MicroRNA Precursor Constructs

Cat. # MIFCZ301A-1 – MIFCZ341A-1

User Manual

Store kit at -20°C on receipt

(ver. 1-061124)

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.

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I. Introduction and Background

A. Purpose of this Manual

This manual provides details and information about microRNA Precursor Constructs in SBI's pMIF lentivectors. This manual does not include information on packaging the pMIF expression constructs into pseudotyped viral particles or transducing your target cells of choice with these particles. This information is available in the user manual Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells, which is available on the SBI website (www.systembio.com). Before using the reagents and material supplied with this system, please read the entire manual.

B. Overview

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).

Initially discovered in *C. elegans* as subtle regulators of cell fate, miRNAs have since been identified in all metazoan organisms, controlling such vital processes as cell proliferation, differentiation, signal transduction, and cell death. The lin-4 and let-7 miRNAs of the nematode were found to modulate gene expression by binding to complementary sites in the 3'-UTR of the target gene's mRNA (Lee, 1993, Olsen, 1999). This binding of microRNAs affords the cell post-transcriptional control over gene expression, and adds another layer to the already complex gene expression regulatory network. Recently, miRNAs were implicated in the biogenesis of human diseases. It was shown that human lung cancer tumors had distinctly lower levels of the let-7 miRNA (Johnson, 2005). This is a critical observation, because the RAS proto-oncogene family has sites that are complementary to let-7, enabling let-7 mediated control of RAS expression. RAS proteins were upregulated in these lung tumors,

suggesting that the perturbation in let-7 expression led to overexpression of RAS and thus oncogenesis.

Viruses which cause human disease also utilize miRNAs to regulate both host and viral gene expression. Pfeffer, et. al (Pfeffer, 2004) found that human cell lines latently infected with Epstein-Barr herpesvirus expressed miRNA of viral origin. Computational analysis of the potential targets of these miRNA revealed predicted interactions with cellular genes involved in proliferation, apoptosis, immune response, and other vitally important pathways. Human immunodefiency virus (HIV) is also predicted to express a small subset of miRNAs that have a broad range of predicted cellular targets (Bennasser, 2004). Conversely, cellular miRNAs expressed in T-cells are predicted to interact with the viral transcripts (Hariharan, 2005).

C. Tools for Functional Study of MicroRNA

As the identification of novel microRNAs continues, hundreds of isolated miRNAs and thousands of predicted conserved miRNAs (John, 2006) have been published. Yet, very few have had their functions experimentally validated. There is an increasing need for robust methods to study the functions of each isolated or predicted microRNA.

The only commercially available product for the functional study of miRNAs is the collections of synthetic miRNA molecules based on predicted mature miRNA sequence. Despite their optimized design criteria, synthetic miRNAs underscore the importance of primary miRNA in its native expressed form. The primary microRNA contains critical biological components involved in mature miRNA expression and cellular processing, it often been processed into several mature miRNA molecules. The second major drawback of synthetic miRNA molecules is that their knockdown effect in the target cell is transient and usually disappears 2-3 days after transfection.

SBI's microRNA Precursor constructs express each individual miRNA precursor in its native context while preserving putative hairpin structures to ensure biologically relevant interactions with endogenous processing machinery and regulatory partners. The lentiviral expression system also makes it possible to have a stable knockdown effect after being introduced into the cells.

D. Unique Features of SBI's 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 100-200 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.

A similar miRNA expression system has been used to create a human miRNA expression library for genetic screening (Mathijs Voorhoeve, 2006). In the library, each retroviral construct contains an approximately 500 bp fragment spanning a given miRNA genomic region under the control of the CMV promoter. The functional study implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors.

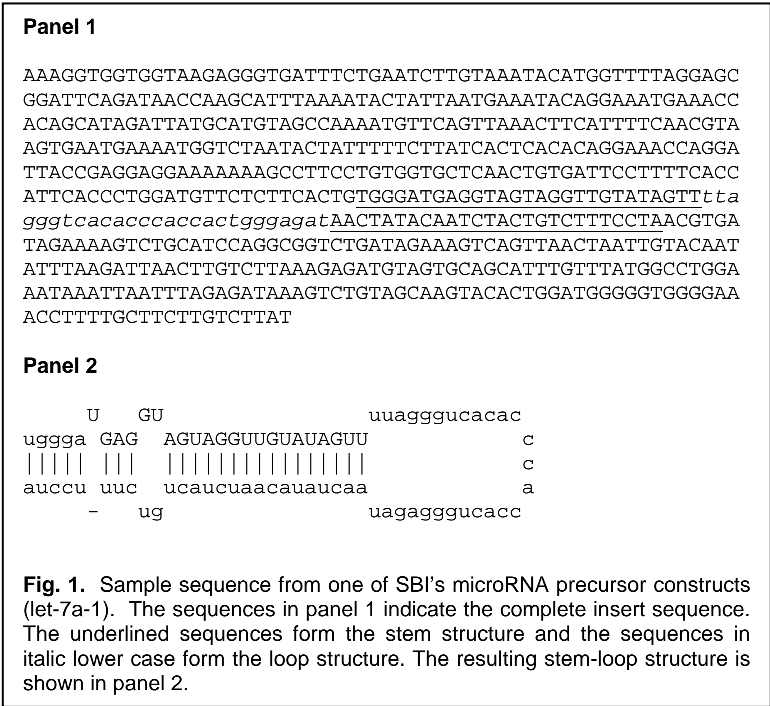

Fig. 1. Sample sequence from one of SBI's microRNA precursor constructs (let-7a-1). The sequences in panel 1 indicate the complete insert sequence. The underlined sequences form the stem structure and the sequences in italic lower case form the loop structure. The resulting stem-loop structure is shown in panel 2.

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 FIV-based expression vectors. Replication incompetent FIV-based vectors are considered a biologically safe alternative to HIV-based vectors as they are derived from a non-human feline virus.

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.

Polycistronic design simplifies identification of transduced cells

The unique organization of the vector results in expression of a polycistronic transcript containing the copGFP fluorescent marker, Zeocin resistance gene, and the precursor microRNA (Figure 2). This direct linkage enables simple identification and enrichment of cells expressing the microRNA precursor.

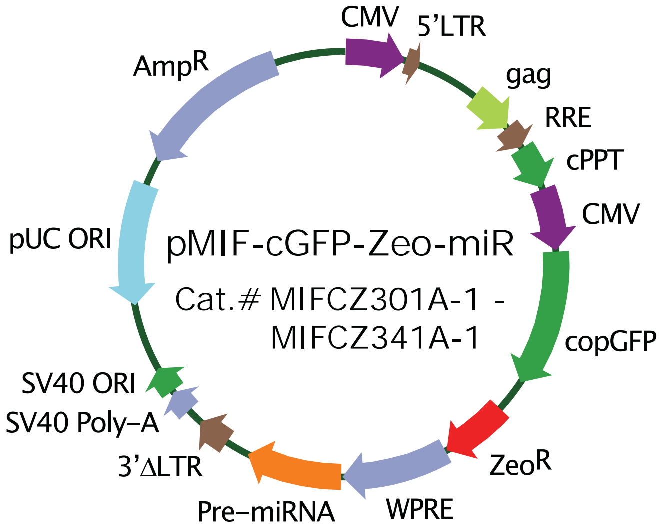

Fig. 2. Map of FIV-based lentiviral plasmid expressing one of the microRNA precursors from SBI's collection.

E. List of Components

MicroRNA Precursor Construct Collection

Catalog #	Accession #	Mature microRNA ID	Vector
MIFCZ301PA-1	MI0000060	hsa-let-7a-1	pMIF-cGFP-Zeo
MIFCZ302PA-1	MI0000061	hsa-let-7a-2	pMIF-cGFP-Zeo
MIFCZ303PA-1	MI0000064	hsa-let-7c	pMIF-cGFP-Zeo
MIFCZ304PA-1	MI0000065	hsa-let-7d	pMIF-cGFP-Zeo
MIFCZ305PA-1	MI0000067	hsa-let-7f-1	pMIF-cGFP-Zeo
MIFCZ306PA-1	MI0000068	hsa-let-7f-2	pMIF-cGFP-Zeo
MIFCZ307PA-1	MI0000077	hsa-mir-21	pMIF-cGFP-Zeo
MIFCZ308PA-1	MI0000083	hsa-mir-26a-1	pMIF-cGFP-Zeo
MIFCZ309PA-1	MI0000086	hsa-mir-28	pMIF-cGFP-Zeo
MIFCZ310PA-1	MI0000087	hsa-mir-29a	pMIF-cGFP-Zeo
MIFCZ311PA-1	MI0000088	hsa-mir-30a	pMIF-cGFP-Zeo
MIFCZ312PA-1	MI0000089	hsa-mir-31	pMIF-cGFP-Zeo
MIFCZ313PA-1	MI0000090	hsa-mir-32	pMIF-cGFP-Zeo

MIFCZ314PA-1	MI0000091	hsa-mir-33	pMIF-cGFP-Zeo
MIFCZ315PA-1	MI0000097	hsa-mir-95	pMIF-cGFP-Zeo
MIFCZ316PA-1	MI0000100	hsa-mir-98	pMIF-cGFP-Zeo
MIFCZ317PA-1	MI0000101	hsa-mir-99a	pMIF-cGFP-Zeo
MIFCZ318PA-1	MI0000102	hsa-mir-100	pMIF-cGFP-Zeo
MIFCZ319PA-1	MI0000103	hsa-mir-101-1	pMIF-cGFP-Zeo
MIFCZ320PA-1	MI0000105	hsa-mir-29b-1	pMIF-cGFP-Zeo
MIFCZ321PA-1	MI0000107	hsa-mir-29b-2	pMIF-cGFP-Zeo
MIFCZ322PA-1	MI0000108	hsa-mir-103-2	pMIF-cGFP-Zeo
MIFCZ323PA-1	MI0000109	hsa-mir-103-1	pMIF-cGFP-Zeo
MIFCZ324PA-1	MI0000111	hsa-mir-105-1	pMIF-cGFP-Zeo
MIFCZ325PA-1	MI0000112	hsa-mir-105-2	pMIF-cGFP-Zeo
MIFCZ326PA-1	MI0000114	hsa-mir-107	pMIF-cGFP-Zeo
MIFCZ327PA-1	MI0000238	hsa-mir-196a-1	pMIF-cGFP-Zeo
MIFCZ328PA-1	MI0000239	hsa-mir-197	pMIF-cGFP-Zeo
MIFCZ329PA-1	MI0000240	hsa-mir-198	pMIF-cGFP-Zeo
MIFCZ330PA-1	MI0000242	hsa-mir-199a-1	pMIF-cGFP-Zeo
MIFCZ331PA-1	MI0000251	hsa-mir-208	pMIF-cGFP-Zeo
MIFCZ332PA-1	MI0000253	hsa-mir-148a	pMIF-cGFP-Zeo
MIFCZ333PA-1	MI0000254	hsa-mir-30c-2	pMIF-cGFP-Zeo
MIFCZ334PA-1	MI0000255	hsa-mir-30d	pMIF-cGFP-Zeo
MIFCZ335PA-1	MI0000262	hsa-mir-147	pMIF-cGFP-Zeo
MIFCZ336PA-1	MI0000265	hsa-mir-7-3	pMIF-cGFP-Zeo
MIFCZ337PA-1	MI0000266	hsa-mir-10a	pMIF-cGFP-Zeo
MIFCZ338PA-1	MI0000267	hsa-mir-10b	pMIF-cGFP-Zeo
MIFCZ339PA-1	MI0000268	hsa-mir-34a	pMIF-cGFP-Zeo
MIFCZ340PA-1	MI0000269	hsa-mir-181a-2	pMIF-cGFP-Zeo
MIFCZ341PA-1	MI0000459	hsa-mir-143	pMIF-cGFP-Zeo

Component	**Conc.**	**Amount**
microRNA Precursor Construct	varies	10 μg

Each construct contains 10 μg of endotoxin-free plasmid DNA that is ready for packaging or transfection. The concentration will vary between constructs and lots. All constructs are shipped in dry ice and should be stored at -20°C upon receipt. Properly stored constructs are stable for 12 months from the date received.

F. Additional Required Materials

Transfection of pMIF Constructs into Target Cells

- Transfection reagent
(Recommended: Lipofectamine™ 2000, Invitrogen, Cat. # 11668-027)

Packaging of pMIF Constructs in Pseudoviral Particles

- pPACKF1 Lentivector Packaging Kit (SBI, Cat. # LV100A-1)
- 293TN Producer Cell Line (SBI, Cat # LV900A-1; or ATCC, 293T/17, Cat # CRL-11268)
- Lipofectamine™ Transfection Reagent (Invitrogen, Cat. # 18324-111)
- Plus™ Reagent (Invitrogen, Cat. # 11514-015)

G. Safety Guidelines

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). Work with FIV-based viruses falls within NIH Biosafety Level 2 criteria. For a detailed description of laboratory biosafety level criteria, consult the following pages on the Centers for Disease Control Office of Health and Safety Web site:

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm
http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Also, you should consult the health and safety guidelines and officers at your institution regarding use and handling of the FIV lentiviral system. In addition, although the system itself has been designed to minimize possible risk, specific recombinant FIV vector constructs may be potentially hazardous, depending on the nature of introduced insert (such as oncogenes, toxins, siRNA to tumor suppressor genes, etc.). For these reasons, it is critical to exercise due caution while working with recombinant lentiviruses.

To ensure safe laboratory handling, you should thoroughly understand the biology of the lentiviral vectors and the specific modifications and design features of the SBI system with which you are working. The original FIV viral vector was developed by Eric M. Poeschla, David J. Looney, and Flossie Wong-Staal in UCSD (Poeschla 2003). Based on this original FIV vector, the pMIF Vectors for cloning and expressing microRNA were developed at SBI. This vector has been modified to remove sequences that overlap with the packaging plasmid to minimize the possibility for homologous recombination and generation of self-replicating viral sequences

when co-transfecting these constructs into packaging cells. SBI's pMIF vectors also have a deletion in the enhancer of the U3 region of 3' ΔLTR to ensure self-inactivation of the lentiviral vector after transduction and integration of the sequences into the genomic DNA of the target cells.

To avoid any possible contamination and maintain a clean laboratory environment we also recommend following these standard safety practices:

- Wear double gloves, face protection, and lab coat at all times.
- Perform work in a limited access area in a Biological Safety Cabinet Class II and post biohazard warning signs.
- Minimize splashes or aerosols with careful pipetting.
- Take precautions with needles, blades, etc.
- Decontaminate work surfaces at least once a day and after any spill of viable material.
- Decontaminate all cultures, stocks, and other biological wastes before disposal using approved decontamination methods, such as autoclaving. Before decontamination the biological materials should be placed in a sealed, durable, leak-proof container for transport from the laboratory.
- Please keep in mind that pMIF vectors are integrated into genomic DNA and could have a risk of insertional mutagenesis.

II. Protocol

A. Applications of SBI's MicroRNA Precursor Constructs

The major advantage of SBI's microRNA precursor constructs compared to synthetic microRNA is that our constructs can be integrated into cells via the lentiviral expression system. With the lentiviral system, it is possible to generate stable cell lines that express specific microRNA. Since microRNAs are believed to be involved in various cellular pathways, these stable cell lines would provide a unique tool that allows you to link microRNA with biological functions.

To make a stable cell line, you first have to package the construct into pseudoviral particles (see Section B). After infecting your target cells with the pseudovirus-containing supernatant, you can select infected cells expressing a high level of copGFP by FACS sorting or enrich the infected cells by Zeocin selection.

Cells that stably express specific microRNA precursors can be also used to monitor other gene expression in order to identify miRNA target genes. Alternatively, the microRNA precursor construct can be cotransfected with a reporter construct that expresses putative microRNA target sequences. By measuring the reporter gene expression in transfected or transduced cells, the putative microRNA target gene can be confirmed.

B. Packaging SBI's Cloned MicroRNA Precursor Constructs

SBI's cloned microRNA precursor constructs can be efficiently packaged into VSV-G pseudotyped viral particles using SBI's pPACKF1 Packaging Plasmid Mix (Cat. # LV100A-1). The provided miRNA precursor expression plasmid was purified using a QIAGEN Endotoxin-free plasmid kit to ensure maximum transfection efficiency. For a detailed packaging protocol, please refer to SBI's pPACK user manual Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells, which is available on the SBI website (www.systembio.com).

C. Transfection of the microRNA Precursor Construct

The microRNA precursor construct can be introduced together with a reporter construct into HeLa or HEK 293 cells using chemical transfection. For example, with these cells, the Lipofectamine™ Reagent (Invitrogen, Cat. # 18324-111) with Plus™ Reagent (Invitrogen, Cat. # 11514-015) system works well. Alternatively, you can use your target cells for this analysis. If you have already established a transfection method for your target cells, use your established conditions. If you do not have an established transfection protocol, we recommend you compare efficiencies of several transfection procedures (*e.g.*, Invitrogen's Lipofectamine™ 2000, Cat. # 11668-027, Clontech's CLONfectin™, Cat. # 631301). It is important to optimize the selected transfection protocol and keep the parameters constant between testing samples and control samples.

III. References

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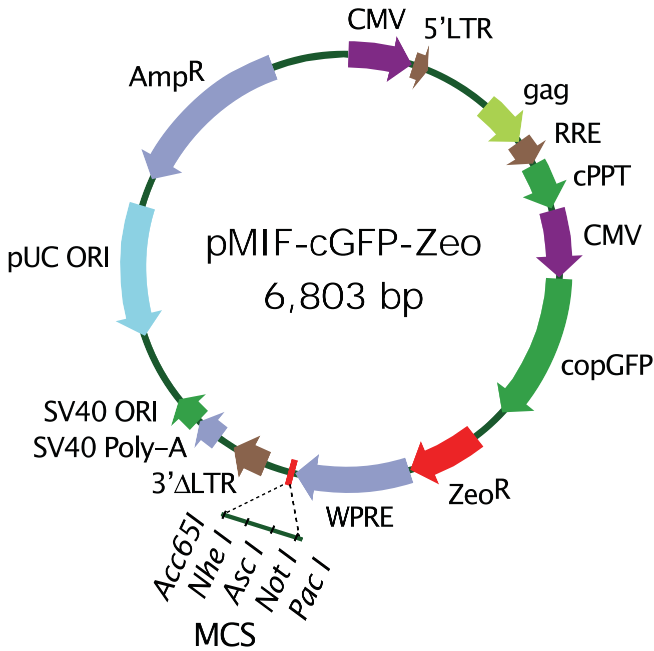
IV. Appendix

A. Map of pMIF-cGFP-Zeo



B. Features of pMIF-cGFP-Zeo

Feature	Location*	Function
CMV-5' LTR	1-415	Hybrid CMV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	762-1011	Packaging signal
RRE	1012-1143	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1150-1390	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1391-1745	Human cytomegalovirus (CMV)--constitutive promoter for transcription of dscGFP
copGFP	1753-2508	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/ transduced cells
ZeoR	2692-3066	Zeocin-resistant marker for selection of the transfected/transduced cells
WPRE	3072-3660	Posttranscriptional regulatory element which enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3816-3999	Required for viral reverse transcription; self-inactivating 3' LTR with deletion in U3 region prevents formation of replication-competent viral particles after integration into genomic DNA
SV40 Poly-A	4087-4218	Transcription termination and polyadenylation
SV40 Ori	4227-4373	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4743-5416(C)	Allows for high-copy replication in *E. coli*
AmpR	5561-6421(C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

* The notation (C) refers to the complementary strand.

C. Properties of the copGFP Fluorescent Protein

The pMIF-cGFP-Zeo 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.

D. Related Products

- **pPACKF1™ Lentivector Packaging Kit (Cat. # LV100A-1)**
  Unique lentiviral vectors that produce all the necessary FIV 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 pPACKF1 and a pMIF microRNA expression construct produce packaged viral particles containing a pMIF microRNA construct.

- **293TN Human Kidney Producer Cell Line** (SBI, Cat. # LV900A-1)
  For packaging of plasmid lentivector constructs.

- **pSIF1-H1-siLuc-copGFP Packaged Positive Transduction Control** (Cat. # LV201B-1)
  The Packaged Positive control lentivector allows you to measure transduction efficiency in target cells based on percent of GFP-positive cells.

- **LentiMag™ Magnetotransduction Kit** (Cat. # LV800A-1)
  A novel, simple, and highly effective approach to increase the number of cells positively transduced with SBI's HIV- and FIV-based lentiviral vectors compared to the standard method of Polybrene®-aided transduction.

- **Lentivector Rapid Titer PCR Kit** (Cat. # LV950A-1 [for human cells], LV951A-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.

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)
1616 North Shoreline Blvd.
Mountain View, CA 94043

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

V. Licensing and Warranty Statement

Limited Use License

Use of the MicroRNA Precursor Construct (*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

This Product is for non-clinical research use only. Use of this Product to produce products for sale or for any diagnostic, therapeutic, clinical (including pre-clinical), veterinary or high throughput drug discovery purpose (the screening of more than 10,000 compounds per day) is prohibited. In order to obtain a license to use this product for these commercial purposes, contact The Regents of the University of California. This Product or the use of this Product is covered by U.S. Patent No. 6,555,107 owned by The Regents of the University of California.

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CMV Promoter

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CopGFP Reporter

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