Murine microRNA Precursor Constructs Cat. #MMIR-xx

User Manual

Store Bacterial streaks at 4˚C

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ver. 1-032812

Contents

I. Introduction and Background

A. Purpose of this Manual

This manual provides details and information about Murine microRNA Precursor Constructs in SBI lentivector (parent SBI vector is pCDH-CMV-MCS-EF1-GFP-T2A-Puromycin; SBI cat# CD513B-1). This manual does not include all the information on packaging the lentivector 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 immunodeficiency 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, many 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 murine 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

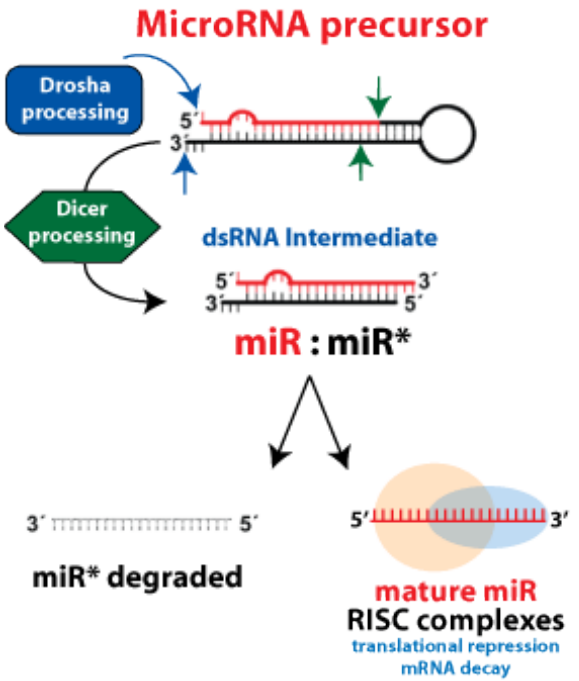
System Biosciences' (SBI's) microRNA Precursor Construct Collection incorporates several unique features that set it apart from any commercially available miRNA collection.

1. Express microRNA precursor transcripts from their native genomic context

The inserts in SBI's murine 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.



2. Lentiviral transduction system is effective and safe

Each of SBI's murine miRNA precursor molecules has been cloned in a lentiviral-based vector. Like other standard plasmid vectors, SBI's miRNA constructs 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, difficult-to-transfect cell lines, or introduced *in vivo.*

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.

3. 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 complicate 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 murine 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.

4. Dual expression design simplifies identification of transduced cells

The unique organization of the vector (Fig 1.) results in expression of another downstream transcript containing the GFP fluorescent and purimycin resistant gene driven by the Human EF1α promoter (see Fig 2.).

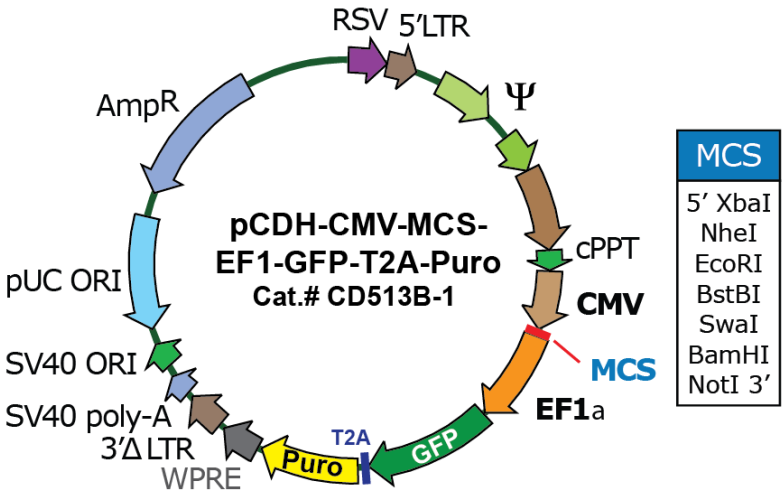


Fig. 1. Map of HIV-based lentiviral plasmid expressing one of the microRNA precursors from SBI's MMIR collection.

The HIV-1 derived 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 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.
- **GFP:** fluorescent marker to monitor cells positive for transfection and transduction.
- **Puromycin resistant gene:** puromycin selection marker to enrich transuded cells and establish stable cell lines.

5. Easily monitor expression-positive cells using GFP fluorescence.

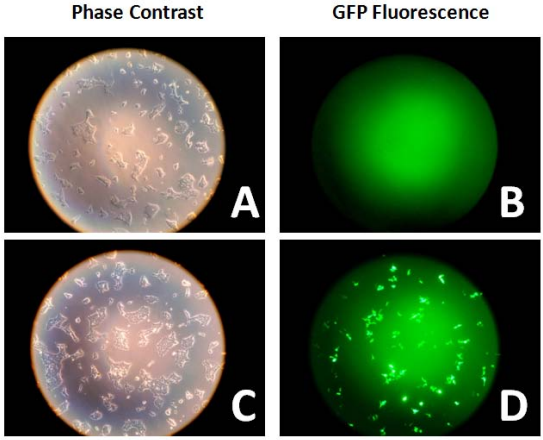
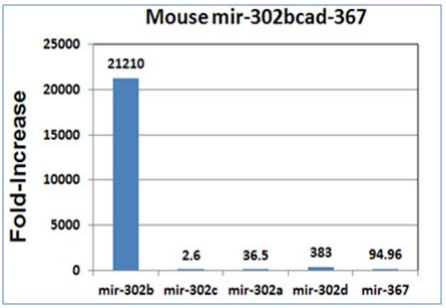


Fig. 2. Transfection and expression of microRNAs can be easily monitored using GFP fluorescence. HEK293 cells were transfected with 500 ng MMIR-465-PA-1 construct DNA or mock controls using Lipofectamine 2000 (Invitrogen). Pictures were taken 24 hrs after transfection. A and B) Mock transfected cells do not appear green. C and D) GFP-positive cells cells appear in the cells transfected with MMIR-465-PA-1.

6. Robust mature miRNAs produced using SBI's Murine MicroRNA Precursor Constructs.



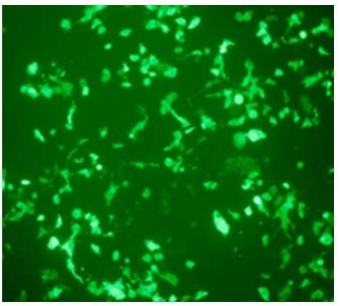


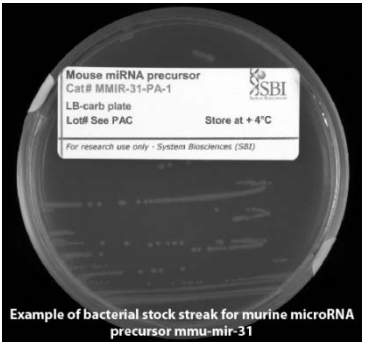
Fig. 3. High levels of microRNAs detected from MMIR constructs. Real-time qPCR with the QuantiMir small RNA quantification system (Cat. # RA420A-1) was used to evaluate expression from the MMIR clones shown to the left. Cells successfully transduced can simply be monitored by fluorescent microscopy.

E. List of Components

- The microRNA precursor constructs are shipped in bacterial stock form (*E. coli*) plated on LB-carbenicillin at 50μg/ml (same as ampicillin, but more stable over time for shipments).

- Upon receipt, individual colonies may be apparent already. If no colonies are visible upon receipt, incubate the LB-carbenicillin overnight at 37ºC. If there are no apparent colonies after overnight incubation, please contact SBI immediately for a replacement (650-968-2200).

- **To propagate your construct in liquid medium, we recommend using LB-carbenicillin at 50 μg/ml and growth at 30 ºC. LB-Ampicillin at 50 μg/ml can also be substituted.**



Example of bacterial stock streak for murine microRNA precursor mmu-mir-31

F. Additional Required Materials

- QIAGEN Endotoxin-free Plasmid Preparation Kit
- PureFection Transfection reagent (SBI, Cat# LV750A-1)
- Viral Packaging supplies (for packaging MMIR constructs into lentiviral particles). We recommend all products from SBI. Please see the SBI website for more details, or contact technical support for assistance.

G. 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'ΔLTR 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/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, 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.

II. Protocols

A. Applications of SBI's Murine 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 of Protocol). After infecting your target cells with the pseudovirus-containing supernatant, you can isolate infected cells expressing a high level of GFP by FACS sorting or by puromycin drug 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. Transfection of the Murine microRNA Precursors

Murine microRNA Precursor plasmids can be transfected into your target cells using the method you usually use for transfecting or electroporating your cells. We also recommend using SBI's PureFection transfection reagent according to the instructions provided in the PureFection user manual.

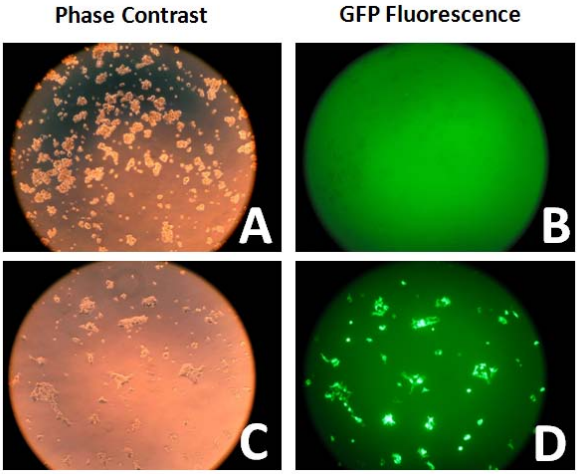


Fig. 4. Sample trasfection data. HEK293 cells were transfected using a standard transfection protocol using either a mock construct (A and B) or a murine MMIR-465-PA-1 construct (C and D).

C. Packaging Murine microRNA Precursors into lentiviral particles

SBI's cloned murine microRNA precursor constructs can be efficiently packaged into VSV-G pseudotyped viral particles using SBI's pPACKH1 Packaging Plasmid Mix (Cat.# LV500A-1). 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).

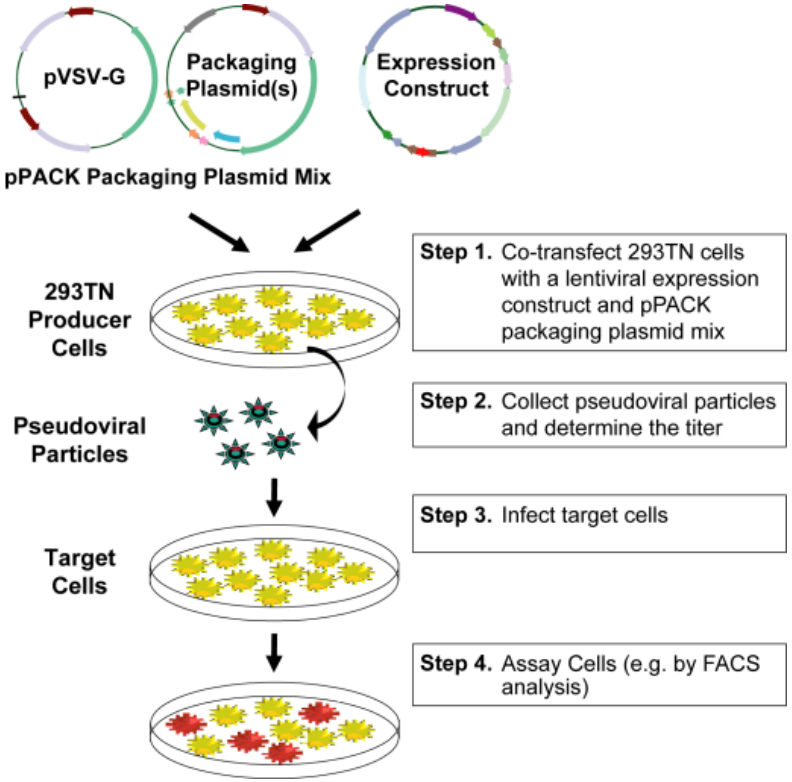


Fig. 5. Example viral packaging protocol.

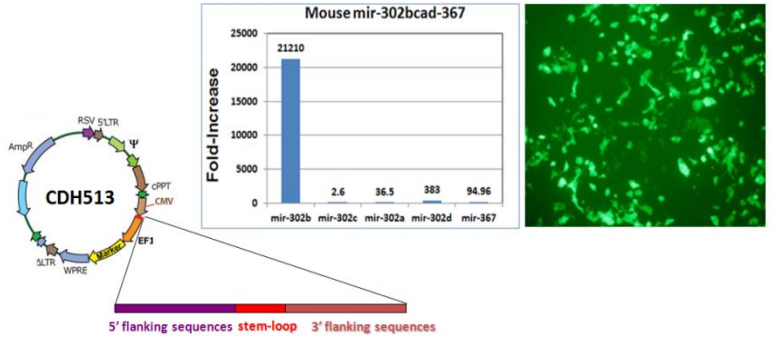


Fig. 6. Sample Transduction data with mouse mir-302bcad-367 cluster. Left is data from SBI's QuantiMir kit qPCR-based microRNA expression profiling. Right is GFP fluorescence.

D. Verifying Expression of the murine microRNA construct

SBI recommends using the QuantiMir kit (Cat# RA420A-1) to verify expression of the microRNA in your target cells. For more details on QuantiMir, please see the SBI website.

E. Sequencing the murine microRNA precursor construct

1. CMV forward primer:5'- GTGGGAGGTCTATATAAGCAG-3'

2. EF1 rev primer: 5' – GCACCCGTTCAATTGCCG – 3'
The sequence read will be the reverse compliment of the "sense" strand miRNA precursor.

3. You can use www.mirbase.org to verify the sequence in the murine microRNA precursor construct.

III. References

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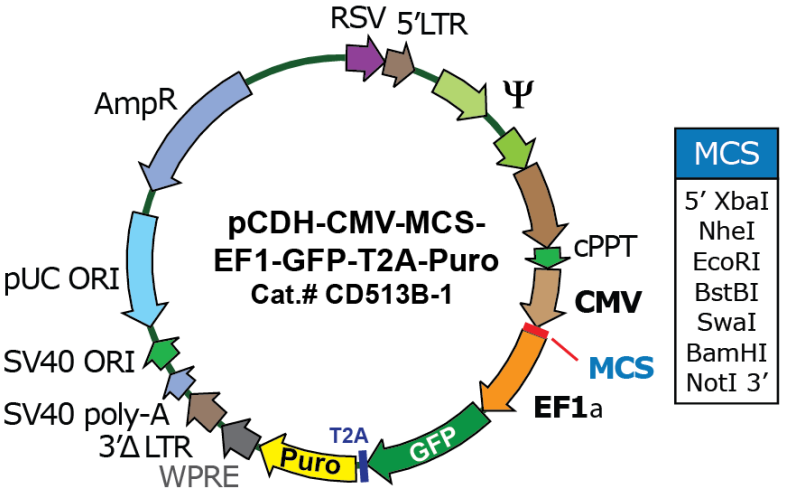
IV. Appendix

A. Map of the murine microRNA precursor vector backbone



B. Properties of the copGFP Fluorescent Protein

The Murine microRNA precursor 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.

V. 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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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

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Use of the Murine 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.

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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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CopGFP Reporter

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SBI has pending patent applications on various features and components of the Product. For information concerning licenses for commercial use, contact SBI.

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