pGreenFire™ Pathway Reporter Lentivectors Cat. # TR0XX Series

User Manual

Contents

I. Introduction and Background

A. Overview

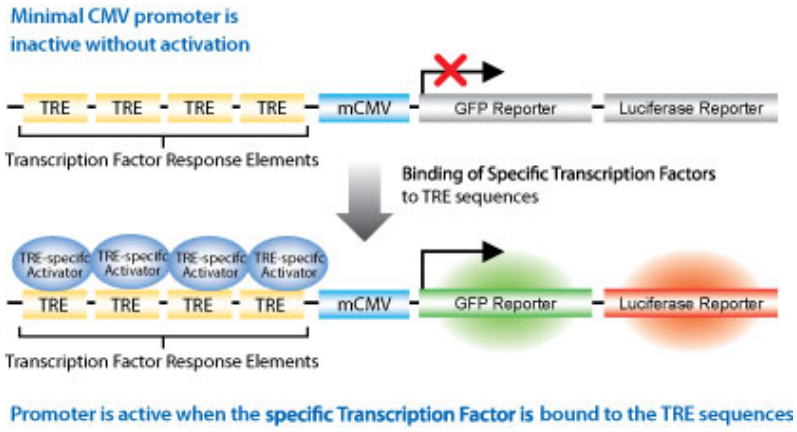
This manual provides information describing how to use the pGreenFire™ Pathway Reporter Constructs and prepackaged virus to generate stable cell lines with the reporter constructs integrated into the host genome. Before using the reagents and material supplied with this product, please read the entire user manual.

B. Lentiviral Pathway Reporter System

Eukaryotic gene expression is regulated by a wide variety of developmental and environmental stimuli. First, an extracellular signaling molecule binds to a specific receptor. The signal is then transmitted through a series of molecular cascades, which activate or deactivate specific transcription factors that regulate gene expression. The expression of any given gene is controlled by multiple transcription factors, which in turn are modulated by multiple signal transduction pathways. Many of these signal transduction pathways converge at transcription factors that bind to specific transcriptional response elements found in the promoters of various genes and

modulate the transcription of these genes. The activation of a signal transduction pathway (*e.g.* by growth factors, drugs, etc.) can therefore be monitored by the expression level of the reporter gene controlled by a promoter containing these response elements.

The pGreenFire reporter constructs contain transcription response elements (TREs) specific for transcription factors that indicate changes in cellular signaling pathways. When a specific transcription factor is present, it binds to the TRE regions and activates transcription of GFP and Luciferase. Therefore, cells expressing the specific transcription factor will fluoresce green and produce luciferase. Cell types that do not express the specific transcription factor to bind to the TRE regions will not have the reporter genes activated.



For example, a cell that has activated the NFkB signaling pathway will have NFkB available in the nucleus to bind to the TRE. When NFkB binds to the TRE region in the pGreenFire reporter vector, GFP will be transcribed and the cells will fluoresce green. Luciferase will also be transcribed and the exact levels can be measured with a standard luciferase assay. Thus, you can both qualitatively and quantitatively measure activation of NFkB signaling.

Advantages of Lentiviral Technology

Lentiviral expression vectors are the most effective vehicles for delivering genetic material to almost any mammalian cell, including non-dividing cells and to model organisms. By packaging the lentiviral transcription reporter vector construct in pseudoviral particles, you can obtain highly efficient transduction and heritable expression of transcriptional reporter constructs even with the most difficult-to-transfect cells, like primary, stem, and differentiated cells. In comparison to retroviral delivery systems, lentivectors enter the cell nucleus without requiring cell replication. Some of the advantages of lentivector technology include:

- Ready-to-use pre-packaged constructs with a wide range of transcriptional response elements for signaling pathway reporting.
- Lentiviral reporter constructs can efficiently transduce nearly all cell types, even those that are difficult-to-transfect such as primary or non-dividing mammalian cells.
- Our lentiviral-based reporter system is a novel approach to study transcriptional regulation and offers many advantages over current transcription reporter systems. Transcription reporter constructs will integrate into the genome and therefore be subject to chromatin regulation (Leung, *et.al.,* 2004). Expression of the reporter gene indicates activation of a given transcriptional response element by the cognate transcription factor in the natural chromosomal environment, rather than in the episomal state in the nucleoplasm as is the case for conventional plasmid-based transcriptional reporter vectors. Tandem copies of integration can be avoided, thus allowing for faithful promoter regulation. Copy number of reporter constructs can be controlled by varying the multiplicity of infection (MOI).

- Construction of stable reporter cell lines is possible with transcriptional reporter lentivectors in just several days without the need for conventional, low efficiency selection of stable transfectants.

- Monitoring of signaling pathways by flow cytometry (FACS) is enabled by GFP reporters.

Biosafety

SBI's pGreenZeo lentivectors are based on the traditional HIV vector backbone. To address biosafety issues, SBI uses a third generation HIV lentiviral vector. (Dull, *et.al.*, 1998, Miyoshi, *et.al.,* 1998, Zufferey, *et.al.,* 1999, Ramezani, *et.al.* 2000). SBI's lentiviral vectors are efficient gene transfer vehicles, as used for research applications, because of their stable integration in non-dividing and dividing cells and long-term transgene expression. SBI's 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.

- Upon integration into the genome, the 5' LTR promoter is inactivated, which prevents formation of replication-competent viral particles.

- The 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.

- The number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (*gag, pol, rev*).

- The corresponding proteins are expressed from different plasmids that lack packaging signals. The packaging plasmids share no significant homology to any of the expression lentivectors, the 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)* are present in the packaged viral genome, as they are expressed from separate plasmids lacking packaging signal. Therefore, the lentiviral particles generated are replication-incompetent.

- For pGreenZeo constructs, produced pseudoviral particles will carry only a copy of your cell specific promoter sequence.

The choice of SBI's lentiviral system for experimental studies is driven by functional considerations, including increased productivity and transduction efficiency. The design of SBI's biosafe vectors has benefited researchers allowing them to conduct experimental studies with lower risk. Currently, SBI's vectors combine improved safety features (that decrease the risk of recombination and vector mobilization) with increased transduction efficiency.

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

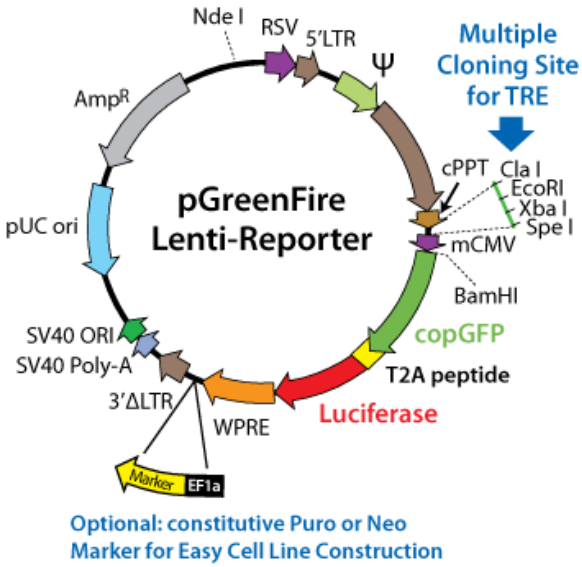
It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and to always follow *standard microbiological practices*, which include:

- Wear gloves and a lab coat when handling the lentiviral vectors, pseudoviral particles, or transduced cells.

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

C. p-GreenFire1 Lenti-Reporter Vectors

The pGreenFire vectors express copGFP reporter gene followed by the self-cleaving T2A peptide and the firefly luciferase gene under the control of a pathway-specific TRE. The WPRE element enhances the expression level of the reporter genes. Puromycin or neomycin resistance under control of the constitutive EF1a promoter is an optional feature which can assist in selection of stably transduced cells. SBI offers a wide range of pathway-specific TREs offered as plasmid constructs or as packaged in VSV-G pseudoviral particles.



For a full list of pGreenZeo reporters available, please see:

http://www.systembio.com/lentiviral-technology/transcription-reporter-vectors/#product_20_tab_1_7

pGreenFire vectors are provided as 10 µg of plasmid DNA.

pGreenFire lenti-reporter constructs must be transduced into target cells as a packaged virus in order for the constructs to function properly. Transfection of the constructs into a target cell keeps the constitutive RSV promoter intact, thus overriding the pathway-specific TRE promoters and leading to nonspecific expression of the reporter genes.

D. pGreenFire1 Prepackaged Virus

In addition to offering the pGreenFire1 reporter constructs as plasmids, SBI also offers packaged pGreenFire1 reporter vectors in VSV-G pseudotyped viral particles. These have been produced by co-transfection of the pGreenFire1 construct and the pPACK-H1 Lentiviral Packaging Plasmid Mix into 293TN producer cells. Following transfection, we collected the media containing the pseudoviral particles and concentrated it with PEGit, then titer with the Global Ultra Rapid Titer kit. For more information on SBI's virus production protocol, see, *Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells*.

The packaged pGreenFire1 viruses are provided as frozen pseudoviral particles. The total number of infection units (ifu) and concentration (the titer) are determined using HT1080 cells and may vary for different lots of each packaged reporter vector. The exact ifu, titer, and volume for each packaged reporter construct are indicated on its corresponding Product Analysis Certificate. The Packaged Lentiviral Reporter Viruses are shipped on dry ice and should be immediately stored at –70°C upon receipt. Avoid thawing and refreezing of pseudoviral particles! Properly stored pseudoviral particles are stable for 6 months from the date received.

E. Additional Required Materials

- **LB Agar and Broth with Ampicillin**
- **MaxEfficiency Stbl2 (Cat. #10268-019) or One Shot OmniMAX2 competent cells** (Cat # **C854003**)
- **Dulbecco's Modified Eagle's Medium (D-MEM)**
  high glucose with sodium pyruvate and glutamine (Invitrogen, Cat. # 11995073)
- **Fetal Bovine Serum** (Invitrogen, Cat. # 16000036)
- **Penicillin/Streptomycin** (Invitrogen, Cat. # 15070063)
- **Trypsin-EDTA** (Sigma, Cat. # T3924)
- **TransDux™** (SBI, Cat. # LV850A-1)
- **Millex-HV 0.45 μm PVDF filters** (Millipore, Cat. # SLHVR25LS)
- **Tissue Culture Plates and Related Tissue Culture Supplies**
- **293TN Human Kidney Producer Cell Line** (SBI, Cat. # LV900A-1)

II. Protocol

A. Key Terms

MOI (multiplicity of infection): The ratio of infectious pseudoviral particles (ifu) to the number of cells being infected. IFU/ # cells = MOI

IFU/ml (infectious units per ml): The relative concentration of infection-competent pseudoviral particles. Also called pseudoviral titer.

Transduction Efficiency: The average copy number of expression constructs per genome of target cell in the infected population.

B. General Comments

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

pGF1-CMV Reporter Construct
This plasmid should be used to estimate transduction efficiency of the lentiviral expression construct into target cells, select the cell type with highest infection efficiency, and to optimize the transduction protocol. Moreover, the presence of copGFP-positive cells indicates that the lentiviral construct can be efficiently expressed in your target cells from the CMV promoter. The construct can also used for calibration of FACS machine for maximum intensity of expression.

pGF-mCMV Reporter
Negative control construct which can be used to transduce target cells under the conditions optimized for the positive control pGZ-CMV construct and determine "background" of GFP fluorescence of target cells with a non-activated CMV promoter.

The transduction efficiency of the pGreenFire1 Packaged Reporter Construct (and your lentiviral expression construct) may vary significantly for different cells and experimental conditions. In order to optimize transduction conditions, we recommend that you use HT1080 (or similar) cells as a positive control in parallel with your target cells and use prepackaged pGF-CMV (TR011VA-1) from SBI. To determine the desired multiplicity of infection (MOI) appropriate for your target cells, you should do several transductions with packaged pGreenFire1 pseudoviral particles at different MOI's (*e.g.* from 0.1 to 5). Results of these test transductions should be used to determine an optimal MOI that yields the optimal percentage of infected cells based on the

percentage of cells expressing the GFP marker. Note that some cell types, such as primary cells may be resistant to infection regardless of the MOI.

Expression of the pGreenFire1 Reporter can be measured directly at about 48-72 hours after transduction. At this time, pGreenFire1 constructs are integrated into the genomic DNA resulting in stably transduced reporter cell lines. Reporter cells can be cloned in order to obtain a uniform population of the GreenFire cell line. Some cells may express the reporter construct in 80-90% of the cells after transduction at an MOI of 1-2. For these "easy-to-transduce" cells, most biological assays can be performed at 48-72 hours after transduction. However, some primary cells may only express the construct in 10-50% of cells, even when transduced at high MOI's. For these "difficult-to-transduce" cells, it is probably desirable to select the cells stably expressing the construct by FACS or clonal selection for experimental assays.

Due to the pathway specificity of the pGreenFire1 reporter, GFP and luciferase expression is only expected to occur in cell types that express the appropriate transcription factors that bind to the TRE regions of the promoter.

C. pGreenFire1 Plasmid Preparation

Transformation of pGreenFire1 lentivector reporter constructs into competent cells

If you have purchased the pGreenFire1 reporter construct as a plasmid, you will need to produce enough for packaging into virus. We recommend using *E.coli* that are RecA- competent cells such MaxEfficiency Stbl2 (Cat. #10268-019) or One Shot OmniMAX2 competent cells (Cat # C854003). Please follow the protocol according to the manufacturer's instructions. The transformed *E.coli* can be grown at 37°C on LB agar with ampicillin overnight.

Plasmid Purification

Lentivector constructs must be grown in liquid culture (LB with Ampicillin) at **30°C**. *E. coli* transformed with lentivector constructs seem to expel the plasmid if grown at 37°C.

SBI recommends the PureLink™ Hi Pure Plasmid Filter Purification Kit from Invitrogen (Cat. # K2100-14) for purification of lentivector plasmids.

D. Production of pGreenFire1 packaged virus

Please refer to the *Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells* manual for a full description of how to package pGreenFire1 into VSV-G pseudotyped viral particles.

E. Transduction of pGreenFire1 into target cells

The following protocol describes the general procedure for the transduction of the pGreenFire1 Reporter Constructs packaged in pseudotyped viral particles into HT1080 cells. This protocol assumes that you will use these guidelines in order to perform transduction of your target cells in parallel using HT1080 cells as a positive control and can be used as a starting point for the optimization for transduction of your particular cell-type.

Day 1

1. Plate 50,000 cells per well in a 24 well plate in cell culture medium.

Day 2

2. Cells should be between 50 to 70% confluent. Aspirate medium from cells.

3. Combine culture medium with TransDux to a 1X final concentration. (For example, add 2.5 µl of TransDux to 500 µl culture medium and then transfer to each well.)

4. Add virus to each well and swirl to mix. (Optional: Add increasing amounts of virus to different wells at varying MOIs (5, 10 and 20, etc.) to optimize the transduction.

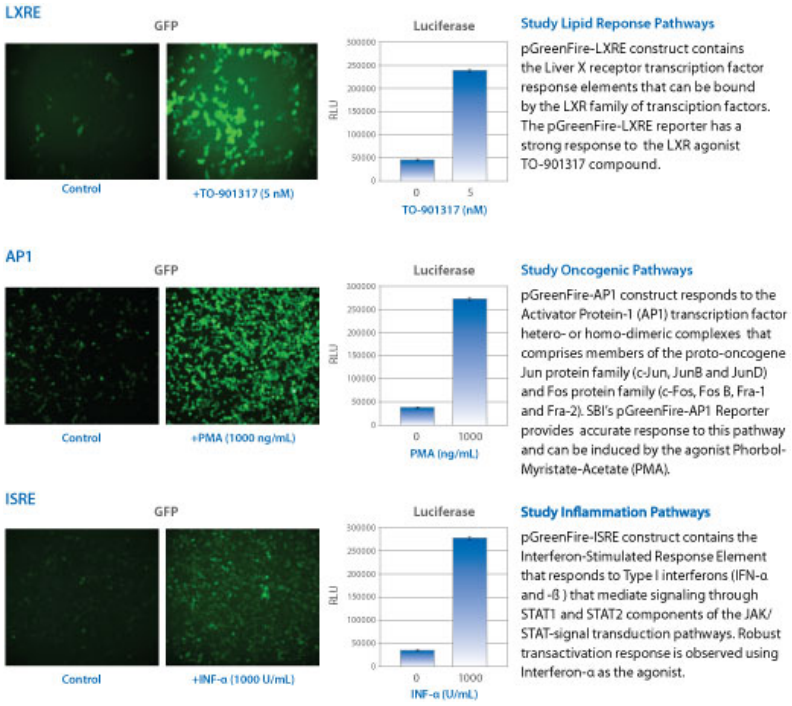
Day 5

5. 72 hours post transduction, the viral genome will be integrated into the host cell genome. Look at the cells for reporter expression if the viral construct has a reporter like GFP.

6. Aspirate off medium. Wash each well with PBS.

7. To establish stabile cell lines, you can now FACs sort for GFP or RFP positive cells. If using an antibiotic selection marker, you can begin your selection procedure.

III. Example Data



IV. Troubleshooting

A. Why are my cells green following transfection for viral packaging?

In order to produce the viral genomic RNA that will be packaged into viral particles, a strong promoter is included in the 5' LTR to drive its expression. Due to the mechanism of lentiviral replication, this promoter in the 5' LTR is not present in the genomic RNA or the resulting integrated provirus. Therefore, GFP is expressed from the viral 5'LTR following transfection, however, following transduction, transcription should only occur from the reporter gene when activated.

B. Inefficient Transduction of Packaged pGreenFire1 Reporter Vector into Target Cells

1. Poor infection efficiency

Target cells have too high or too low density

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

Target cell line may be difficult to transduce
Use a higher concentration (less fold dilution) of pseudoviral particles. Optimize the transduction protocol and use as positive control cells HT1080 cell line.

Polybrene® added during infection stage
If Polybrene® is toxic to the target cells, switch to TransDux.

Loss of pseudoviral titer during storage
Ensure storage of the Packaged Reporter Vector at –70°C. Each freeze-thaw cycle causes reduction of the titer by 20-30%. Use a fresh stock for transduction. Do not keep the stock longer than 6-12 months.

Volume of infecting supernatant is too high
Keep the volume as low as possible to achieve maximal adsorption of viral particles to the cells.

2. Transduction affects target cell viability

Packaged Reporter Vector affects target cell growth
Use a shorter transduction time to minimize the toxic effect to the target cells. Try replacing with a similar target cell type.

Polybrene® is toxic for target cells
Switch to TransDux™.

3. No Expression of positive control pGF1-CMV reporter in target cells

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.

V. References

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VI. Appendix

A. pGF1-CMV Features

Feature	Location *	Function
RSV-5' LTR	1-415	Hybrid RSV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	567-919	Packaging signal
RRE	1066-1309	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1798-1899	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1943-2271	Human cytomegalovirus (CMV)--constitutive promoter for transcription of dscGFP and zeoR
copGFP-T2A-FLuc	2329-4848	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/ transduced cells; a destabilizing (ds) peptide on the C-end shortens the half life time of the mature protein to 1 hour
WPRE	4859-5399	Posttranscriptional regulatory element which enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	5538-5771	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	5843-5974	Transcription termination and polyadenylation
SV40 Ori	5983-6129	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	6499-7172(C)	Allows for high-copy replication in *E. coli*
AmpR	7317-8177(C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

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

B. Properties of the copGFP

The pGreenZeo 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*.). A unique feature of the copGFP protein is the presence of an additional destabilizing (ds) peptide on the C-end of the protein which shortens the half life time of the mature protein without additional transcription to 1 hour. 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 lentiviral constructs into cells.

C. Related Products

pPACK Lentivector Packaging Kits
HIV-Based: pPACKH1 Packaging Kit (Cat. # LV500A-1)

Unique lentiviral vectors that produce all the necessary lentiviral proteins and the VSV-G envelope glycoprotein from vesicular stomatitis virus required to package pGreenZeo/pRedZeo lentiviral constructs into pseudoviral particles.

LentiSuite for HIV-based Systems (Cat. # LV300A-1)
Contains pPACK plasmids, 293TN producer cells, PEG-it virus concentration solution, and the Ultra Rapid Titering Kit

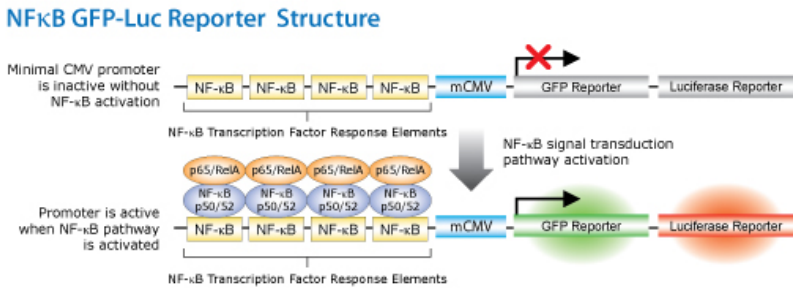
NFkB Reporter Cell Lines

NFkB/ 293/GFP (Cat. # TR800A-1)

NFkB/ Jurkat/ GFP (Cat. # TR850A-1)

NFkB/ 293/ GFP-Luc (Cat. # TR860A-1)

These reporter cell lines have been transduced with the NFkB-pGreenFire constructs and clonally selected.



D. 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:

Phone: (650) 968-2200
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Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

System Biosciences (SBI)
265 North Whisman Rd.
Mountain View, CA 94043

VII. Licensing and Warranty

Use of the pGreenFire1™ Pathway Reporter Lentivector (*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.

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

HIV Vector System

This product is for non-clinical research use only. Use of this Product to produce products for resale or for any diagnostic, therapeutic, clinical, veterinary, or food purpose is prohibited. In order to obtain a license to use this Product for these commercial purposes, contact the Office of Research and Technology Ventures at the Dana-Farber Cancer Institute, Inc. in Boston, Massachusetts, USA. This Product or the use of this Product is covered by U.S. Patents Nos. 5,665,577 and 5,981,276 (and foreign equivalents) owned by the Dana-Farber Cancer Institute, Inc.

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