat. #6965)
  e. pLVpro-MSCV-EI-ZsGreen1 Vector (Cat. #6966)
  f. pLVpro-EF1 α -ZsGreen1 Vector (Cat. #6967)

*1 a and b are suitable for stably obtaining high-titer lentiviruses. To obtain high-titer lentiviruses with this product, we strongly recommend using *Trans*IT-VirusGEN or *Trans*IT-293 Transfection Reagent.

*2 d, e, and f are lentivirus vector plasmids that express fluorescent protein ZsGreen1, and are convenient for use as positive controls to check transfection efficiency and the biological titer of lentivirus. Depending on the type of cell to be infected, select the promoter.

C. Media for culturing mammalian cells

- Lenti-X 293T Cell Line and HT-1080 cell line culture medium
  Use Dulbecco's Modified Eagle's Medium (DMEM) containing high-concentration glucose (4.5 g/L) with 10% FBS
- Culture medium and additives for target cells
- Penicillin/Streptomycin solution (10,000 units/ml Penicillin G sodium salt, 10,000 μg/ml Streptomycin sulfate)
- Trypsin-EDTA
- Dulbecco's phosphate buffered saline (DPBS)
- Cell Cryopreservation solution

D. Culture equipment

- Cell culture plates (100 mm dish, 12-well plates, or flasks, etc.)
- Sterile culture tubes (1.5 ml, 2.0 ml, 15 ml, etc.) and vials for virus cryopreservation
- 0.45 μm filter for filtration of virus suspension

Note：When performing filtration, use a filter with low protein binding, such as a PolyVinylidene DiFluoride (PVDF), cellulose acetate, or polyethersulfone (PES) filter. Do not use a cellulose nitrate filter, as it binds to the surface proteins on the lentivirus envelope and destroys the virus.

- Other equipment and facilities necessary for cell culture

E. Lentivirus titration

We strongly recommend measuring the lentiviral titer to ensure accurate, reproducible gene transduction.

Lenti-X qRT-PCR Titration Kit (Cat. #631235) uses qRT-PCR to perform measurement quickly and easily (Quinn, *et al*., 1997). This kit allows the measurement of viral titer in about 4 hours by using real-time PCR using the intercalator method. The Lenti-X p24 Rapid Titer Kit (Cat. #632200) uses ELISA to measure the amount of p24 capsid protein in the viral supernatant. The measured amount of p24 capsid protein correlates with the viral titer.

Measuring viral titer by the following methods helps determine the right time to collect viral supernatant. Lenti-X GoStix™ Plus (Cat. #631280/631281) allows rapid determination of the viral titer in 10 minutes, using 20 μl of culture supernatant, by detecting lentivirus p24.

F. Lentivirus purification

We recommend purifying the lentivirus to remove cellular contaminants that could inhibit gene transduction. Lenti-X Maxi Purification Kit (Cat. #631233/631234) uses an efficient gravity-flow column protocol for obtaining high-purity, intact lentiviruses from crude supernatants.

G. Lentivirus concentration

Lenti-X Concentrator (Cat. #631231/631232) allows you to concentrate viral titer100-fold without ultracentrifugation. Using concentrated lentiviruses makes it possible to infect target cells with high multiplicity of infection (MOI) (for details, see "Appendix：Supplemental Protocol").

H. Polybrene for enhancing gene transduction by lentiviruses

Adding polybrene (hexadimethrine bromide; Sigma-Aldrich, No. H9268) is an effective way to promote gene transduction by recombinant lentiviruses. Polybrene is a polycation that reduces charge repulsion between the virus and cell membrane. The optimal concentration of polybrene for the target cells (concentration at which infectivity becomes maximal and toxicity becomes minimal) needs to be experimentally determined within the concentration range of 2 to 12 μg/ml. When working with cells that could be adversely affected by polybrene or with hematopoietic cells, consider using the RetroNectin® reagent instead.

I. RetroNectin for enhancing gene transduction by lentiviruses

RetroNectin (Recombinant Human Fibronectin Fragment) (Cat. #T100A/B) is a recombinant fibronectin fragment (CH-296) that can greatly improve the efficiency of gene transduction by retroviruses and lentiviruses. By coating the tissue culture plate (non-treated plate), RetroNectin provides a substrate to which both virus and cell can bind. Simultaneous binding of virus and cell to this substrate promotes gene transduction by increasing contact between cell and virus. RetroNectin is particularly useful when you are working with non-adherent cells (lymphocytes, lymphocyte cell strains, etc.), cells resistant to gene transduction (hematopoietic stem cells, etc.), or cells that are particularly sensitive to polybrene.

V. Construction of pLVpro lentivirus vector plasmid carrying the desired gene

1. To amplify the plasmid DNA, transform into an *Escherichia coli* host strain, e.g., *E. coli* HST08 Premium Electro-Cells (Cat. #9028)*, and use a commercially available plasmid purification kit to purify it.

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

2. Using standard cloning technology, insert the desired gene into the multi-cloning site (MCS) of the pLVpro Lentivirus Vector Plasmid. You can also use In-Fusion® Snap Assembly Master Mix (Cat. #638943, etc.), which allows easy cloning of PCR products to any linear vector.
   Sequence of the pLVpro Lentivirus Vector Plasmid can be downloaded from the Takara Bio web catalog.

   Note： The desired gene (cDNA or gene segment) is inserted into the sequence including the ATG start codon and a stop codon. The expression level can be improved by adding a Kozak consensus ribosome binding site (Kozak, 1987). A poly-A signal is unnecessary for gene segments or cDNA. The insertion of poly-A signal between virus LTRs causes polyadenylation to occur during viral genome transcription, and may impede production of functional recombinant virions (Cofin, *et al*., 1997).

3. Prepare plasmid DNA suitable for transfection of the packaging cells.

   Reference: We recommend using NucleoBond Xtra Midi Plus/Maxi Plus (Cat. #740412.10, 740416.10, etc.) and NucleoBond Xtra Midi/Maxi (Cat. #740410.10, 740414.10, etc.). After using the NucleoBond kit, the plasmid DNA solution is further centrifuged at 14,000*g* for 10 minutes and the supernatant is collected to obtain high-purity plasmid DNA suitable for transfection.

VI. Production of recombinant lentivirus from pLVpro Lentivirus Vector Plasmid

Protocol: Production of recombinant lentivirus using Lenti-X 293T cells and LVpro Packaging Mix

- To obtain high-titer lentiviruses with LVpro Packaging Mix, use Lenti-X 293T cells and strictly follow the protocol described below. In particular, follow requirements for (1) culture size and volume, (2) DNA amount and quality suitable for transfection, and (3) incubation time.
- The following protocol has been optimized with pLVpro Lentivirus Vector Plasmid and LVpro Packaging Mix for gene transduction into Lenti-X 293T cells and production of recombinant lentiviruses.
- Perform all steps in a safety cabinet. In order to handle lentiviruses, you will need a biosafety-level facility approved for use with lentiviruses. Recombinant lentiviruses packaged with HIV-1–derived vector have infectivity toward human cells. Please take proper safety measures.

VI-1. Lenti-X 293T cell culturing

Add Lenti-X 293T cells (5.0×10^6 cells/10 ml/dish*) into 100-mm cell culture dish and culture it overnight in a 5% CO_2 incubator at 37℃.
Use DMEM culture medium containing 10% FBS. You can also use DMEM culture medium with 10% FBS and 1% Penicillin-Streptomycin.

* If you are using CalPhos Mammalian Transfection Kit as transfection reagent, culture the cells in a 2.5×10^6 cells/10 ml/dish.

VI-2. Transfection (the day after cell culturing)

Lenti-X 293T cells are transfected with pLVpro Lentivirus Vector Plasmid and LVpro Packaging Mix.
We recommend using one of the following transfection reagents:

a. *Trans*IT-VirusGEN Transfection Reagent
b. *Trans*IT-293 Transfection Reagent
c. CalPhos Mammalian Transfection Kit

Transfection protocols for each of these reagents are shown below.

a. Protocol for *Trans*IT-VirusGEN Transfection Reagent

(For detailed procedure, see the manual for *Trans*IT-VirusGEN Transfection Reagent)

1. Transfer *Trans*IT-VirusGEN Transfection Reagent to room temperature and vortex to mix before use.
2. In a 2.0-ml tube, mix the serum-free DMEM and the plasmid DNA in the following proportions, and mix well with gentle pipetting.

Reagent	Amount
LVpro Packaging Mix	7 µl
0.5 µg/µl pLVpro Vector	10 µl
Serum-free DMEM	1,000 µl
Total	1,017 µl

3. To the mixture prepared in Step 2, add 30 µl of *TransIT*-VIrusGEN Transfection Reagent, mix with gentle pipetting, and let stand at room temperature for 15 to 60 minutes.
4. Drop all of the mixture from Step 3 into the Lenti-X 293T cells cultured the day before and continue to culture in a 5% CO_2 incubator at 37℃.

b. Protocol for *Trans* IT-293 Transfection Reagent

(For detailed procedures, see manual for *Trans*IT-293 Transfection Reagent)

1. Transfer *Trans*IT-293 Transfection Reagent to room temperature and vortex to mix before use.
2. In a 2.0-ml tube, mix the serum-free DMEM and the plasmid DNA in the following proportions, and mix with gentle pipetting.

Reagent	Amount
LVpro Packaging Mix	7 µl
0.5 µg/µl pLVpro Vector	10 µl
Serum-free DMEM	1,500 µl
Total	1,517 µl

3. To the mixture in prepared Step 2, add 45 µl of *Trans*IT-293 Transfection Reagent, mix with gentle pipetting, and let stand at room temperature for 15 to 30 minutes.
4. Drop all of the mixture from Step 3 into the Lenti-X 293T cells cultured the day before and continue to culture in a 5% CO_2 incubator at 37℃.

c. Protocol for CalPhos Mammalian Transfection Kit

1. Transfer 2 × HEPES-Buffered Saline, 2 M Calcium Solution, and Sterile H_2O to room temperature.
2. Mix plasmid DNA and Calcium Solution in a 15 ml tube in the following proportions:

Reagent	Amount
LVpro Packaging Mix	7 µl
0.5 µg/µl pLVpro Vector	10 µl
2 M Calcium Solution	87 µl
Sterile H_2O	595 µl
Total	699 µl

3. Add 699 µl of 2 × HEPES-Buffered Saline to the solution prepared in Step 2, place a lid on the tube, and shake up and down vigorously 15 times to mix.
4. Let stand at room temperature for 5 minutes.

   Note: Do not incubate for more than 5 minutes and move on to the next step immediately. Longer incubation causes the calcium phosphate-DNA crystals to become too large, which can reduce the transfection efficiency.

   Note: When using CalPhos Mammalian Transfection Kit, formation of calcium phosphate crystals can be confirmed by examination with a microscope.

5. Drop all of the mixture from Step 4 into the Lenti-X 293T cells cultured the day before and continue to culture in a 5% CO_2 incubator at 37℃.

VI-3. Changing the medium

Change the medium with 10 ml of fresh DMEM containing 10% FBS about 24 hours after transfection.

VI-4. Collection of lentivirus solution

1. Collect the culture supernatant containing the lentivirus about 48 hours after transfection.
2. Filter the collected culture supernatant with a 0.45 µm filter for use as lentivirus solution.

   Note: Lentivirus solution can be stored long-term at -80℃. We recommend making aliquots for storage to avoid repeated freeze-thaw cycles, as it can decrease viral titer (Higashikawa, *et al*., 2001).

VII. Titration of recombinant lentivirus

A. Various titration methods

To obtain consistent transduction results using a known MOI, it is necessary to titrate your lentivirus stocks. Freshly harvested virus stocks can be titrated immediately, or frozen in aliquots at –80℃. Note that each freeze-thaw cycle can reduce the functional titer of the virus stock. Viral titers will depend heavily on the cell type and method used for titration. There may also be significant differences between transduction efficiency in cells used for titration (e.g., HT-1080) and in the type of target cells transduced. However, titrations are important for determining the relative virus content of stocks prepared from different vectors, and for:

- Determining the optimal transduction conditions
- Adjusting the MOI to control the viral copy number of transduced cells
- Determining the maximum number of cells that can be infected by a virus stock

Titration can be accomplished using different methods, depending on the presence of a selectable or fluorescent marker:

- **qRT-PCR**
  The Lenti-X qRT-PCR Titration Kit allows titration of viral RNA in the supernatant in about 4 hours using the intercalator method. This method can be used irrespective of probes and the type of lentivirus vector used, which makes it useful for comparing the titers of various vectors and titration of virus stock.

- **p24 ELISA**
  Lenti-X p24 Rapid Titer Kit measures the amount of p24 capsid protein in the virus supernatant by the ELISA method. The amount of p24 correlates with the viral titer. It takes about 4 hours to perform this assay.

- **Flow cytometry**
  With a lentivirus vector carrying a fluorescent protein, transduction efficiency can be determined by measuring the fluorescence level with flow cytometry.
  (See "B. Measuring biological titer with flow cytometry" on the following page)

- **Instant lentivirus test**
  With Lenti-X GoStix Plus, the lentivirus titer can be measured easily by adding 20 μl of virus supernatant and Chase Buffer to GoStix. The GoStix detect lentiviral p24 with only 20 μl supernatant, and can be used to determine whether virus production is within a usable range or for selecting the best time to harvest your virus.

B. Measuring biological titer with flow cytometry

The biological titer of recombinant lentivirus is calculated based on detection of expression of the transduced gene. The following protocol describes how to determine the biological titer of pLVpro-MSCV-ZsGreen1 Vector (Cat. #6965) carrying fluorescent protein gene ZsGreen1.

B-1. HT-1080 cell culturing

Transfer HT-1080 cells into 12-well cell culture plates (2.5×10^4 cells/ml/well), and culture in a 5% CO_2 incubator at 37℃. Use DMEM medium containing 10% FBS.

B-2. Infection with lentivirus (the day after cell culturing)

1. Add polybrene to DMEM medium containing 10% FBS (0.5 µl of 8 mg/ml polybrene solution per 450 µl of culture medium).
2. Replace the medium of HT-1080 cell culture in B-1 with 450 µl of the medium containing polybrene.
3. Prepare serial dilution of the lentivirus solution with DMEM containing 10% FBS. The degree of dilution will depend on the viral titer. We recommend having a range from 20-fold to 2,000-fold dilution.
4. Infect the HT-1080 cells from Step 2 by adding 50 µl of diluted lentivirus solution (final polybrene concentration 8 µg/ml; final viral dilution rate, 200- to 20,000-fold)
5. Culture overnight in a 5% CO_2 incubator at 37℃.

B-3. Changing the medium

1. Discard the medium containing the virus and replace with 1 ml of fresh DMEM containing 10% FBS the following day.
2. Culture for 2 days in a 5% CO_2 incubator at 37℃.

B-4. Evaluation with flow cytometer

Three days after infection, detach cells with Trypsin/EDTA to recover, and measure the ratio of ZsGreen1 positive cells using a flow cytometer.

B-5. Calculation of viral biological titer

Calculate the biological titer (IFU/ml) by plugging the ZsGreen1 positive ratio into the formula shown below. The ZsGreen1 positive ratio should be between 1.0 to 20.0%.

Titer (IFU/ml) = number of infected cells × ZsGreen1 positivity rate (%)/100 × virus dilution rate/fluid volume at time of infection (0.5 ml)

Note: For precise calculation of biological titer, an accurate cell count at the time of infection can be obtained by counting the HT-1080 cells prepared for cell counting on the day of infection with lentivirus.

VIII. Methods for transduction of target cells with recombinant lentiviruses

Methods for transducing lentivirus vector into target cells include the polybrene method, static infection method, centrifugal infection method, and the RBV-spin method using RetroNectin (Cat. #T100A/B). It is important to select the optimal method because transduction efficiency and cell viability are affected by the method used.

VIII-1. Polybrene method

The usual practice when transducing genes into adherent cell strains (HT-1080, HeLa, etc.) is to use polybrene. The optimum final concentration of Polybrene may be determined preliminarily but is generally between 2–12 μg/ml. Excessive exposure to Polybrene (>24 hr) can be toxic to cells. Refer to the following protocol for determining the optimal conditions for transduction into your target cells.

1. Culture the target cells the day before transduction.
2. Use freshly-prepared virus stock, or titrated frozen lentivirus stock. Mix the thawed lentivirus gently.

   Note: Do not use a vortex mixer. The viral titer decreases each time the stock is frozen and thawed.
3. Prepare appropriate cell culture medium.
4. Calculate and add polybrene so that its final concentration at transduction (Step 6) is 4 μg/ml.
5. Dilute the lentivirus with medium to obtain the desired MOI. If the virus titer is unknown, use a serial dilution of the lentivirus stock. Make sure that the entire amount of virus solution does not exceed 1/2 of the total volume of medium used for transduction.
6. Add the diluted virus solution prepared in Step 5 to the target cells and culture at 37℃ in a 5% CO_2 incubator for 8 to 24 hours.

   Note: Centrifuging the plate before incubation at 37℃ at 1,200*g* for 60 to 90 minutes at 32℃ or room temperature significantly improves the infection efficiency. You can shorten the transduction time to 6 to 8 hours to avoid exposing the cells to polybrene for long periods of time.
7. Remove the transduction medium containing the virus and add fresh medium.

   Caution: Discarded medium contains infectious lentivirus and should be discarded properly.
8. Culture the infected cells until sufficient transgene expression can be confirmed (usually 24 to 48 hours).
9. Recover the cells for analysis.

VIII-2. Static infection method

For transduction of non-adherent cell strains (SupT1, J45.01, etc.) or when using a viral vector with high titer, infection can also be performed by the Static infection method.

1. Seed the target cells after adjusting the volume of target cell medium so that the lentivirus solution can be added.
2. Use freshly-prepared virus stock, or titrated frozen lentivirus stock. Mix the thawed lentivirus gently.

   Note: Do not agitate in a vortex mixer. The viral titer will decrease each time the stock is frozen and thawed.
3. Dilute the lentivirus with medium to obtain the desired MOI. If the virus titer is unknown, use a serial dilution of the lentivirus stock. Make sure that the entire amount of virus solution does not exceed 1/2 of the total volume of medium used in transduction.
4. Add the viral supernatant to the cells and culture at 37℃ in a 5% CO_2 incubator for 8 to 24 hours.
5. Add an equal volume of fresh medium.
6. Culture the infected cells until sufficient transgene expression can be confirmed (usually 24 to 48 hours).
7. Recover the cells for analysis.

VIII-3. Centrifugal infection method

Centrifugal infection method is effective for transduction of cell strains that adhere weakly to polybrene or non-adherent cell strains that have low infection efficiency with the Static infection method. Moreover, you can acheive highly efficient infection of human peripheral blood mononuclear cells (PBMCs) by the Supernatant method (centrifugal infection) using RetroNectin.

1. Seed the target cells after adjusting the volume of cell culture medium so that the lentivirus solution can be added.

   Note: For the Supernatant method using RetroNectin, culture the cells in RetroNectin–coated plates (See VIII-4).
2. Use freshly-prepared virus stock, or titrated frozen lentivirus stock. Mix the thawed lentivirus gently.

   Note: Do not agitate in a vortex mixer. The viral titer will decrease each time the stock is frozen and thawed.
3. Dilute the lentivirus with medium to obtain the desired MOI. If the virus titer is unknown, use a serial dilution of the lentivirus stock. Make sure that the entire amount of virus solution does not exceed 1/2 of the total volume of medium used in transduction.
4. Add the virus supernatant to the cells and centrifuge the plate at 1,200*g* for 60 to 90 minutes at 32℃ or room temperature.
5. Culture in a 5% CO_2 incubator at 37℃ for 8 to 24 hours.
6. Add an equal volume fresh medium.
7. Culture the infected cells until sufficient transgene expression can be confirmed (usually 24 to 48 hours).
8. Recover the cells for analysis.

VIII-4. RetroNectin Bound Virus (RBV)-spin method

Using RetroNectin can markedly improve transduction efficiency for cells that are difficult to transduce or that are sensitive to polybrene. Particularly for transduction of PBMCs, use of the RBV spin method will help eliminate the effects of factors other than the virus vector at the time of infection, because there is little effect on cell proliferation, as well as little carryover of the virus solution.

A. Preparation of RetroNectin-coated plates

1. Thaw the RetroNectin solution and mix it until it is homogeneous (avoid vortexing). Dilute with sterile PBS to a concentration of 20 to 100 μg/ml.[*1]

   *1 To avoid loss of RetroNectin, do not filter-sterilize RetroNectin solution diluted with PBS.

2. Add diluted RetroNectin solution to the plate[*2] to a concentration of 0.25 to 0.5 ml/cm^2 (enough to cover the bottom of the plate), spread it out over the plate, and let it stand at room temperature for 2 hours or at 4℃ overnight. If using a 24-well plate, add diluted RetroNectin solution (0.5 ml per well; if using a 6-well plate, add 2 ml per well).

   *2 Be sure to use a non-treated plate.

3. Remove the diluted RetroNectin solution, and block with an appropriate amount of 2% BSA/PBS solution. Allow the plate to stand at room temperature for 30 minutes.[*3] If using a 24-well plate, add 0.5 ml per well of blocking solution; if using a 6-well plate, add 2 ml per well.

   *3 If the solution will be used right away, there is no need for blocking with 2% BSA/PBS solution. In this case, repeat Step 4 (washing with PBS or HBSS/HEPES) twice.

4. Remove the BSA solution and wash once with an appropriate amount of PBS or HBSS/HEPES（Hank's Balanced Salt Solution supplemented with 2.5% (v/v) 1 M Hepes）and store the plate after removing the wash solution. This plate will be used as the RetroNectin–coated plate.[*4]

   *4 If blocking has been performed with 2% BSA/PBS solution, the coated plate can be stored for 1 week at 4℃ after sealing with Parafilm.

B. RBV-spin method (centrifugal infection)

Note: When a multi-well plate is used, the transduction rate may differ depending on the position of the well. We recommend using wells positioned as close to the center of the plate as possible.

1. Use freshly prepared virus stock or titrated frozen lentivirus stock. Mix the thawed lentivirus gently.
   Note: Do not agitate in a vortex mixer. The viral titer will decrease each time the stock is frozen and thawed.
2. Dilute the lentivirus with medium to obtain the desired MOI. If the virus titer is unknown, use a serial dilution of the lentivirus stock.
3. Add 125 to 250 μl/cm2 of the virus supernatant to a RetroNectin–coated plate.
4. Centrifuge at 4℃ and 2,000*g* for 2 hours to cause the viral particles to adhere to the RetroNectin.
5. Remove the virus solution, but do not allow the plate to dry out, and add an appropriate amount of PBS or PBS containing 0.1 to 2% albumin (BSA or HSA) (Do not remove the solution until immediately before infecting the cells with the virus).
6. Prepare the target cell suspension. The appropriate cell concentration will differ depending on the size and proliferation rate of the target cells.

7. Remove the solution from the virus-bound plates prepared in Step 3 above, and rapidly add the cell suspension. Centrifuge for 1 minute at 500 *g* to promote contact between the target cells and the virus vector.
8. Culture at 37℃ in a 5% CO_2 incubator.
9. Culture the infected cells until sufficient transgene expression can be confirmed (usually 24 to 48 hours).
10 Recover the cells for analysis.

IX. Reference Data

IX-1. Comparison of recombinant lentiviral titers prepared with *Trans*IT-293 vs. *Trans*IT-VirusGEN Transfection Reagent

High-titer lentiviruses can be obtained using *Trans*IT-293 or *Trans*IT-VirusGEN Transfection Reagent together with the LVpro Packaging Mix and pLVpro Lentivirus Vector Plasmid. Titer comparison data for use of *Trans*IT-293 vs. *Trans*IT-VirusGEN Transfection Reagent are shown in Fig. 2, below.

< Experimental method >

Lentiviruses were prepared by co-transfecting Lenti-X 293T cells with ZsGreen1–expressing lentivirus vector plasmid and packaging plasmid under conditions shown below.

	Lentivirus Vector Plasmid	Transfection Reagent
1	pLVpro-MSCV-ZsGreen1 Vector	*Trans*IT-293
2	pLVpro-MSCV-EI-ZsGreen1 Vector	*Trans*IT-293
3	pLVpro-EF1 α -ZsGreen1 Vector	*Trans*IT-293
4	pLVpro-MSCV-ZsGreen1 Vector	*Trans*IT-VirusGEN
5	pLVpro-MSCV-EI-ZsGreen1 Vector	*Trans*IT-VirusGEN
6	pLVpro-EF1 α -ZsGreen1 Vector	*Trans*IT-VirusGEN

Note: In all cases, LVpro Packaging Mix was used as packaging plasmid.

The biological titer of the lentiviruses obtained was evaluated in HT-1080 cells (Fig. 3). Comparable high-titer lentiviruses were obtained with both *Trans*IT-293 and *Trans*IT-VirusGEN Transfection Reagent.

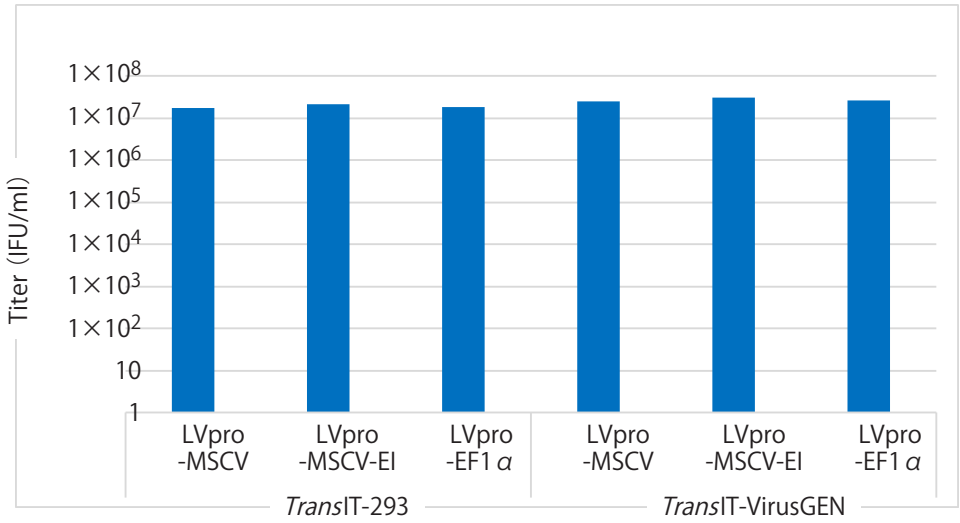

Fig. 2. Comparison of lentiviral titers obtained with *Trans*IT-293 vs. *Trans*IT-VirusGEN Transfection Reagent.

IX-2. Comparison of recombinant lentiviral titers prepared with LVpro Packaging Mix vs. other company's kit

Data comparing the lentiviral titers obtained with LVpro Packaging Mix and pLVpro Lentivirus Vector Plasmid or another company's lentivirus vector plasmids and packaging mix are shown below.

< Experimental method >

Lentivirus was obtained by co-transfecting Lenti-X 293T cells with ZsGreen1–expressing lentivirus vector plasmid and packaging plasmid as shown below.

	Lentivirus Vector Plasmid	Packaging Plasmid	Transfection Reagent
1	Company A's vector*	Company A's	Company A's
2	Company A's vector*	LVpro Packaging Mix	*Trans*IT-VirusGEN
3	pLVpro-MSCV-ZsGreen1	Company A's	Company A's
4	pLVpro-MSCV-ZsGreen1	LVpro Packaging Mix	*Trans*IT-VirusGEN

* Vector plasmid contains ZsGreen1 gene in Company A's vector with CMV promoter.

The biological titer of the lentiviruses was evaluated in HT-1080 cells. Compared to case 1 in which all Company A's products were used, a 10 times higher lentivirus titer was obtained when Company A's lentivirus vector plasmid was combined with LVpro Packaging Mix and *Trans*IT-VirusGEN (case 2), as well as when pLVpro Lentivirus Vector Plasmid was combined with Company A's packaging plasmid and transfection reagent (case 3). Moreover, the lentivirus with the highest titer was obtained when all Takara Bio products were used (case 4) (Fig. 3).

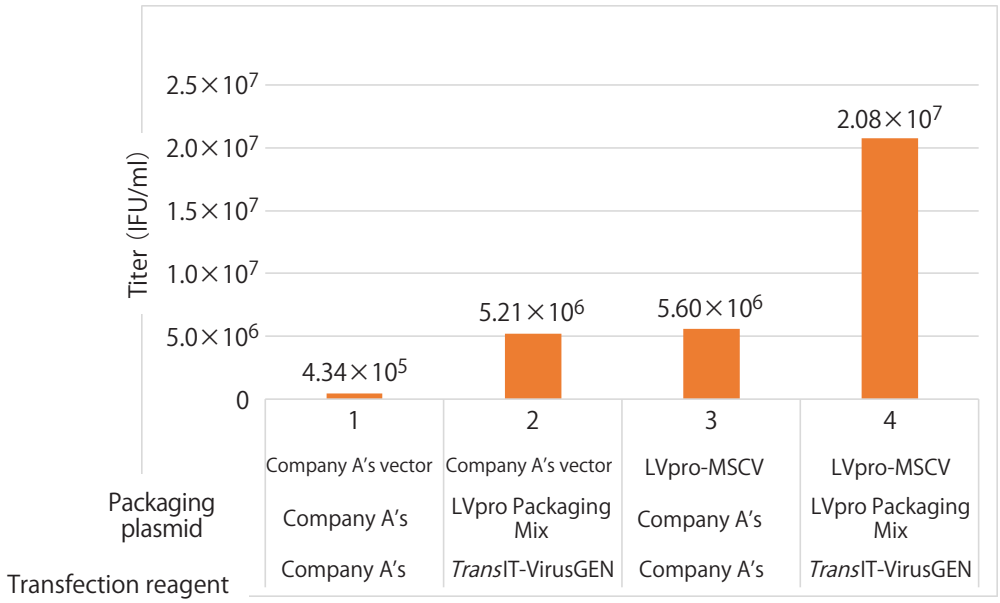

Fig. 3. Comparison of lentiviral titers prepared with LVpro Packaging Mix and other company's kit.

IX-3. Comparison of transduction methods using lentivirus vectors

Use of RetroNectin greatly improves the efficiency of transduction by lentiviruses. It is particularly useful when you are working with floating cells (lymphocyte cell strains, etc.) or with cells that are difficult to transduce (lymphocytes, hematopoietic stem cells, etc.). The RBV-Spin method using RetroNectin is recommended for hard-to-transduce cells.

Lentivirus transduction efficiency obtained using the RBV-Spin method or polybrene method in human peripheral blood mononuclear cells (PBMCs) are shown below.

< Experimental method >

The ZsGreen1–expressing lentivirus vector was diluted 30-fold, and genes were transduced into PBMCs by the RBV-Spin method or polybrene method. Cells were recovered 3 days after infection, and measured for CD8-positive and ZsGreen1-positive rate using a flow cytometer. The positive rate shown with the RBV-Spin method is higher than that shown with the polybrene method — about 20% vs. 10% (Fig. 4).

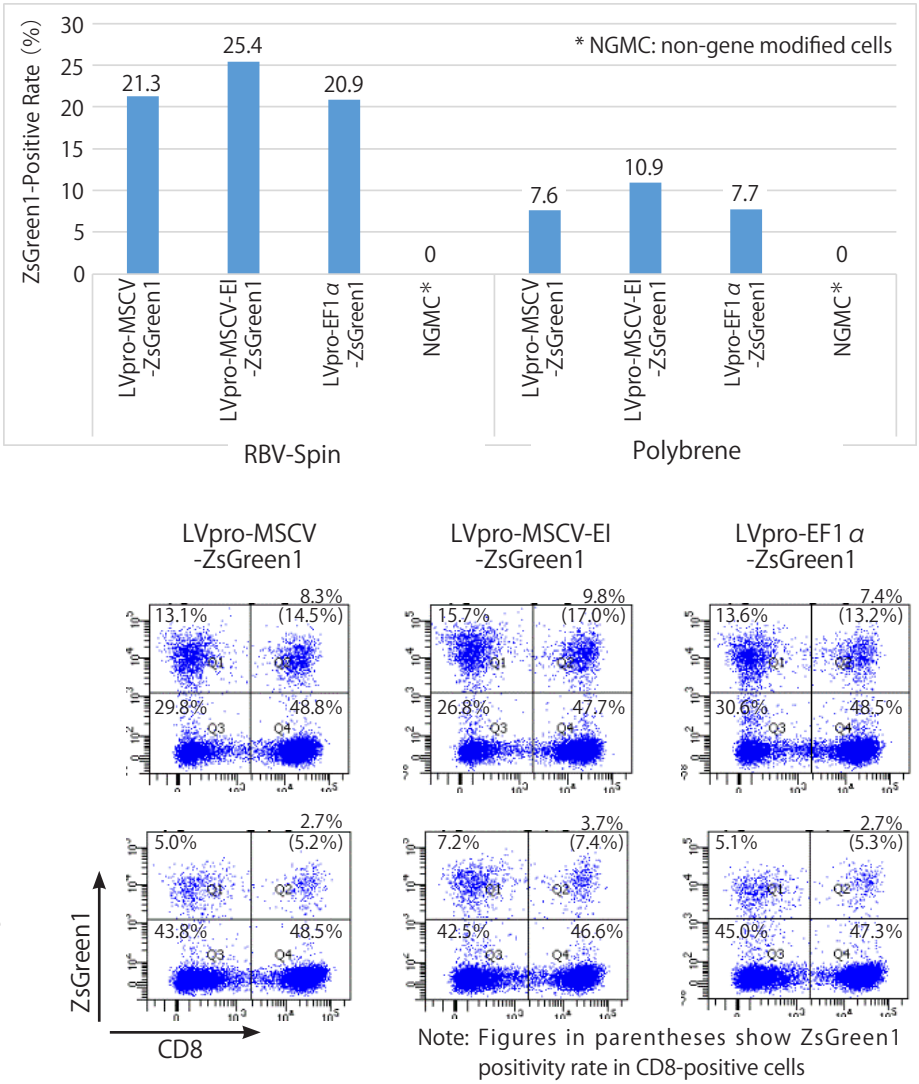

Fig. 4. Comparison of lentiviral transduction efficiency obtained using RetroNectin or polybrene.

IX-4. Comparison of transduction efficiency of lentivirus vector plasmid with and without WPRE2

The pLVpro Lentivirus Vector Plasmid in this product contains WPRE2, but WPRE2 can have a negative effect on certain cell types. The WPRE2 in these plasmids can easily be removed from the plasmid by using restriction enzyme *Sph*I. Data comparing the presence or absence of WPRE2 in the pLVpro Lentivirus Vector Plasmid are shown below.

< Experimental Method >

pLVpro Lentivirus Vector Plasmid was treated with *Sph*I (Cat. #1246A) to remove WPRE2. Lentivirus was obtained by co-transfecting the ZsGreen1-expressing lentivirus vector plasmid minus WPRE2 and LVpro Packaging Mix. The ZsGreen1-expressing lentivirus vector obtained was serially diluted and transduced into five different cell types, using the polybrene method, static infection method, or Supernatant method using RetroNectin (see VIII-3. Centrifugal infection method), and then the biological titer was measured.

Figures 5 and 6 show the biological titers and the mean fluorescent intensity (MFI) of the pLVpro lentivirus vector with WPRE2, compared to those for lentivirus vector without WPRE2 (shown as 100%). For almost all cell types, the biological titer is higher for the pLVpro lentivirus vector with WPRE2, but with some cell types, a decrease in MFI of ZsGreen1 was observed.

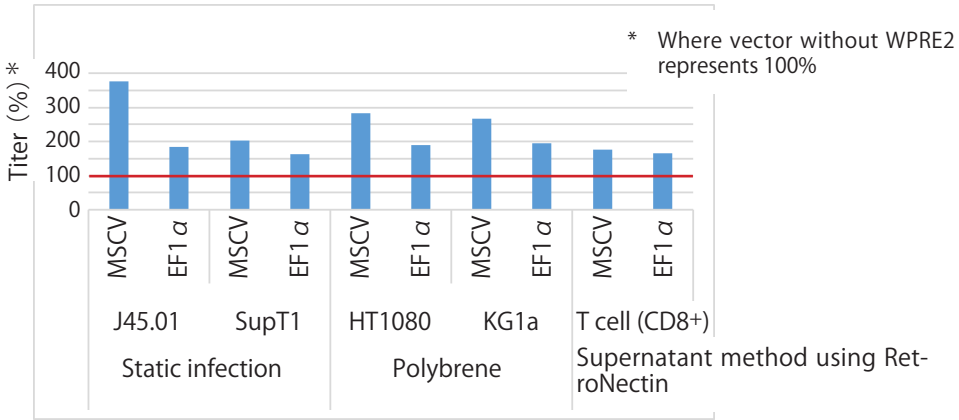

Fig. 5. Comparison of titer obtained from lentivirus vector plasmid with and without WPRE2

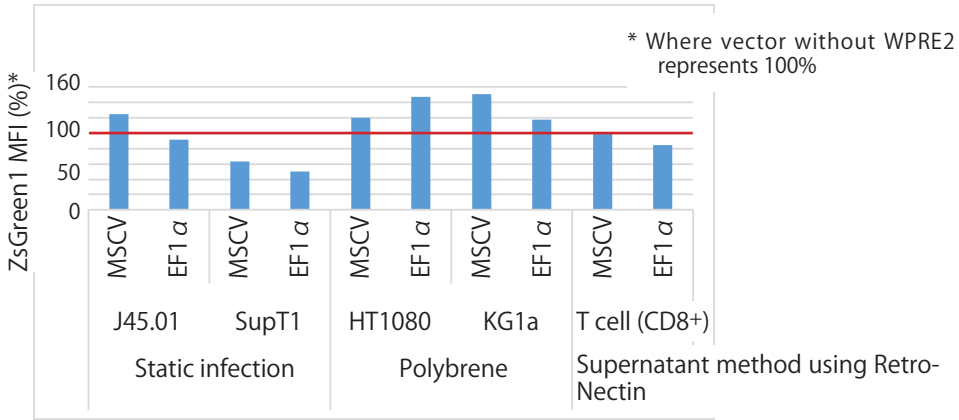

Fig. 6. Comparison of MFI for lentivirus vector plasmid with and without WPRE2

IX-5. The selection of promoter

There are three types of promoters for the pLVpro Lentivirus Vector Plasmid: MSCV-U3, MSCV-EI, and EF1 α. Because transduction efficiency and gene expression differ depending on the type of cells that are transduced and the target gene, it is recommended that you select the promoter best suited to your purpose.

A comparison of promoter activity of each cell type is shown below.

< Experimental method >

Lentiviruses were obtained by co-transfection with ZsGreen1-expressing pLVpro Lentivirus Vector Plasmid and LVpro Packaging Mix. The ZsGreen1-expressing lentivirus vector was serially diluted, and each type of cell was transduced with the polybrene method, static infection method, or Supernatant method using RetroNectin (see VIII-3. Centrifugal infection method). The ZsGreen1 positive rate of the recovered cells was measured with a flow cytometer (Fig. 7).

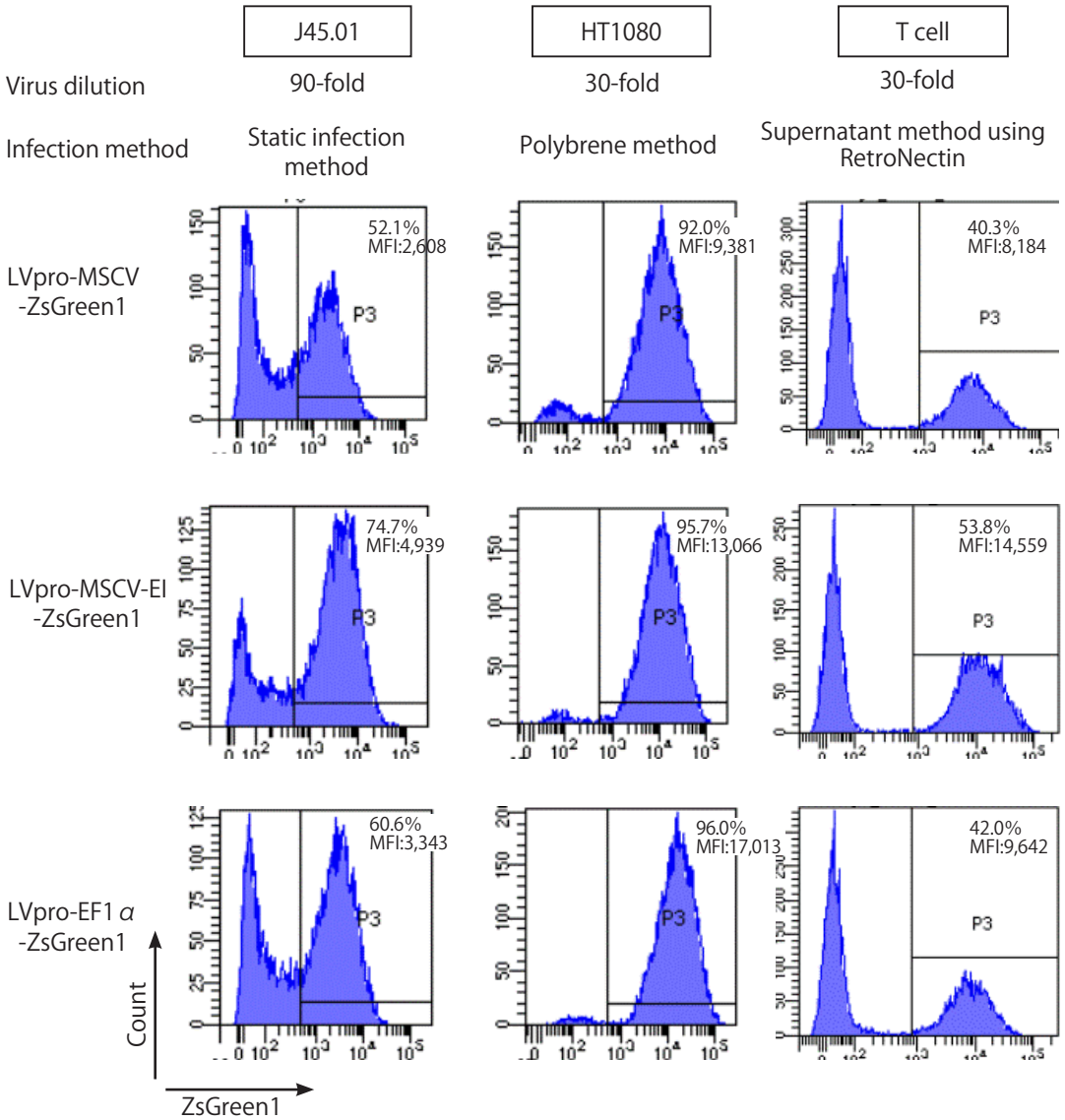

Fig. 7. Comparison of promoter activity obtained with pLVpro Lentivirus Vector Plasmid.

X. Appendix：Supplemental Protocol

< Virus concentration >

Lenti-X Concentrator (Cat. #631231) is a reagent for easy, quick, and efficient concentration of lentivirus solution without ultracentrifugation.

1. Add Lenti-X Concentrator to lentivirus solution and mix.
   (For 30 ml of the solution, add 1/3 Concentrator, or 10 ml)
2. Incubate at 4℃ for 30 min to overnight.
3. Centrifuge at 1,500*g* for 45 minutes at 4℃.
4. After removal of the supernatant, pipette up and down to resuspend in PBS, etc., in 1/10 to 1/100 volume (for 10- to 100-fold concentration).

XI. References

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XII. Related Products

[Transfection reagents]
CalPhos™ Mammalian Transfection Kit (Cat. #631312)

[Packaging cells]
Lenti-X™ 293T Cell Line (Cat. #632180)

[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)

[Recombinant lentivirus purification]
Lenti-X™ Maxi Purification Kit (Cat. #631233/631234)

[Recombinant lentivirus concentration]
Lenti-X™ Concentrator (Cat. #631231/631232)

[Increasing infection rate with recombinant virus]
RetroNectin® (Recombinant Human Fibronectin Fragment) (Cat. #T100A/B)