



Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells

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LV601B-1, LV900A-1**

User Manual

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I. Introduction and Background

A. Purpose of this Manual

This manual provides information describing how to package lentivector expression constructs in pseudoviral particles and use of packaged expression constructs for transduction of target cells. Specifically, it provides critical instructions on how to package an FIV-based or HIV-based Lentivector Expression construct in VSV-G pseudotyped viral particles by co-transfecting 293TN Producer Cells with a Lentivector Expression construct and the pPACKF1™ (for FIV-based constructs) or pPACKH1™ (for HIV-based constructs) Packaging Plasmid Mix. Recommendations are also provided for selection and use of FIV-based and HIV-based lentivector systems for transducing a wide range of target cells. This manual does not include information about construction of expression constructs in lentiviral expression vectors. Information on making constructs using these vectors is available in the user manuals for each of SBI's Lentivector Cloning and Expression Vectors. User manuals, which are provided with each of the Lentivector products, can also be accessed on the SBI website (<http://www.systembio.com>). Before using the reagents and material supplied with this product, please read the entire user manual.

B. Lentiviral Expression Systems

Lentiviral expression vectors are the most effective vehicles for transducing and stably expressing different effector molecules (siRNA, cDNA, DNA fragments, antisense, ribozymes, etc.) or reporter constructs in almost any mammalian cell—including non-dividing cells and whole model organisms (Cann, 2000). As with standard plasmid vectors, it is possible to introduce lentiviral constructs in plasmid form into the cells with low-to-medium efficiency and get transient expression of effectors (reporters) using conventional transfection protocols. However, by packaging the lentiviral expression construct into pseudoviral particles, you can obtain highly efficient transduction (up to 100%)—even with the most difficult to transfect cells, such as primary, stem, and differentiated cells. The expression construct transduced in cells is integrated into genomic DNA and provides stable, long-term expression of siRNA, cDNA or reporter gene. Endogenously expressed siRNA effectors provide long-term silencing of the target gene and allow the researcher to generate cell lines and transgenic organisms with a stable knockdown phenotype for functional studies. Expression of full-length cDNAs from integrated viral constructs is a unique tool to study gain-of-function effect for cellular phenotypes. Stably integrated transcriptional reporter constructs are a novel approach to the study of transcriptional regulation in the natural chromosomal environment and the monitoring of specific signaling pathways. Moreover, lentiviral delivery does not produce the non-specific cell responses typically associated with chemical transfections or use of an adenoviral delivery system (Gould, 2003, Cann, 2000).

SBI offers both FIV-based and HIV-based lentiviral expression systems. Both systems consist of three main components:

- (1) The lentiviral expression vector (e.g., shRNA construct in pSIF1-H1-Puro™ or cDNA construct in pCDF lentivector)
- (2) The lentiviral packaging plasmids (e.g., pPACKH1™ Packaging Plasmid mix)
- (3) A pseudoviral particle producer cell line (e.g., 293TN cells)

The lentiviral expression vector contains the genetic elements required for packaging, transduction, stable integration of the viral expression construct into genomic DNA, and expression of the siRNA, cDNA, or reporter. The lentiviral packaging plasmids provide all of the proteins essential for transcription and packaging of an RNA copy of the expression construct into recombinant pseudoviral particles. For production of a high titer of pseudoviral particles, producer cells (e.g., HEK 293 cells) need to be transiently co-transfected with the expression and packaging vectors. Expression constructs packaged in pseudoviral particles are secreted by producer cells in culture media and can be used directly to transduce expression constructs into target cells. Following transduction into the target cells, this expression construct is reverse transcribed and integrated into the genome of the target cell, providing a high level of expression of effector or reporter molecules.

The most popular lentiviral expression system is HIV based (Federico, 2003; Heiser, 2004; Machida, 2003). Despite improved biosafety features, third generation HIV cloning vectors still pose a potential biohazard risk due to the possible recombination with endogenous viral sequences to form a self-replicating HIV virus. SBI's FIV-based Expression system addresses these issues since they are derived from a feline immunodeficiency virus (Curran, 2002; Sauter, 2001; Loewen, 2003). Both of SBI's HIV-based and FIV-based lentivector systems meet Biosafety Level 2 (BSL-2) based on the criteria published by the Centers for Disease Control (for details see section F).

C. SBI's Expression Lentivectors

SBI offers a wide range of FIV-based and HIV-based lentivectors for cloning and expression of siRNA, cDNA and transcriptional reporters. SBI's lentivectors are a third generation of lentivectors developed for gene therapy applications (Poeschla, 2003; Sodroski, J.G., 1997, 1999; Federico, 2003; Heiser, 2004; Machida, 2003). Appendix A shows several examples of vectors for expression of siRNA (pSI), cDNA (pCD) and transcriptional reporters (pTR). These lentivectors have similar functional maps and include the following common features:

- **Hybrid CMV-5'LTR** (for FIV-based vectors) and **hybrid RSV-5'LTR promoter** (for HIV-based vectors) provide a high level of expression of full-length pseudoviral constructs in 293 producer cells.
- Genetic elements (**cPPT, GAG, LTRs**) necessary for the packaging, transduction, and stable integration of the viral expression construct into genomic DNA.
- **SV40 origin** for stable propagation of the lentiviral plasmid in 293 producer cells.
- The **pUC origin** for high copy replication and maintenance of the plasmid in *E.coli* cells.
- The **ampicillin resistance** gene for selection in *E.coli* cells.
- **WPRE element** enhances stability and translation of the CMV-driven transcripts.
- The **SV40 polyadenylation signal** enables efficient termination of transcription and processing of recombinant transcripts.

For siRNA Expression vectors:

- H1 expression cassette located in 3' Δ LTR provides constitutive and efficient RNA polymerase III-dependent transcription of shRNA transcript in a wide range of cell lines.
- CMV promoter promotes a high level of expression of copGFP (fluorescent reporter), puromycin-N-acetyl transferase (drug selectable marker) or truncated H2Kk protein (cell surface marker) for detection and selection of transduced cells.

For cDNA Expression vectors:

- CMV promoter drives a high level of expression, in a wide variety of cell lines, of a gene of interest (cDNA) which may be cloned into the multiple cloning site (MCS) located downstream of CMV promoter.
- Optional second expression cassette with puromycin resistance gene or copGFP reporter under the control of a constitutive elongation factor 1 (EF1) promoter for selection or FACS analysis of transduced cells.

For Transcriptional Reporter vectors:

- CMV promoter is replaced with a hybrid TRE-mCMV promoter to drive expression of a reporter gene (dscopGFP, luciferase or β -galactosidase) depending on presence of specific transcriptional factor which binds to TRE element and activates transcription from the mCMV promoter.

For detailed descriptions of SBI's Expression Lentivectors, please refer to the User Manual for each specific lentivector or visit SBI's web site at <http://www.systembio.com>.

D. Packaging of Expression Constructs into Pseudoviral Particles

Currently, the most efficient technology for producing a high titer of infectious, replication-incompetent lentiviral particles is based on transient, coordinated expression of a lentiviral expression construct and all necessary packaging proteins delivered into producer cells by simultaneous transfection with lentiviral expression and packaging plasmids. When expressed in packaging cells, the highly-efficient hybrid CMV/5'LTR (or RSV/5'LTR) promoter of the expression construct produces large numbers of the expression construct transcript that contains all of the functional elements (*i.e.*, Psi, RRE, and cPPT) required for efficient packaging. The expression construct transcript is efficiently packaged into VSV-G pseudotyped viral particles with helper proteins produced by the pPACK plasmids. Pseudoviral particles generated by producer cells within 48 – 72 hr can be concentrated, frozen, and used in later experiments.

To facilitate packaging, SBI offers a 293TN producer cell line that was optimized for effective production of a high titer of pseudoviral particles by introduction of the SV40 large T antigen and neomycin gene product.

The pPACKF1 Packaging Plasmid Mix consists of a combination of two plasmids—pFIV-34N and pVSV-G (see maps in Appendix B). The pFIV-34N plasmid contains the structural (*gag*), regulatory (*vif*, *gp4*, *rev*, *nef*) and replication (*pol*) genes which code for the proteins required to produce the lentivirus. The viral *env* gene, which encodes the envelope protein that defines the tropism (*i.e.*, the range of infectable cells), is deleted in the pFIV-34N construct. The pVSV-G plasmid expresses the envelope glycoprotein of vesicular stomatitis virus (VSV-G) from the CMV promoter, thus replacing lentiviral *env* gene. Viral particles, VSV-G protein pseudotyped, mediate viral entry through lipid binding and plasma membrane fusion and can infect both mammalian and non-mammalian cells (Burns, 1993).

The pPACKH1 Packaging Plasmid Mix consists of an optimized mixture of three plasmids—pPACKH1-GAG, pPACKH1-REV and pVSV-G (see maps in Appendix C). Similar to the FIV-based Packaging Plasmid Mix, the pPACKH1 plasmids provide all necessary structural, replication, and integration proteins and helper functions to produce VSV-G pseudotyped particles.

For more detailed descriptions and sequence information regarding our cloning and packaging lentivectors, visit our web site at <http://www.systembio.com>.

E. Delivery of Packaged Lentivector Constructs into Target Cells

Pantropic VSV-G pseudotyped viral particles containing the RNA copy of the lentivector expression construct can be efficiently used to deliver and stably express effector and reporter sequences in a wide range of mammalian target cells. In order to provide guidelines for the use of lentivector delivery systems, we compared transduction efficiencies of FIV-based and HIV-based vectors in different cell types. Fig. 1 shows a comparison of transduction efficiencies of FIV-based and HIV-based lentivector systems for 27 different cell lines, including primary and stem cells.

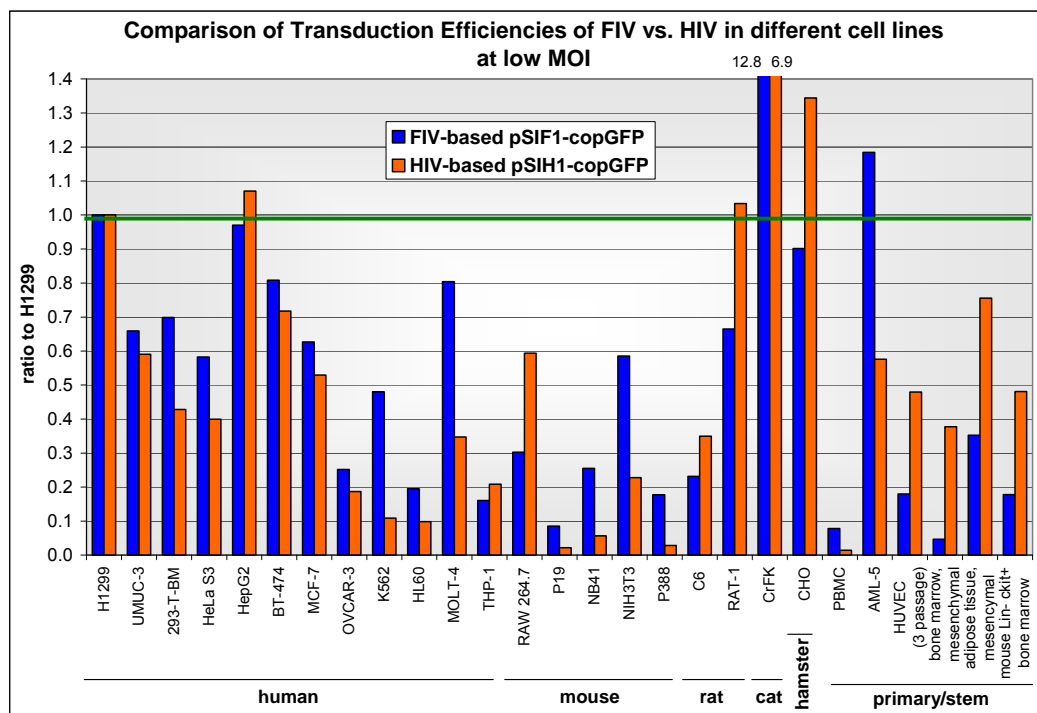


Fig. 1. Comparison of transduction efficiencies for FIV-based and HIV-based packaged positive transduction controls for 27 different cell lines based on FACS analysis percentage of GFP-positive cells at low Multiplicity of Infection (MOI).

These data clearly demonstrate that unlike commonly used cancer cell lines (like H1299, HeLa, HeK295, HepG2, etc.) that can be effectively transduced by lentivectors, some cell types (mouse Lin- ckit+ bone marrow, P19, PBMC, HL60, P388) are more resistant to infection. More efficient transduction of more “resistant” cell types may be possible by using a higher concentration of pseudoviral particles per cell in order to achieve the same MOI, as demonstrated in Appendix C, but not in all cases. It is important to mention that FIV-based and HIV-based lentivectors have different tropism. For example, the FIV-based system is more effective at infecting several of the tested mouse cell lines (P19, NB41, NIH3T3, P38) and some of the blood cells (MOLT-4, K562, T-cells from AML patient). The HIV-based system is more effective at infecting stem and primary cells (HUVEC, bone marrow, adipose).

Pseudotyped lentiviruses have been successfully used to infect many other cell types, including neuronal, dendritic, endothelial, retinal, pancreatic, hepatic, aortic smooth muscle cells, airway epithelia, skin fibroblasts, macrophages, etc. Lentivectors have been successfully used also for direct *in vivo* delivery and expression of transgenes in muscle, brain, airway epithelium, liver, pancreas, retina, and skin. For a more complete list of cells or tissues, which have been successfully transduced with lentivectors, please see the Reference Section.

The pseudoviral particles can infect (or transduce) target cells and express effector or reporter molecules but cannot replicate within target cells because the viral structural genes are absent and the LTRs are designed to be self-inactivating upon transduction. Following transduction into the target cells, the expression cassette is reverse transcribed and integrated into the genome of the target cell. After integration, the expression cassette continuously and stably produces high levels of effector or reporter molecules in target cells. Target cells stably expressing the effector molecule can be isolated using the selectable marker contained in the expression vector construct (e.g., puromycin or copGFP).

F. Safety Guidelines

SBI's Expression lentivectors together with the pPACK packaging plasmids comprise the third-generation lentiviral expression system. The HIV-based lentivectors are based on the vectors developed for gene therapy applications by Dr. J. G. Sodroski (US patent #5,665,577 and # 5,981,276).

The original FIV expression system was developed by Eric M. Poeschla, David J. Looney, and Flossie Wong-Staal at UCSD (Poeschla, 1998; Poeschla 2003). The feline immunodeficiency virus (FIV) was originally isolated from cat blood. Despite common close exposure of humans to FIV through contact with domestic cats (including bites, scratches, etc.), no human infection or disease has ever been associated with FIV (Poeschla, 2003).

Both FIV-based and HIV-based lentivector systems are designed to maximize their biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The CMV promoter (in FIV-based vectors) and RSV promoter (in HIV-based vectors) upstream of 5'LTR in the lentivector allow efficient Tat-independent production of viral RNA, reducing the number of genes from HIV-1 that are used in this system.
- Number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (*gag*, *pol*, *rev*), and the corresponding proteins are expressed from different plasmids (for HIV-based packaging plasmids) lacking packaging signals and share no significant homology to any of the expression lentivectors, pVSV-G expression vector, or any other vector, to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (*gag*, *pol*, *rev*) will be present in the packaged viral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.
- Pseudoviral particles will carry only a copy of your expression construct.

Despite the above safety features, use of HIV-based vectors falls within NIH Biosafety Level 2 criteria due to the potential biohazard risk of possible recombination with endogenous viral sequences to form self-replicating virus, or the possibility of insertional mutagenesis. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at <http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm>. It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and always follow standard microbiological practices, which include:

- Wear gloves and lab coat all the time when conducting the procedure.
- Always work with pseudoviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area are to be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.

G. List of Components

pPACK Lentivector Packaging Kits

The pPACK Packaging Plasmid Mix is an optimized mixture of the packaging plasmids in an amount sufficient for 10 co-transfections with a lentivector expression construct in 10-cm tissue culture plates (or, alternatively, 75 cm² flasks). The positive control expression construct with copGFP reporter and shRNA sequence targeting Firefly luciferase is provided in an amount sufficient for 6 control co-transfections with the pPACK packaging plasmid mix.

pPACKF1™ Lentivector Packaging Kit (Cat. # LV100A-1)

for packaging FIV-based lentivector expression constructs

200 µl pPACKF1 Packaging Plasmid Mix: Mixture of pFIV-34N and pVSV-G plasmids (0.5 µg/µl)

20 µl pSIF1-H1-siLuc-copGFP Positive Control Plasmid (0.5 µg/µl)

pPACKH1™ Lentivector Packaging Kit (Cat. # LV500A-1)

for packaging HIV-based lentivector expression constructs

200 µl pPACKH1 Packaging Plasmid Mix: Mixture of pPACKH1-GAG, pPACKH1-REV, and pVSV-G plasmids (0.5 µg/µl)

20 µl pSIH1-H1-siLuc-copGFP Positive Control Plasmid (0.5 µg/µl)

- The pPACK Plasmid Mixes and copGFP Positive Control Plasmid are shipped on dry ice or blue ice and should be stored at -20°C upon receipt. Properly stored plasmids are stable for 12 months from the date received.

Packaged Positive Transduction Controls

Packaged VSV-G pseudotyped Positive Transduction Controls are used to estimate and optimize transduction efficiency of lentivector expression constructs and packaged GeneNet siRNA Libraries. The packaged positive controls with copGFP reporter are provided in an amount sufficient to infect $\geq 2 \times 10^5$ cells at an MOI of 1. The constructs contain an shRNA targeting Firefly Luciferase. ***Proceed with Section II.D for the transduction control protocol.***

pSIF1-H1-siLuc-copGFP Packaged Positive Transduction Control (FIV-based)

(Cat. # LV201B-1)

100 – 500 µl pSIF1-H1-siLuc-copGFP Packaged Control, $\geq 2 \times 10^5$ ifu (may vary by lot)

pSIH1-copGFP Packaged Positive Transduction Control (HIV-based)

(Cat. # LV600A-1)

100 – 500 µl pSIH1-copGFP Packaged Control, $\geq 2 \times 10^5$ ifu (may vary by lot)

pSIH1-H1-siLuc-copGFP Packaged Positive Transduction Control (HIV-based)

(Cat. # LV601B-1)

100 – 500 µl pSIH1-H1-siLuc-copGFP Packaged Control, $\geq 2 \times 10^5$ ifu (may vary by lot)

- The Packaged Controls are shipped on dry ice and should be immediately stored at -70°C upon receipt. **Avoid thawing and refreezing of pseudoviral particles!** Each freeze-thaw cycle causes reduction of the titer by 20-30%. Properly stored pseudoviral particles are stable for 6 months from the date received.

293TN Producer Cell Line (Cat. # LV900A-1)

The 293TN Human Kidney cell line is optimized for effective production of a high titer of pseudoviral particles and stably expresses the SV40 large T antigen and neomycin gene products.

0.5 – 2 ml 293TN Producer Cell Line, $\geq 2 \times 10^6$ cells (may vary by lot)

- The 293TN Cell Line is shipped on dry ice and should be stored at -80°C or liquid nitrogen (gas phase) upon receipt. Properly stored 293TN cells are stable for 1-12 months from the date received, depending on storage conditions.

H. Additional Required Materials**SBI products:**

- **293TN Human Kidney Producer Cell Line** (SBI, Cat. # LV900A-1; or ATCC, 293T/17, Cat # CRL-11268)
- **Peg-It Virus concentration solution** (SBI, Cat. # LV810A-1)
- **Lentivector Expression Construct** (see <http://www.systembio.com> for a full list)
Expression constructs should be purified with a **QIAGEN Endotoxin-free Plasmid Purification Kit**. The following kit combinations can be used for Midi scale preparation of endotoxin-free DNA:
 - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Maxi Kit, Cat. # 12362
 - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Buffer Set, Cat. # 19048
 Please visit the QIAGEN website to download the specialized protocol that is not contained in the user manual:
 - <http://www1.qiagen.com/literature/protocols/pdf/QP15.pdf>

Additional reagents:

- **Dulbecco's Modified Eagle's Medium (D-MEM)**
(high glucose with sodium pyruvate and L-glutamine; Invitrogen, Cat. # 11995073)
- **Fetal Bovine Serum** (Invitrogen, Cat. # 16000036)
- **Puromycin** (Sigma, Cat. # P8833)
- **Penicillin/Streptomycin** (Invitrogen, Cat. # 15070063)
- **Trypsin-EDTA** (Sigma, Cat. # T3924)
- **Polybrene[®]** (hexadimethrine bromide; Sigma, Cat. # H9268)
- **Millex-HV 0.45 μm PVDF filters** (Millipore, Cat. # SLHVR25LS)
- **Tissue Culture Plates and Related Tissue Culture Supplies**
- **Lipofectamine[™] Reagent** (Invitrogen, Cat. # 18324-111)
- **Plus[™] Reagent** (Invitrogen, Cat. # 11514-015)
- **Sterile TE Buffer** (10 mM Tris pH 8.0, 0.1 mM EDTA pH 8.0)
- **H1299 human lung carcinoma cell line** (ATCC, Cat. # CRL-5803)

II. Protocol

A. Procedure Outline and General Comments

The flowchart below outlines the general steps required for packaging of both FIV-based and HIV-based expression constructs and transduction and expression of the viral expression construct in target cells. In the diagram below, using the HIV-based system as an example, the positive control pSIH1-H1-siLuc-copGFP plasmid, which expresses the copGFP reporter, is used as the expression construct. To construct a lentiviral expression construct for your experiment, refer to the user manual provided with each specific lentivector.

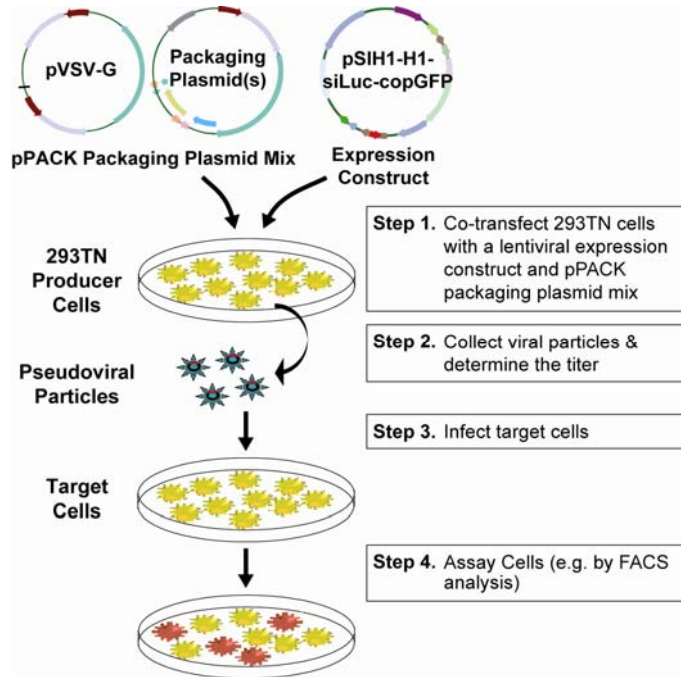


Fig. 2. Procedure for transient production of pseudoviral particles and transduction of effector expression construct into target cells.

The lentiviral expression system was designed to simplify all necessary steps in production of pseudoviral particles and transduction of an expression construct into target cells. For general information and background on working with lentiviral technology, we recommend the General Reviews listed in the Reference Section, particularly, Federico, 2003, Cann (2000) and Buchschacher et al. (2000).

To ensure optimal results, follow these general guidelines during your experiments:

- **Lentiviral expression construct quality:** To generate your specific lentiviral expression construct, refer to the protocol in the user manual provided with the vector. Transfection efficiency significantly depends on the quality of plasmid DNA. We recommend purifying plasmid DNA with a QIAGEN Endotoxin-free Plasmid Kit (see Section I.F) or by CsCl centrifugation. You will need 2 µg of lentiviral expression construct in sterile TE buffer with a concentration ranging from 0.2 – 2 µg/µl for each transfection in a 10-cm culture plate (or 75 cm² flask).
- **Maintaining 293TN cell line:** The 293TN cell line is a highly transfectable derivative of the HEK293 cell line with constitutive expression of SV40 T-antigen and neomycin resistance gene. The 293TN cells should be grown at 37°C in a humidified chamber with 5% CO₂ in D-MEM medium supplemented with 4 mM L-glutamine, 1.5 g/l glucose, 100 units/ml penicillin G, 100 µg/ml streptomycin (90%), and heat-inactivated fetal bovine serum (10%). With a doubling time of less than 24 hours, the 293TN cells should be split every 1 – 2 days when they reach 70 – 80% confluency. For subculturing, detach the cells with 0.25% trypsin, 0.03% EDTA at 37°C, add fresh culture medium, and split at a ratio of 1:3 – 1:5.
- **pSIF1-H1-siLuc-copGFP or pSIH1-H1-siLuc-copGFP Expression Vector:** Included in the pPACK Packaging Kit as a positive control to optimize and troubleshoot your packaging protocol. Specifically, this plasmid should be used as a reference when quantifying viral titer and assaying expression of your

construct. If you use a Lentivector without a copGFP reporter and expression of copGFP will not interfere with your biological assay, you can mix the copGFP construct with your expression construct at a 1:100 ratio and use it as internal positive control at every stage of your experiment. The vectors also express an shRNA targeting Firefly Luciferase. Alternatively, you may determine titer by RT-PCR using one of SBI's UltraRapid Lentiviral Titer Kits (LV960A-1 [for human cells]; LV960B-1 [for mouse cells]).

- **Transfection with Lipofectamine™ and Plus™ reagent:** The protocol provided in the experimental section is based on our experience with lentivectors and 293TN cells, and is similar to the one provided for Lipofectamine™ (Invitrogen). To maximize transfection efficiency, you may need to optimize transfection conditions. For a detailed protocol, optimization of transfection conditions, and for scaling-up pseudoviral production, please refer to Invitrogen's Lipofectamine™ Reagent Manual.
- **Pseudoviral titer:** The relative titer is measured based on flow cytometry or by fluorescent microscopy and is always specific to a particular cell line. For titering, we use H1299 cells. Below are some key terms used in the protocol:

ifu/ml (infectious units/ml)—the relative concentration of infection-competent pseudoviral particles;

MOI (multiplicity of infection)—the ratio of infectious pseudoviral particles (ifu) to the number of cells being infected. For example, if 1×10^6 cells are to be infected at an MOI of 0.1, then 1×10^5 ifu should be added to the cells;

Transduction Efficiency—the average copy number of expression constructs per genome of target cell in the infected (transduced) population.

B. Pseudovirus Production

The following protocol describes the generation of pseudoviral particles containing a copy of your lentivector expression construct. The yield of recombinant lentiviral particles typically produced under these optimized conditions is 10 ml of $1 - 3 \times 10^6$ ifu/ml for the FIV system and $2 - 5 \times 10^6$ ifu/ml for the HIV system per 10-cm culture plate (or 75 cm² flask) when measured by transduction of H1299 cells. This amount of pseudoviral particles is generally sufficient to infect $5 - 10 \times 10^6$ target cells at an MOI equal to 1. We recommend including the copGFP expression construct as an external or internal positive control in all of your experiments.

1. Plate 3×10^6 293TN cells in a fresh 10-cm plate (or 75 cm² flask) the day before transfection in 9 ml of D-MEM medium, supplemented with glutamine and heat-inactivated serum (10% FBS) without antibiotics. Disperse the cells by thorough pipetting to ensure even distribution. At the moment of transfection, the cells should reach 50 – 70% confluency.
2. Mix 20 μ l (10 μ g) of the pPACK Packaging Plasmid Mix with 2 μ g (2 – 25 μ l depending on concentration) of your lentivector expression construct, then dilute the plasmid mixture into 400 μ l D-MEM medium without serum and antibiotics. Add 20 μ l of Plus™ Reagent, mix, and incubate at room temperature for 15 min.
3. Dilute 30 μ l of Lipofectamine™ Reagent into 400 μ l of D-MEM medium without serum and antibiotics. Mix gently.
4. Dropwise, add diluted Lipofectamine™ Reagent (from step 3) to DNA/Plus™ Reagent complex (from step 2), then mix gently by inversion and incubate at room temperature for 15 min.
5. Add the DNA/ Plus™ Reagent/ Lipofectamine™ Reagent complex (from step 4) to the plate from step 1, and mix complexes with medium gently by tilting the plates a few times and incubate at 37°C in a CO₂ incubator for 48 hours.
6. We recommend collecting supernatants at 48 hours post-transfection (Step 5). Usually, the peak of virus production is achieved at 48 hours. Collect all 10 ml of the pseudovirus-containing medium in a 15-ml sterile, capped conical tube, and centrifuge at 3000 rpm at room temperature for 5 minutes to pellet cell debris. Following centrifugation, transfer the viral supernatant into a fresh 15-ml sterile, capped conical tube. Then aliquot the supernatant into sterile 1.5-ml microfuge tubes and store them at -80°C.

If you choose to concentrate the viral particles, we recommend following the User Manual of the SBI's PEG-*it*™ Virus Precipitation Solution (Cat. # LV810A-1).

Caution: You are working with infectious pseudoviral particles at this stage. Please follow the recommended guidelines for working with BSL-2 safety class (see Section I.G for more details).

Note: The supernatant containing the pseudoviral particles can be used directly to determine the pseudoviral titer, and directly used to infect target cells *in vitro* as long as the target cells can survive in conditioned medium. You can also aliquot and store the supernatant at -80°C without cryoprotectant.

Freezing and thawing may result in 20 – 30% loss of viral titer with each cycle. Alternatively, to obtain a higher titer, you may concentrate pseudovirus by any of the following suggested methods:

- SBI's PEG-*it*TM Virus Precipitation Solution (Cat. # LV810A-1) (*recommended*)
- Centrifuge the supernatant at 50,000 x g for 90 min at +4°C, then resuspend the pseudoviral-containing precipitate in a smaller volume of D-MEM
- Sucrose cushion centrifugation.

C. Pseudoviral Titer Estimation

We recommend that you estimate the titer of the pseudovirus-containing supernatant before proceeding with transduction experiments for the following reasons:

- To ensure that pseudoviral stock is viable
- To determine the percentage of target cells which can be transduced with pseudoviral stock
- To control the number of copies of integrated viral constructs per target cell

The protocol for measuring of relative titers assumes that the positive control expression plasmid—the control copGFP plasmid—was mixed with your expression construct as an internal control at a ratio of 1:100 and is packaged into pseudoviral particles. In an alternative approach, the copGFP control plasmid can be packaged separately but in parallel with your construct, as an external control. In this scenario, the control plasmid would be used to check and optimize the transfection/packaging steps (see Section B). If you are not using the copGFP plasmid as an internal control, refer to the Alternative Methods note immediately following the titering procedure.

To determine the relative pseudoviral titer, you will need to transduce the packaged lentivector expression construct into the H1299 cell line in the presence of Polybrene (4 – 8 µg/ml) for 3 – 8 hours, allow 72 hours for expression to start, and then count the number of cells expressing copGFP by fluorescent microscopy or by FACS.

To check your titer, you can also choose a cell line appropriate for your experimental system. The lentiviral constructs should effectively transduce most of the commonly used mammalian cell lines (see Section E). Relative titers may vary up to 10 to 20-fold depending on the chosen cell line.

Day 1.

1. For each viral stock, plate H1299 cells in a 24-well plate at a density of $0.6 - 1 \times 10^5$ cells per well 24 hours prior to viral infection. Add 1 ml of complete D-MEM medium (with serum and antibiotics) and incubate cells at 37°C with 5% CO₂ overnight.

Day 2.

2. Prepare complete D-MEM medium plus 10% FBS with Polybrene to a final concentration of 5 µg/ml. Prepare an extra well as a control.

Note: Polybrene[®] is a polycation that neutralizes charge interactions to increase binding between the pseudoviral capsid and the cellular membrane. The optimal concentration of Polybrene depends on cell type and may need to be empirically determined (usually in the range of 2 – 10 µg/ml). Excessive exposure to Polybrene (>12 hr) can be toxic to some cells.

3. Remove culture medium and replace with 0.5 ml of complete D-MEM medium with 10% serum and Polybrene (from Step 2). For each pseudoviral stock, use three wells. Infect H1299 cells by adding 1 µl of viral stock into the first well (dilution factor of 500), 10 µl of viral stock into the second well (dilution factor of 50), and 100 µl of viral stock into the third well (dilution factor of 5). For one additional well (mock well control) add 0.5 ml of D-MEM medium with Polybrene (from Step 2). Incubate cells at 37°C with 5% CO₂ overnight.

Day 3.

4. Remove culture medium and replace with 1 ml of complete D-MEM medium (without Polybrene). Incubate the cells at 37°C with 5% CO₂ overnight.

Day 4.

5. Split the cells 1:3 to 1:5 if necessary, depending on the type of cells, and incubate in complete D-MEM for an additional 24 hours.

Day 5.

- Count the fraction of fluorescent cells by FACS analysis. You may also visualize the cells for copGFP fluorescence, but the results may be inaccurate due to inconsistencies in counting methods. Use an average of the fraction of green-glowing cells in 5 – 10 random fields of view to estimate the overall fraction of fluorescing cells on the plate (*i.e.*, the fraction of infected cells). Multiply the fraction of infected cells by 1.5×10^5 (in this example, the expected number of H1299 cells on the plate at the moment of infection), and by the corresponding dilution factor, then divide into 0.5 ml to determine the relative titer of the pseudovirus in the supernatant.

Alternative Methods: The viral titer can also be estimated by real time PCR using SBI's UltraRapid Lentiviral Titer Kit (Cat. # LV960A-1 [human cells] or Cat. # LV960B-1 [mouse cells]).

If you are using an expression vector with puromycin resistance, titer can be determined by puromycin selection. Split the counted number of cells into aliquots in serial dilutions ($1/10$; $1/10^2$; $1/10^3$; $1/10^4$) and culture them in complete D-MEM medium containing puromycin ($1 \mu\text{g/ml}$). Continue to incubate cells for one week. Every second day, replace the selection medium with fresh medium. After at least a week, determine the fraction of infected cells by calculating the number of colonies present at a given cell dilution multiplied by the cell dilution factor and divided into the number of cells taken for selection. Then calculate relative viral titer as previously described. Please note that the titer determined by puromycin selection is usually significantly less than the titer determined by counting GFP-positive cells, and it also depends on cell type and selection conditions.

For Lentivector constructs with the H2Kk reporter, titer can be determined by staining transduced cells with FITC-H2Kk antibodies, followed by FACS analysis or fluorescent microscopy (for details, see <http://www.miltenyibiotech.com>).

D. Transduction of the Packaged Lentivector Expression Construct

General considerations:

- **The transduction efficiency of the expression construct** varies significantly for different cells and experimental conditions, including virus concentration, exposure time to virus, and growth area of cells. In order to optimize transduction conditions, we recommend that you use either a titrated pseudoviral stock containing the positive control copGFP construct or your packaged expression construct mixed with the copGFP construct (for details, see Section A). To determine the concentration of pseudoviral particles required to provide the desired multiplicity of infection (MOI) for your target cells, you should do several transductions with different concentrations of packaged pseudoviral particles containing the control plasmid. Results of these test transductions should be used to determine an optimal concentration that yields the optimal percentage of infected cells based on copGFP fluorescence. Note that some cell types, e.g. some suspension cultures, may be rather resistant to infection (see Section I.E).
- **Expression of the lentiviral construct** can be measured directly at about 48 – 72 hours after transduction (“transient transduction”). Selecting stably transduced cells requires additional time after transduction. For example, puromycin selection usually requires an additional two weeks. The decision to use “transiently transduced” cells or selected cells depends on the nature of your target cells, biological assay, etc. Some infected, actively dividing cells (e.g., 293, HT1080, HeLa, etc) may express the construct in 100% of cells at an MOI of 1. For these “easy-to-transduce” cells, most biological assays can be performed at 48 – 72 hours after transduction. However, some cells may only express the construct in 10 – 50% of cells, even when transduced with a high concentration of infection-competent pseudoviral particles. For these “difficult-to-transduce” cells, it is probably desirable to select the clones stably expressing the Lentivector construct for experimental assays.
- **SBI’s Expression Lentivectors** contain a deletion in the 3’LTR which leads to self-inactivation of the lentiviral vector after reverse transcription and integration into genomic DNA. Although more than one copy of a lentiviral construct may be integrated into the genome of a single cell, the lentiviral construct cannot produce infectious viral particles. However, in spite of these safety features, please remember that you are working with transducible pseudoviral particles. Although the particles are replication-incompetent, they are infection-competent, so the expression cassette which they carry will infect, integrate, and express in any mammalian cells. Please follow the recommended guidelines for working with BSL-2 safety class (see Section I.F for more details).

The following protocol provides a general recommendation for transduction of your lentivector expression construct packaged in pseudotyped viral particles into target cells. Use these guidelines as a starting point for determining optimal conditions for your cells and experiments.

Day 1.

1. Plate target cells in a 24-well plate at a density of 0.5×10^5 cells per well 24 hours prior to viral infection. Add 0.5 ml of complete optimal medium (with serum and antibiotics) and incubate cells at 37°C with 5% CO₂ overnight.

Note: It is possible to use other plate formats for transduction as well. In this case, the amount of cells should be adjusted depending on the growth area of the well or plate.

Day 2.

2. Prepare a mixture of complete medium with Polybrene at a final concentration of 5 µg/ml. Remove media from plate wells and replace with 0.5 ml of this Polybrene/media mixture per well (for 24-well plate).

Note: Note: Mix the virus with the medium gently by rotation or inversion. Do not vortex.

Note: Polybrene® is a polycation that neutralizes charge interactions to increase binding between the pseudoviral capsid and the cellular membrane. The optimal concentration of Polybrene depends on cell type and may need to be empirically determined (usually in the range of 2 – 10 µg/ml). Excessive exposure to Polybrene (>12 hr) can be toxic to some cells.

- Infect target cells by adding the prepared virus. We suggest using several amounts of pseudoviral stock (example: 1 μ l, 5 μ l and 10 μ l of virus). In addition, we recommend including a transduction well with a copGFP positive control construct virus and other appropriate positive and negative control construct virus preparations. Incubate cells at 37°C with 5% CO₂ overnight. For extremely fast-growing and metabolizing cell lines, such as 293T, use 3% FBS in the medium.

Day 3.

- Remove the culture medium and replace with 1 ml of complete medium (without Polybrene). Incubate the cells at 37°C with 5% CO₂ overnight.

Day 4.

- By day 4, the culture will be confluent (depending on cell type). Split it 1:3 to 1:5, depending on the type of cells, and continue incubating for 48 hours in complete D-MEM.

Day 6.

- The infected target cells can be analyzed for transient expression of the expression construct using an appropriate biological assay. If you have used an internal copGFP control, determine the percentage of infected cells by counting fluorescing cells by flow cytometry. Alternatively, the infected cells can be identified by selecting and counting based on selection marker genes contained in the expression construct (e.g., puromycin), but the titer determined by puromycin selection is usually less than the titer determined by copGFP selection.

III. Troubleshooting

A. Low Viral Titer (10^5 ifu/ml)

1. Poor Transfection Efficiency

293TN Cells have too high or too low density

Plate fewer or more cells in order to have about 50 – 70% confluency at transfection stage.

Lentivector expression construct DNA preparation is of poor quality

Purify plasmid DNA using a QIAGEN Endotoxin-free Plasmid Purification Kit or by phenol/chloroform extraction followed by a CsCl gradient centrifugation.

Plasmid DNA/Lipofectamine™/Plus Reagent ratios are incorrect

Optimize the ratios using the guidelines provided in the Lipofectamine™ protocol.

2. Inefficient Production of the pseudovirus

293TN Cells are of poor quality

Optimize growth conditions, check growth medium, and don't grow 293TN cells for more than 20 passages. Check for mycoplasma contamination. Do not overgrow the cells (do not allow the cells to reach more than 90% confluency in order to keep the culture continuously in logarithmic growth phase).

Pseudoviral supernatant harvested too early or too late

Harvest supernatant every 12 hours starting 24 hours after transfection for 2 – 3 days (24, 36, 48, 60, 72 hours), then titer each batch.

Lentiviral expression construct is too large

The packaging limit for the lentiviral system is 8.5 kb from 5' LTR to 3' dLTR. However, the efficiency of packaging drops significantly at greater than 2 kb of cDNA insert length. For a 3 kb insert, the titers could be 10-fold lower than for a 1 kb insert.

Truncated viral RNA transcript

Re-check the lentivector construct sequence to confirm the absence of a polyadenylation (ATAAA) site between the LTR elements.

B. Inefficient Transduction of Packaged Lentivector Expression Constructs

1. Poor Infection Efficiency

Your stock contains low titer of virus

Optimize infection protocol by using standard pre-packaged pseudoviral stocks of copGFP positive control which can be purchased from SBI (see Appendix F, Related Products).

Volume of infecting supernatant is too high

Keep volume as low as possible to achieve maximal adsorption of viral particles to the cells.

The assay is performed too early

Normally, the maximal expression of integrated provirus is expected to develop by 72 hours after infection. However, some cells display delayed expression. Try the assay at a later time, such as 96 hours.

CMV promoter is not functional in target cells

Replace the CMV promoter with the elongation factor 1 (EF1) promoter in the expression construct.

Target cell line may be difficult to transduce

Check titer with 293TN or H1299 cells. Optimize the transduction protocol. Use a higher MOI.

Concentration of Polybrene added during titration is too high

Add and optimize Polybrene concentration in the range of 4 – 10 μ g/ml.

Loss of pseudoviral titer during storage

Aliquot and store pseudoviral stock at -80°C . Each freeze-thaw cycle drops the titer about 30%. Use a fresh aliquot for transduction.

The cell might methylate some toxic sequences within 10 – 14 days

2. Infection Affects Target Cell Viability

Pseudoviral stock medium affects target cell growth

Dilute the stock medium or concentrate the pseudovirus by centrifugation to minimize the amount of medium added to the target cells. We recommend using SBI's PEG-*it*TM Virus Precipitation Solution (Cat. # LV810A-1).

Polybrene is toxic for target cells

Optimize the concentration and exposure time to Polybrene during the transduction step. It is also possible to transduce the cells without Polybrene, though at a higher MOI.

3. No Expression of Expression Construct

The CMV or H1 promoter is not functional in target cells

We have observed this in primary cells, but the only way to solve this problem is to change the type of target cells or replace the CMV promoter with the EF1 promoter and H1 promoter with the U6 promoter.

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V. Appendix

A. Examples of SBI's Expression Lentivectors

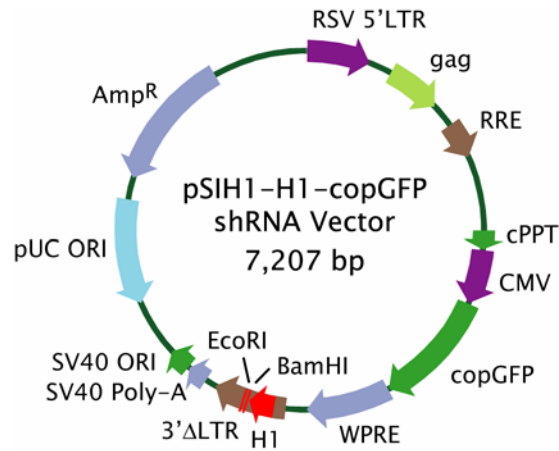


Fig. 3. HIV-based shRNA Cloning and Expression Lentivector.
Included as a positive control in the pPACKH1 packaging kit to monitor packaging efficiency.

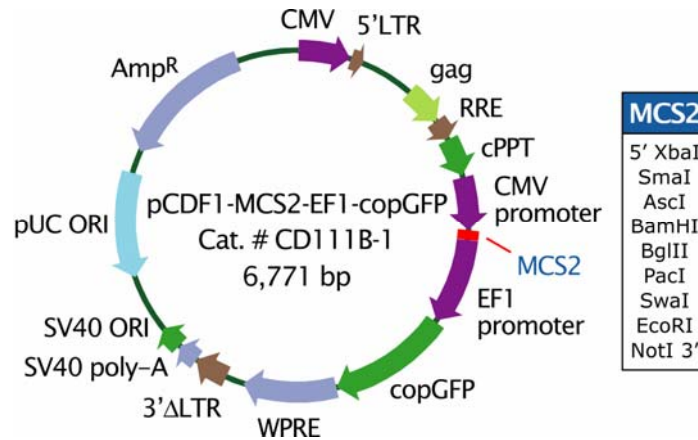


Fig. 4. FIV-based cDNA Expression Lentivector.

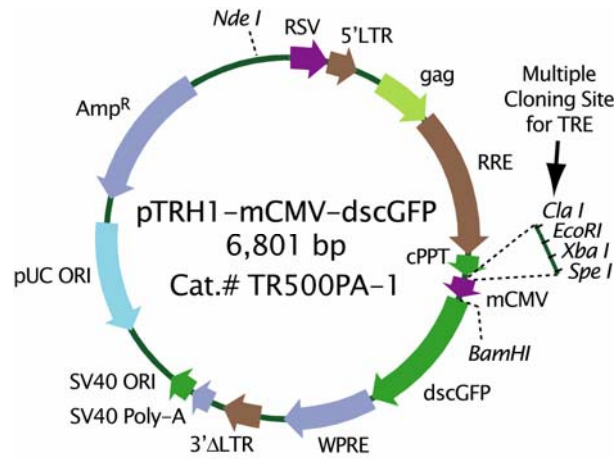


Fig. 5. HIV-based PathNet™ Transcriptional Reporter Lentivector.

B. Functional Maps of Packaging Plasmids for FIV-based Lentivector System

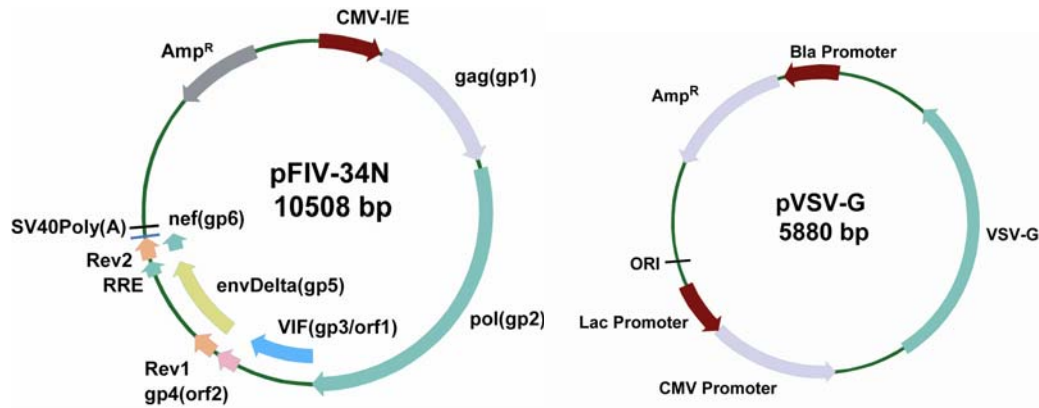


Fig. 6. Functional maps of plasmids in pPACKF1 Packaging Plasmid Mix—pFIV-34N packaging plasmid (left) and pVSV-G pseudotyping plasmid (right).

C. Functional Maps of Packaging Plasmids for HIV-based Lentivector System

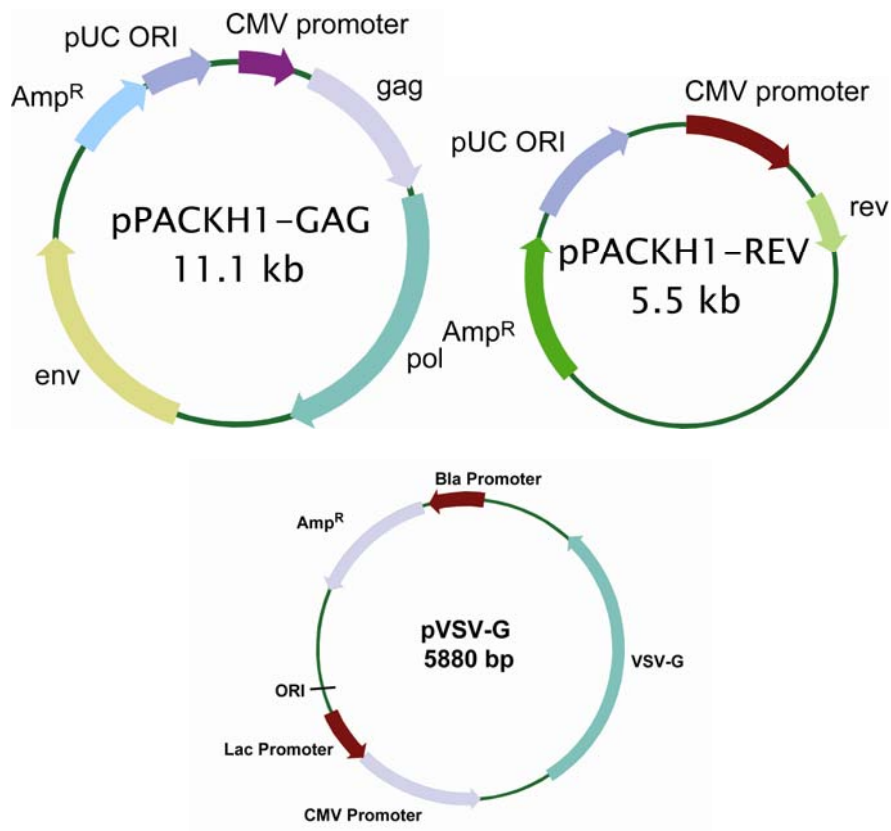
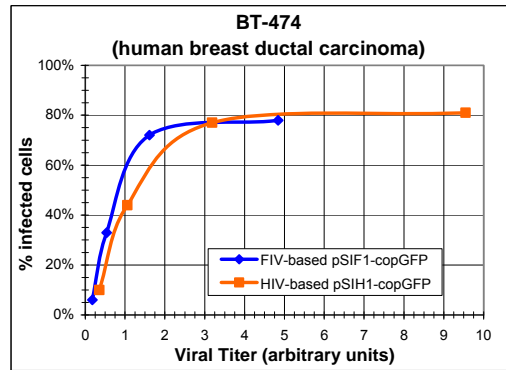
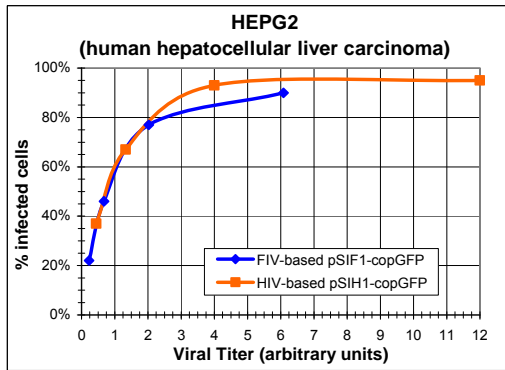
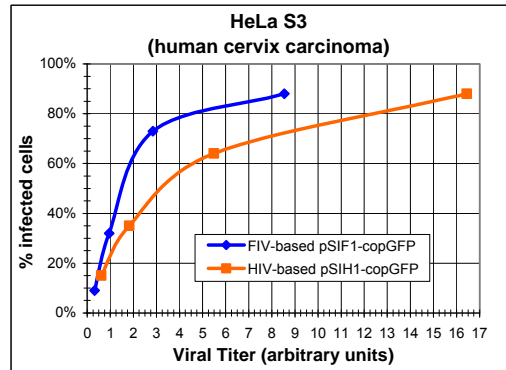
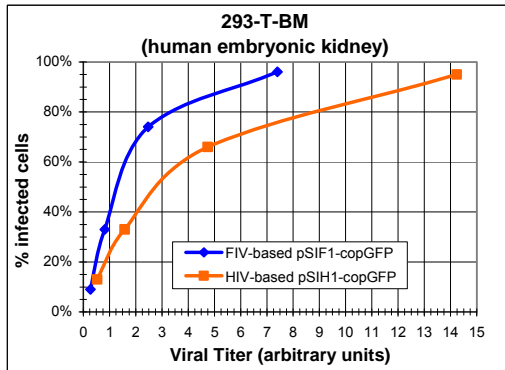
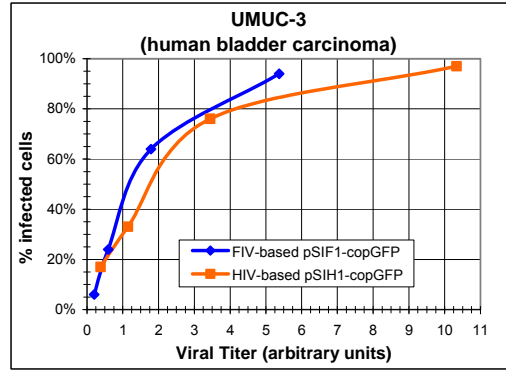
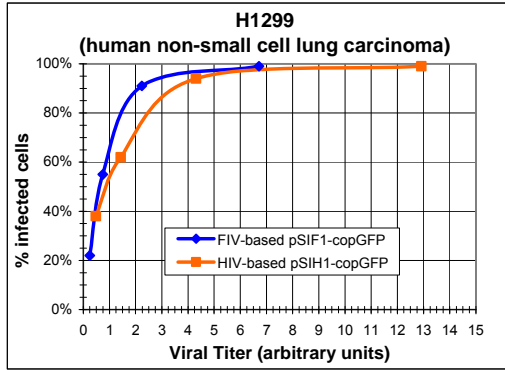


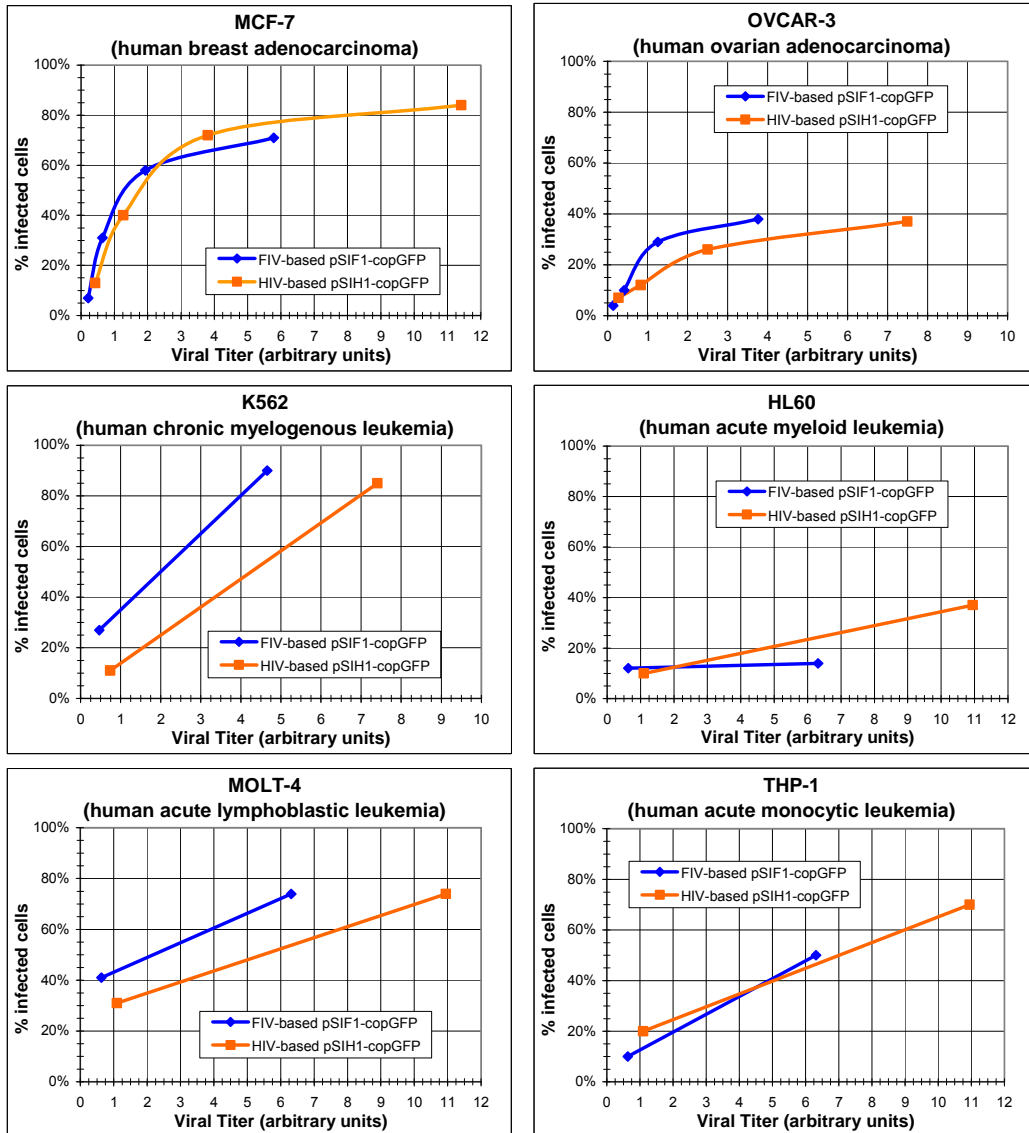
Fig. 7. Functional maps of plasmids in pPACKH1 Packaging Plasmid Mix—pPACKH1-GAG (top left) and pPACKH1-REV (top right) packaging plasmids, and pVSV-G pseudotyping plasmid (bottom).

D. Transduction Efficiencies of Different Cell Lines with Increasing Relative Concentration of Viral Particles for FIV-based and HIV-based Lentivectors

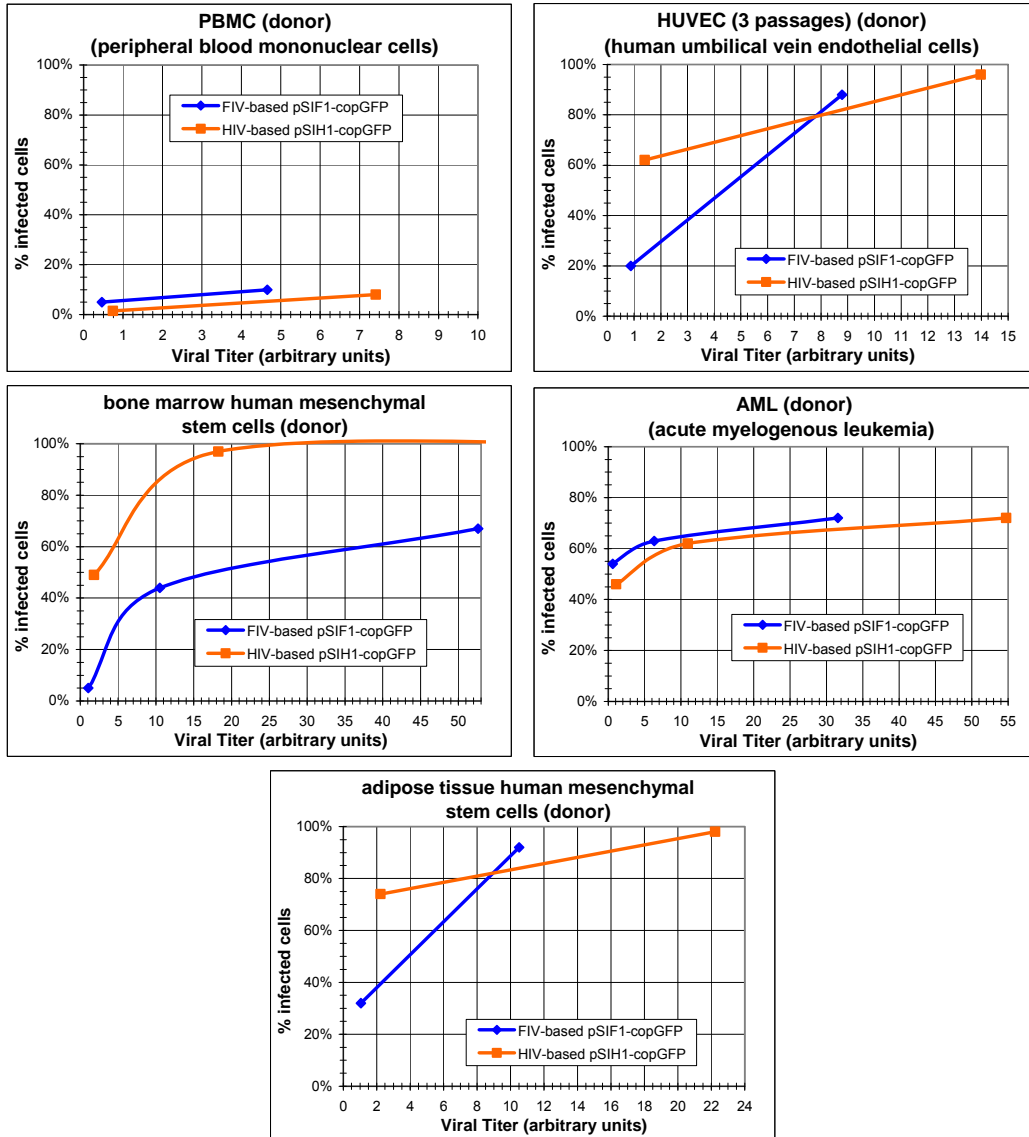
Human Cell Lines



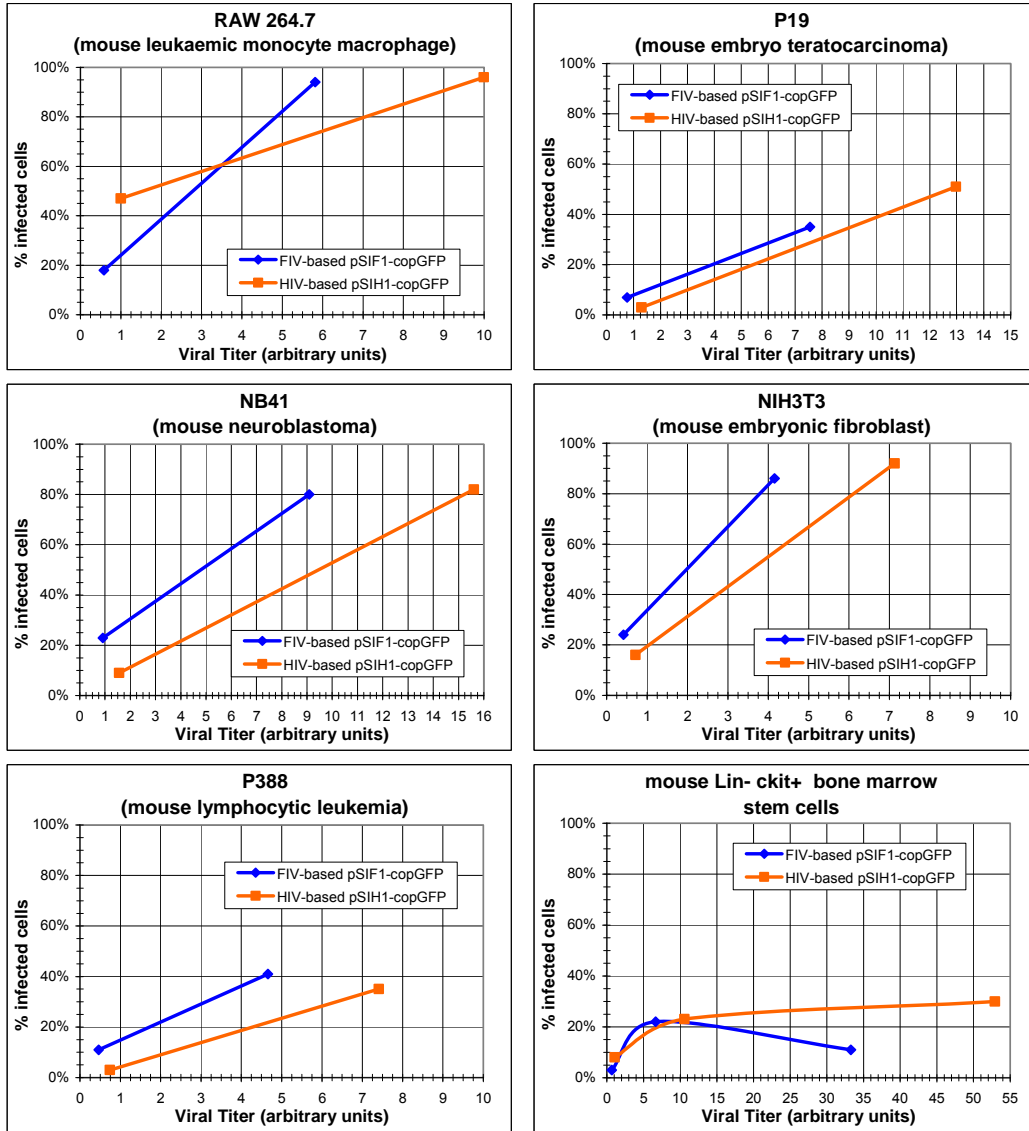
Human Cell Lines (cont'd)



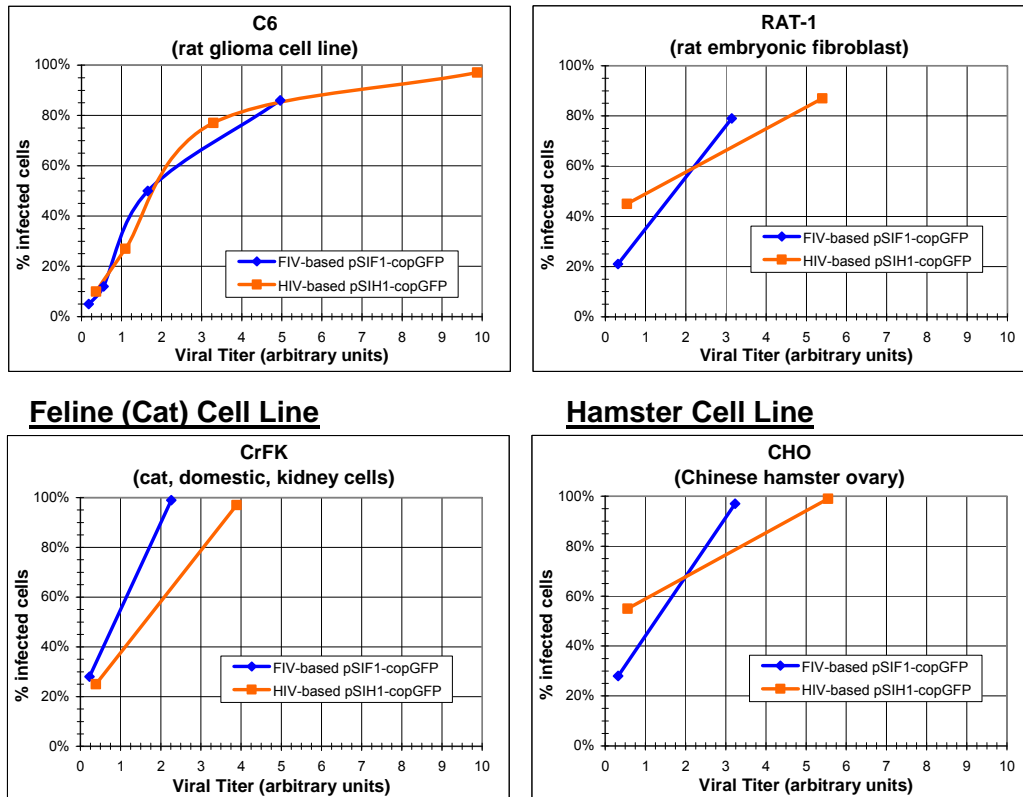
Human Primary/Stem Cell Lines



Mouse Cell Lines



Rat Cell Lines



E. Properties of the CopGFP Fluorescent Protein

The pSIF1-H1-siLuc-copGFP, pSIH1-copGFP, and pSIH1-H1-siLuc-copGFP Vectors contain the full-length copGFP gene with optimized human codons for high level of expression of the fluorescent protein from the CMV promoter in mammalian cells. The copGFP marker is a novel natural green monomeric GFP-like protein from copepod (*Pontellina sp.*). The copGFP protein is a non-toxic, non-aggregating protein with fast protein maturation, high stability at a wide range of pH (pH 4 – 12), that does not require any additional cofactors or substrates. The copGFP protein has very bright fluorescence that exceeds at least 1.3 times the brightness of EGFP, the widely used *Aequorea victoria* GFP mutant. The copGFP protein emits green fluorescence with the following characteristics:

emission wavelength max – 502 nm	quantum yield – 0.6
excitation wavelength max – 482 nm	extinction coefficient – 70,000 M ⁻¹ cm ⁻¹

Due to its exceptional properties, copGFP is an excellent fluorescent marker which can be used instead of EGFP for monitoring delivery of expression constructs into cells.

F. Related Products

- **UltraRapid Lentiviral Titer Kit** (Cat. # LV960A-1 [human cells], LV960B-1 [mouse cells])
Allows you to measure copy number (MOI) of integrated lentiviral constructs in genomic DNA of target cells after transduction with constructs made in any of SBI's FIV or HIV-based Lentivectors or GeneNet™ siRNA Libraries.
- **NF- κ B/293/GFP Transcriptional Reporter Cell Line** (Cat. # TR800A-1)
This human embryonic kidney (HEK)-293-based cell line with a 300-fold NF- κ B-dependent activation of a copGFP reporter gene—3 times more sensitive than competitor cell lines—makes analysis of Nuclear Factor kappa B (NF- κ B) pathway activation more sensitive and reliable.
- **LentiMag™ Magnetotransduction Kit** (Cat. # LV800A-1)
A novel, simple, and highly effective approach to increase transduction efficiency with SBI's lentiviral vectors compared to the standard method of Polybrene®-aided transduction.
- **PEG-*it*™ Virus Precipitation Solution** (Cat. # LV810A-1)
Simple and highly effective means to concentrate lentiviral vector inocula produced with SBI's pPACK Lentivector packaging systems.
- **Cloning and Expression Lentivectors** (many)
Choose from FIV and HIV-based shRNA, miRNA, cDNA, or TRE cloning and expression vectors. For a list of currently available vectors, please visit our website at www.systembio.com.

G. 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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1616 North Shoreline Blvd.
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Phone: (650) 968-2200
(888) 266-5066 (Toll Free)

Fax: (650) 968-2277

E-mail:

General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

VI. Licensing and Warranty Statement

Limited Use License

Use of the pPACK Lentivector Packaging Kit, Packaged Positive Transduction Control, or 293TN Producer Cell Line (*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.

The purchaser of the Product is granted a limited license to use the Product under the following terms and conditions:

The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use.

The Product may not be resold, modified for resale, or used to manufacture commercial products without prior written consent of SBI.

This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research.

FIV Vector System

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HIV Vector System

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CMV Promoter

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