



Lenti-miR™ Virus Library

Cat. #s PMIRHPLVA-1, PMIRHPLVAHT-1

User Manual

Store kit at -80°C on receipt

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

(ver. 1-2008.06.10)

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**** This Product shall be used by the purchaser for internal research purposes only and distribution is strictly prohibited without written permission by System Biosciences.**

I. Introduction and Background

A. Purpose of this Manual

This manual provides information about the design and production of the Lenti-miR™ virus library and detailed instructions on its use. The Lenti-miR™ virus library contains all of SBI's microRNA precursor clones as a pooled, ready-to-infect, VSV-G pseudotyped lentiviral preparation. For more detailed information regarding the individual clones that comprise the library, please refer to the user manual for the PMIRHxxxPA-1 product line, this is available online at www.systembio.com. Before using this reagent and associated materials provided in this kit, please read the entire manual.

B. MicroRNA Background

The term microRNA (miRNA) describes a novel class of small, non-coding RNAs which regulate gene expression post-transcriptionally by disrupting translation or directing cleavage of complementary mRNAs. These 17-26 nucleotide (nt) single-stranded miRNA molecules are synthesized as primary transcripts (pri-miRNA) that are often polycistronic, containing a small number of clustered miRNA units. Following transcription, and while the transcript still remains nuclear, the Drosha RNase III nuclease processes the pri-miRNA into ~70nt stem loop precursors (pre-miRNA). These pre-miRNA molecules are transported to the cytoplasm by a complex of proteins which includes the dsRNA binding protein Exportin-5, where they are processed to their final mature form by another RNase III nuclease, Dicer (Lee, 2002, Yi, 2003). It is here in the cytoplasm that mature miRNAs ultimately affect the protein levels of their target mRNAs by binding to complementary regions, and either inhibiting translation, or directing mRNA cleavage (Kim, 2005), see FIG 1.

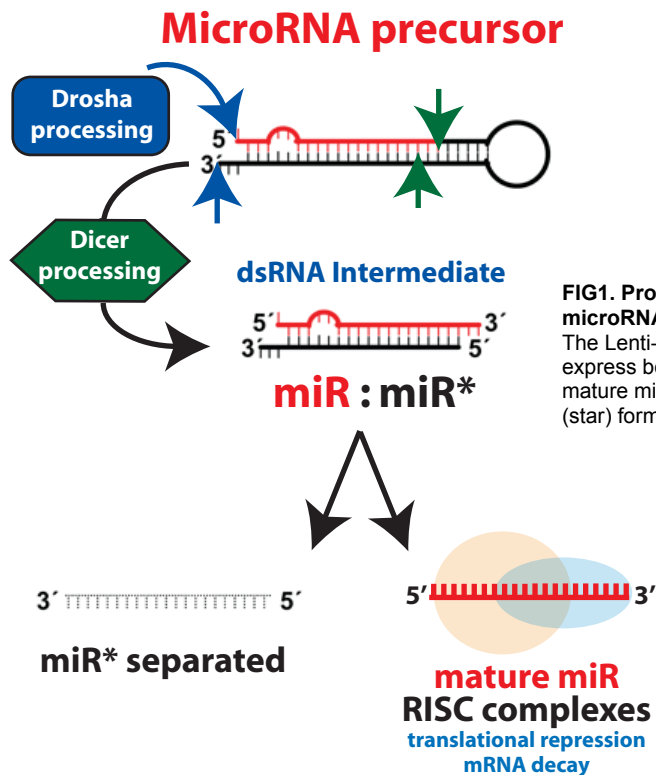


FIG1. Processing of microRNA precursors. The Lenti-miR library will express both the both the mature miR and the miR* (star) forms.

C. Product Description

The Lenti-miR™ virus library contains a pool of SBI's microRNA precursor clones in a ready-to-infect lentiviral preparation. Each virus within the pool will express an individual miRNA precursor in its native context while preserving putative hairpin structures to ensure biologically relevant interactions with endogenous processing machinery and regulatory partners. Details of the individual microRNA precursor clone construction and preparation of the virus pool are described in **Appendices A and B**, respectively.

Also included in this kit are a positive control virus (CD511VB-1) to assess transduction efficiency in your cells of interest and the oligonucleotide primers that may be used to identify the microRNA precursor sequence responsible for the observed phenotypes.

D. Intended use

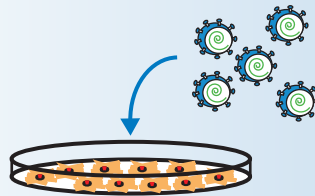
The Lenti-miR™ virus library is a tool that enables the study of phenotypic effects associated with the over-expression of individual microRNAs. The lentivirus preparation is pseudotyped with VSV-G that allows for broad cellular tropism. Hard-to-transfect mammalian cell lines, primary cells, non-dividing cells and even whole animal studies are possible. Transduced cells exhibiting the phenotypes of interest are isolated by selection or sorting. The microRNA or microRNAs responsible for generating the phenotypes of interest may be recovered through simple genomic PCR using lentivector-specific primers followed by direct sequencing of microRNA precursor clones.

Lenti-miR™ Virus Library Phenotypic Screens

Simultaneously over express SBI's full complement of Human MicroRNAs

Infect

- Cell lines
- Stem cells
- Animal tissues



Select

- cells exhibiting phenotype
- Tumor Invasion
 - Cell differentiation
 - Stress responses
 - Senescence
 - Apoptosis



Identify

- MicroRNA effectors
- Isolate desired cells
 - Recover genomic DNA
 - Amplify microRNA clone
 - Directly sequence

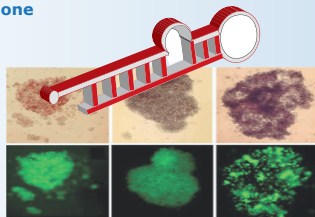
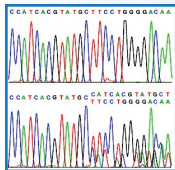
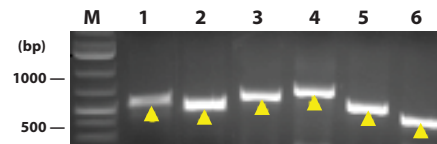
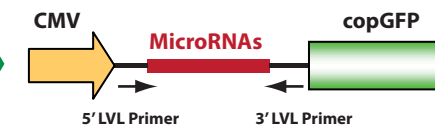


FIG 2. An overall experimental workflow for virus transduction, phenotypic selection is shown at left. Schematic for the recovery of the MicroRNA effectors is shown at bottom.

Identify MicroRNA Effectors



PCR to recover the exogenous pre-microRNA sequences

II. Protocol

A. General considerations:

- **The transduction efficiency of the Lenti-miR™ library** varies significantly for different cells and experimental conditions. In order to optimize transduction conditions, we recommend that you use the positive control CD511VB-1 virus provided in this kit to determine the optimal MOI for your cells of interest (see below).
- **Determining the Optimal MOI**
MOI is defined as the number of transducing units per cell. A number of factors can influence determination of an optimal MOI including the nature of your mammalian cell (actively- versus non-dividing), its transduction efficiency, your application of interest. If you are transducing the Lenti-miR virus library into your cells for the first time, we recommend using a range of MOIs (e.g. 1, 2, 5, 10) to determine the MOI required to obtain optimal expression for your particular application. Efficiency of transduction of your cell type with the positive control virus (CD511VB-1) or the Lenti-miR virus library can be monitored by copGFP expression.
- **Expression of the microRNA precursors** can be measured directly at about 48-72 hours after transduction. For more information, refer to the QuantiMir small RNA quantitation system's User manual (www.systembio.com).
- **Transient versus Stable screen?** Depending on the nature of your phenotypic screen, you may use the Lenti-miR virus library to transiently or stably transduce your cells. Transduced cells can be sorted by flow cytometry based upon copGFP expression.
- **Biosafety considerations.** Although these viral particles are replication-incompetent, they can infect mammalian cells and integrate into the host cell genome. For detailed biosafety information see **Appendix F**. Please follow all relevant Federal, State and Local regulations for working with BSL-2 class viruses.

B. Transduction of Lenti-miR Virus Library

The following protocol provides procedures for transduction of the Lenti-miR virus library into target cells and is optimized for adherent cell lines such as HeLa or MCF7 in a 6-well plate format. Use these guidelines as a starting point for determining optimal conditions for your cells and experiments. To use a different culture dish, adjust the number of cells, volumes of reagents and quantities of virus in proportion to the surface area of the dish/well (see Table below).

Tissue Culture Dish	Surface area per well (cm ²)	Suggested medium volume per well (ml)
100mm	56	10
60mm	20	4
35mm	8	2
6 well	9.4	2
12 well	3.8	1
24 well	1.9	0.5
96 well	0.3	0.2

Day 1.

1. Plate target cells in a 6-well plate at a density of 1×10^5 cells per well 24 hours prior to viral infection in 2 ml of complete medium with serum and antibiotics. Incubate cells at 37°C with 5% CO₂ overnight. Typically cells will be 30-50% confluent at the time of infection.

Day 2.

2. Dilute an appropriate amount (depending on the optimal MOI) of virus into 0.5 ml of complete medium. Add Polybrene (example LentiBrene, SBI cat# LV811A-1) to a final concentration of 5-8 µg/ml.

IN THIS EXAMPLE :

- 100,000 cells/well were seeded on Day 1.
- Assume doubling overnight, yields 200,000 cells/well on Day 2.
- To infect at an MOI of 5, use 1×10^6 IFUs virus per well.

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 the culture medium from cells. Infect target cells by adding the viral stock dilutions to the wells. For one well (mock well control) add 0.5 ml of D-MEM medium with Polybrene. Incubate cells at 37°C with 5% CO₂ overnight.

Day 3.

4. Remove the culture medium and replace with 2 ml of complete medium (without Polybrene). Incubate the cells at 37°C with 5% CO₂ overnight.

C. Phenotypic Selection

Day 4 to 6: By 48 to 72 hours post-transduction, copGFP expression will be apparent in infected cells.

- **Transient transduction screen:**

Begin your phenotypic analysis and select cells exhibiting the desired phenotypes.

- **Stable transduction screen:**

Begin your FACS sort for copGFP positive cells to enrich for stably transduced cells. Establish a stably-transduced cell population before performing your phenotypic analysis.

D. Recovery of Effector MicroRNA Clone Sequences

Materials required and not supplied in kit:

For Purification of genomic DNA

- DNeasy Tissue Kit (QIAGEN, Cat. # 69504 or equivalent)

For PCR Amplification

- Standard PCR kit (TITANIUM Taq PCR Kit, Clontech Cat# 639210 or equivalent)
- Thermal Cycler (DNA Engine, MJ Research, Cat. # PTC-200 or equivalent)
- 2.5% 1X TAE Agarose gel

1. Purify Genomic DNA from Selected Cells from Lenti-miR Phenotypic Screen

Prepare genomic DNA from selected cells according to manufacturer's recommendations. Measure the yield of DNA by spectrophotometer. You should expect to isolate 5-10 µg of genomic DNA from approximately 1×10^6 cells. Dilute the DNA sample in deionized water to a concentration of 0.2 µg/µl.

2. An sample PCR reaction for microRNA effector clone sequence amplification is provided below.

- A. Prepare a PCR Master Mix for all reaction tubes, plus one additional tube. Combine the following components in the order shown:

41	µl	Deionized H ₂ O
5	µl	10X PCR buffer
1	µl	50X dNTP mix (10 mM of each dNTP)
1	µl	LVL Primer Mix (10 µM of each primer)
1	µl	Purified Genomic DNA (~200ng)
1	µl	50X Taq DNA polymerase
<hr/>		
50	µl	Total volume

Note: Include no DNA negative control.

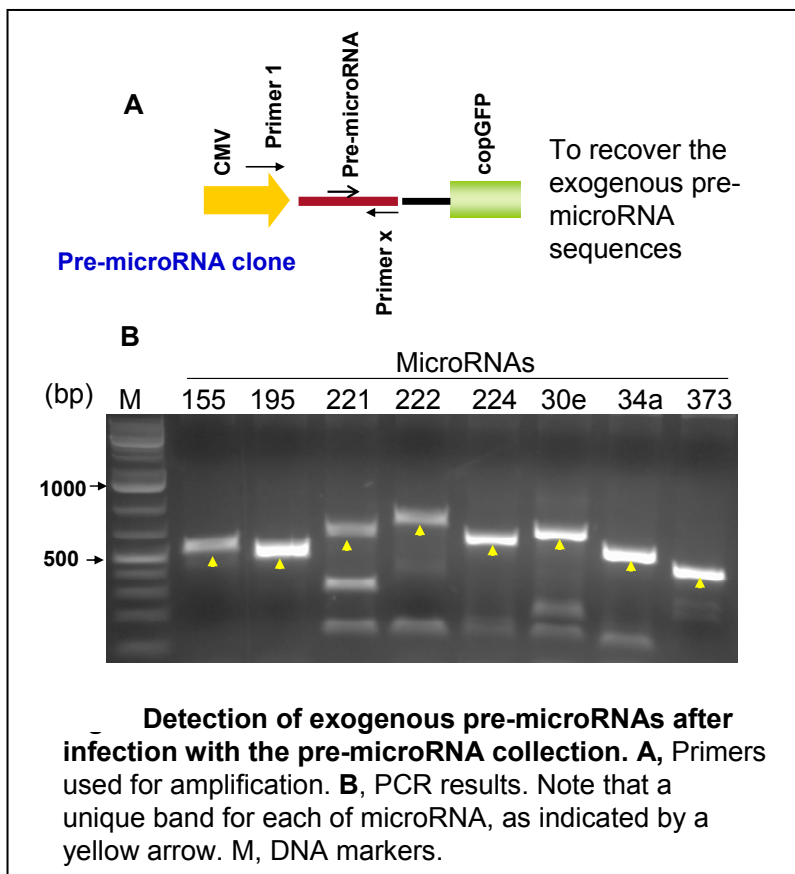
- B. Mix contents by vortexing, and spin the tube briefly in a microcentrifuge.
- C. Perform PCR amplification according to these guidelines:
- 94°C for 2 min
 - (94°C for 30 sec; 60°C for 30 sec) for 25 cycles
 - 68°C for 1 min
 - 15°C hold

- D. When the program is completed, analyze a 10-µl sample from each tube and suitable DNA size marker (200 bp-2 kb) on a 1.5% agarose/EtBr gel in 1X TAE. Note that each microRNA precursor clone within the library collection varies in length.

Therefore the expected amplicon size may range from 500 to 600 bp (see sample data on the following page).

- E. Sequence the effector microRNA amplicons recovered after gel purification using either the Forward or Reverse PCR primer (PCR primer sequences are provided in Appendix C.)

An example of a successful microRNA effector sequence amplification is shown below.



III. Troubleshooting

1. Poor Infection Efficiency

Too high a volume of infecting supernatant

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

The assay is carried out too early

Normally, the maximal expression of integrated provirus is expected to develop by 48 hours after infection. However, some cells display delayed expression. Try the assay at a later time, such as 72 or 96 hours.

Target cell line may be difficult to transduce

Optimize the transduction protocol. Use a higher MOI.

Wrong amount of Polybrene added during titration

Add and optimize Polybrene concentration in the range of 4-10 $\mu\text{g/ml}$.

Loss of pseudoviral titer during storage

Store pseudoviral stock at -80°C . Each freeze-thaw cycle drops the titer 2-4 fold. Use a fresh aliquot for transduction.

2. Infection Affects Target Cell Viability

High MOI may be toxic to some cell types. Reduce the amounts of virus used.

Polybrene is toxic for target cells

Optimize the concentration and exposure time to Polybrene during the transduction step.

3. No Expression of microRNA precursors

The CMV promoter is not functional in target cells

It is a very rare case, but the only way to solve this problem is to change the type of target cells.

4. No PCR product Amplified

The amount of genomic DNA in your sample is too low. Repeat purification of genomic DNA from infected cells, or measure the concentration of genomic DNA again using a smaller dilution factor.

5. Sequence of microRNA effector is ambiguous

If direct sequencing of the recovered and PCR-amplified microRNA effector does not produce a single sequence, your screen may have identified two or more co-effector microRNAs that may contribute to your phenotype. Your cells may have been transduced by more than one virus. The observed phenotype may be due to expression of one or more microRNAs. To identify and separate the individual microRNA effectors:

- A. Clone the amplicon(s) into a suitable PCR-cloning vector (TA or TOPO plasmids, Invitrogen).
- B. Sequence at least 10 individual clones to identify candidate effector microRNAs.
- C. Test individual candidates for the phenotype of interest. If no individual microRNA reproduces the observed phenotype, test combinations of the candidates identified above. All individual microRNA precursor clones are available from SBI.

IV. APPENDIX

A. Lenti-miR MicroRNA Precursor Constructs

System Biosciences' (SBI's) microRNA Precursor Construct Collection incorporates several unique features that set it apart from any commercially available miRNA collection.

Express microRNA precursor transcripts from their native genomic context

The inserts in SBI's **microRNA Precursor Construct Collection** represent more than just the mature microRNA sequences listed in Sanger's miRBase (<http://microrna.sanger.ac.uk/sequences/>). Each construct in SBI's collection consists of the stem loop structure and 300-500 base pairs of upstream and downstream flanking genomic sequence. This unique feature ensures that the microRNAs expressed from SBI's clones act as naturally as possible. The native structure ensures interaction with endogenous RNA processing machinery and regulatory partners, leading to properly cleaved microRNAs.

Lentiviral transduction system is effective and safe

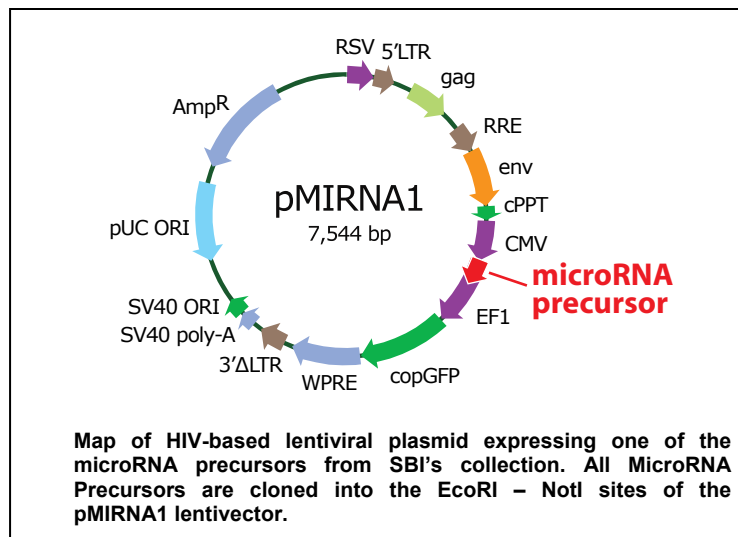
Each of SBI's miRNA precursor molecules has been cloned in a lentiviral-based vector. Like other standard plasmid vectors, SBI's miRNA construct can be used for transient expression of miRNA using conventional transfection protocols. Moreover, its lentiviral backbone allows each miRNA construct to be packaged in pseudoviral particles and introduced into non-dividing or difficult-to-transfect cell lines. In particular, all of the miRNAs have been cloned in SBI's HIV-based expression vectors. Replication incompetent HIV-based vectors are considered biologically safe.

RNA Polymerase-II promoter ensures robust expression from single copy integrants

While pol-III promoters are required to express very short siRNA and shRNA, the primary microRNAs can be expressed using conventional pol-II promoters (Stegmeier, 2005). The traditionally utilized pol-III promoters (H1 and U6) are constitutively expressed in all cell types, which complicates studies where knockdown of a gene product is lethal. Different pol-II promoters can alternatively provide either spatially or temporally defined expression (Shin, 2006). SBI's microRNA Precursor Clone Collection expresses each miRNA precursor from the CMV promoter. This robust strong viral promoter ensures a high level of expression from a single copy of integrated miRNA construct.

Dual expression design simplifies identification of transduced cells

The unique organization of the vector (**below**) results in expression of a another downstream transcript containing the copGFP fluorescent marker driven by the Human EF1 α promoter.



The HIV-1 derived pMIRNA1/pCDH vectors contain the following common features:

- **WPRE element**—enhances stability and translation of the CMV-driven transcripts.
- **SV40 polyadenylation signal**—enables efficient termination of transcription and processing of recombinant transcripts.
- **Hybrid RSV-5'LTR promoter**—provides a high level of expression of the full-length viral transcript in producer 293 cells.
- **Genetic elements (cPPT, GAG, LTRs)**—necessary for packaging, transducing, and stably integrating the viral expression construct into genomic DNA.
- **SV40 origin**—for stable propagation of the pMIRNA1 plasmid in mammalian cells.
- **pUC origin**—for high copy replication and maintenance of the plasmid in *E.coli* cells.
- **Ampicillin resistance gene**—for selection in *E.coli* cells.
- **copGFP**—fluorescent marker to monitor cells positive for transfection and transduction.

Properties of the copGFP Fluorescent Protein

The pMIRNA1 Vector contains 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), and 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;

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

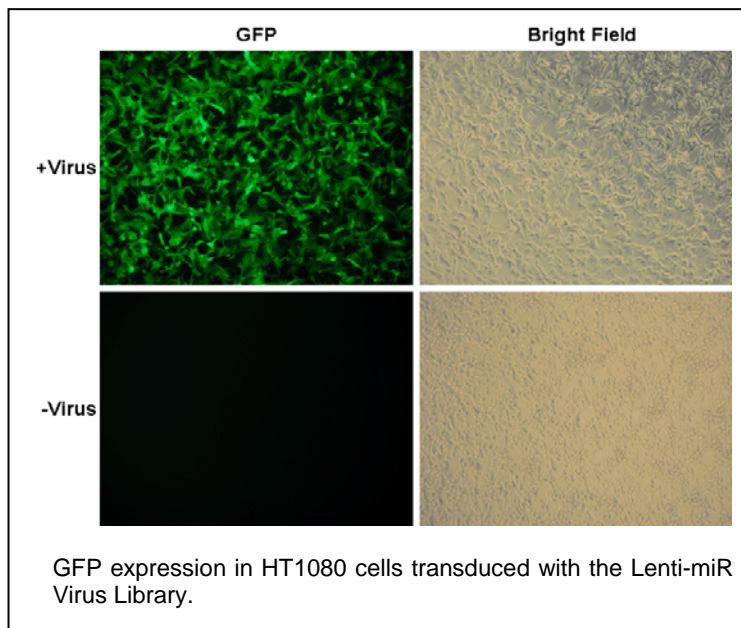
Due to its exceptional properties, copGFP is an excellent fluorescent marker which can be used instead of EGFP for monitoring delivery of lentivector constructs into cells.

B. Production and Titering of Lenti-miR Virus Library

The Lenti-miR Virus Library was prepared as follows. The full set of SBI's human precursor microRNA expression clones was pooled and packaged into VSV-G pseudotyped viral particles by co-transfecting 293TN Producer Cells (SBI Cat #LV900A-1) with the library clones and pPACKH1 (SBI Cat #LV500A-1) according to the procedures described in the "Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells". Virus was concentrated with PEG-it Virus Precipitation Solution (SBI Cat #LV810A-1) according to the instructions in the User Manual. Infectious titers were determined on HT1080 cells using the UltraRapid Lentiviral Titering Kit for Human Cells (SBI Cat#LV960A-1) according to the recommended procedures. Additional information and User Manuals for each of these products are available on our website: www.systembio.com.

Sample Transduction Data

Easily monitor infection efficiency by GFP expression.



C. List of Kit Components

- Aliquots of pooled Lenti-miR virus library
- Tube of positive infection control virus (CD511VB-1)
- Tube of LVL PCR Primer Mix (10 μ M of each primer)
 - o pCDH-5.1: 5' - GCC TGG AGA CGC CAT CCA CGC TG - 3'
 - o pCDH-3.1: 5' - GAT GTG CGC TCT GCC CAC TGA C - 3'

D. Related Products

- **pPACKH1™ Lentivector Packaging Kit (Cat. # LV500A-1)**
Unique lentiviral vectors that produce all the necessary HIV viral proteins and the VSV-G envelope glycoprotein from vesicular stomatitis virus required to make active pseudoviral particles. 293TN cells (SBI, Cat. # LV900A-1) transiently transfected with the pPACKH1 and a pMIRNA1 microRNA expression construct produce packaged viral particles containing a microRNA construct.
- **293TN Human Kidney Producer Cell Line (SBI, Cat. # LV900A-1).**
For packaging of plasmid lentivector constructs.
- **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.
- **LentiBrene (Cat. # LV811A-1).**
- **Lentivector UltraRapid Titer PCR Kit (Cat. # LV960A-1 [for human cells], LV960B-1 [for 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.
- **QuantiMir small RNA quantitation system (Cat. # RA420A-1)**
Easily profile miRNAs using a single cDNA synthesis step and real-time qPCR.
- **MicroRNA Precursor Clone collection (cat#s pMIRHxxxPA-1)**
SBI's collection of individual microRNA precursor expression clones in lentivectors.

E. 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:

System Biosciences (SBI)
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Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

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).

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' Δ LTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter (in HIV-based vectors) and CMV promoter (in FIV-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 SBI's lentivectors 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/bmb14/bmb14s3.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, leak proof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.
- Please keep in mind that pMIRNA1 vectors are integrated into genomic DNA and could have a risk of insertional mutagenesis.

V. References

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VI. Licensing and Warranty Statement

Limited Use License

Use of the Lenti-miR virus library (*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.

HIV Vector System

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