pSIF-H1 shRNA Cloning and Expression Lentivectors

(Cat. #s SI100C-1, SI101B-1)

User Manual

Store kit at -20°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. 4-080514)

Contents

A. Introduction and Background

1. Purpose of this Manual

This manual provides details and information necessary to clone an shRNA template into the pSIF-H1 shRNA Cloning and Expression Vectors (pSIF1-H1-Puro and pSIF1-H1-copGFP Vectors). Specifically, it provides critical instructions on designing and synthesizing shRNA templates, cloning the shRNA templates into the H1 expression cassette of pSIF-H1 Vectors, and verifying final vector constructs. This manual does not include information on packaging the pSIF-H1 vector construct 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 web site (www.systembio.com). Before using the reagents and material supplied with this system, please read the entire manual.

2. Lentiviral shRNA Expression System

Short double-stranded RNAs with sizes 19-29 bp can efficiently mediate gene silencing in mammalian cells by guiding sequence-specific degradation of target mRNA sequences (Bernstein 2001, Hammond 2000). Synthetic double-stranded siRNA molecules can be introduced into cells to suppress gene expression transiently. Alternatively, shRNA templates can be cloned into an shRNA expression vector—such as SBI's FIV-based or HIV-based RNAi Cloning and Expression Lentivectors—and expressed in the cells of choice.

Lentiviral expression vectors are the most effective vehicles for delivering genetic material to almost any mammalian cell—including non-dividing cells and whole model organisms. As with standard plasmid vectors, it is possible to introduce shRNA lentivector constructs in plasmid form into the cells with low-to-medium efficiency using conventional transfection protocols. However, by packaging the lentiviral shRNA construct in pseudoviral particles, you can obtain highly efficient transduction and heritable expression of siRNA—even with most difficult to transfect cells, like primary, stem, and differentiated cells. The expression construct transduced in cells is integrated into genomic DNA and provides stable, long-term expression of the target gene. Endogenously expressed siRNA effectors provide long-term silencing of the target gene and allow the researcher to generate cell lines and transgenic organisms with a stable knockdown phenotype for functional studies

The lentiviral siRNA expression system consists of three main components:

(1) The lentiviral expression vector (*e.g.*, pSIF1-H1-Puro™)

(2) The lentiviral packaging plasmids (*e.g.*, pPACKF1™ Packaging Plasmid mix)

(3) A pseudoviral particle producer cell line (*e.g.*, 293TN cells)

The lentiviral expression vector contains the genetic elements responsible for packaging, transduction, stable integration of the viral expression construct into genomic DNA, and expression of the siRNA effector sequence. The packaging vector provides all the proteins essential for transcription and packaging of an RNA copy of the expression construct into recombinant viral particles. For production of a high titer of viral particles, producer cells

(*e.g.*, HEK 293 cells) need to be transiently co-transfected with the expression and packaging vectors. Expression constructs packaged in pseudoviral particles are secreted by producer cells in culture media and could be used directly to transduce expression construct in target cells. Following transduction into the target cells, this expression construct is reverse transcribed, integrated into the genome of the target cell, and provides a high level of expression of siRNA. For a detailed description of SBI's Lentivector expression system, please refer to the Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells user manual.

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

3. pSIF shRNA Expression Lentivectors

The pSIF siRNA expression system is a third generation of FIV-based expression lentivectors developed for gene therapy applications (Poeschla, 2003). See section F for safety guidelines. The pSIF vectors (see detailed functional map in Appendix) provide the following features:

- H1 expression cassette provides constitutive and efficient RNA polymerase III-dependent transcription of shRNA transcripts in wide range of cell lines.
- CMV promoter promotes high level of expression of copGFP (fluorescent reporter) or puromycin-N-acetyl transferase (drug selectable marker) for detection and selection of transduced cells.
- Hybrid CMV-5'LTR promoter provides a high level of expression of the full-length viral construct in 293 cells.
- Genetic elements (cPPT, GAG, LTRs) necessary for packaging, transducing, and stable integration of the viral expression construct into genomic DNA.
- SV40 origin for stable propagation of the pSIF plasmid in 293 producer cells.
- The pUC origin for high copy replication and maintenance of the plasmid in *E.coli* cells.
- The ampicillin-resistance gene for selection in *E.coli* cells.
- WPRE element enhances stability and translation of the CMV-driven transcripts.
- The SV40 polyadenylation signal enables efficient termination of transcription and processing of recombinant transcripts.

The pSIF1-H1-Puro Vector (Cat. # SI100C-1) contains a puromycin resistance gene to enable drug selection of target cells stably expressing the siRNA. The pSIF1-H1-copGFP Vector (Cat. # SI101B-1) contains a copGFP gene. CopGFP is a novel fluorescent protein,

derived from copepod plankton (Panalina sp.), which is similar to EGFP but has a brighter color. This gene serves as a fluorescent reporter for the transfected or transduced cells.
Two approaches have been developed for *in vivo* expression of siRNA from plasmid and viral vectors. In one approach, the sense and anti-sense strands are transcribed separately from two independent promoters and form the siRNA duplex (Lee 2002, Miyagishi 2002). With the second approach, a single-stranded shRNA sequence with a fold-back stem-loop structure (also known as a "hairpin") is expressed from a single promoter (Abbas-Terki 2002, Qin 2003, Wiznerowicz 2003). This sequence is then converted into double-stranded siRNA after intracellular processing cleaves the loop (Brummelkamp 2002, Paddison 2002). In both approaches, the siRNA molecules are transcribed from constitutive RNA polymerase III promoters (*i.e.*, U6 and/or H1) and terminated with TTTTT (T_5) sequences (Tuschl 2002). The U6 and H1 promoters are different in size but contain the same conserved sequence elements (Myslinski 2001).

The pSIF-H1 Vectors are designed to express a single-stranded shRNA sequence with a fold-back stem-loop structure (also known as a "hairpin") from a RNA polymerase III H1 promoter (Abbas-Terki 2002, Qin 2003, Wiznerowicz 2003). The hairpin-type siRNA (shRNA) template oligonucleotides need to be cloned into unique BamHI/EcoRI sites located just downstream of an H1 promoter (Figure 1). The pSIF-H1 vectors are provided in ready-for-ligation linearized form that has been predigested with BamHI and EcoRI, and purified to remove the stuffer fragment. The linearized vector contains two unique 5' overhangs to facilitate directional cloning of shRNA template oligos with minimal self-ligation background (Figure 1). When the shRNA construct is expressed from constitutive H1 promoter and terminated with the TTTTT sequence, the shRNA transcript folds into the hairpin structure, which is recognized by the DICER enzyme, cleaved to form a functional ds siRNA and transferred to a RISC complex for selective digestion of complementary target mRNAs ((Brummelkamp 2002, Paddison 2002) (Figure 2). Two PCR primers are designed for regions flanking the shRNA insert in order to provide a simple way for screening of plasmid clones for the presence of shRNA inserts by PCR (Figure 2).

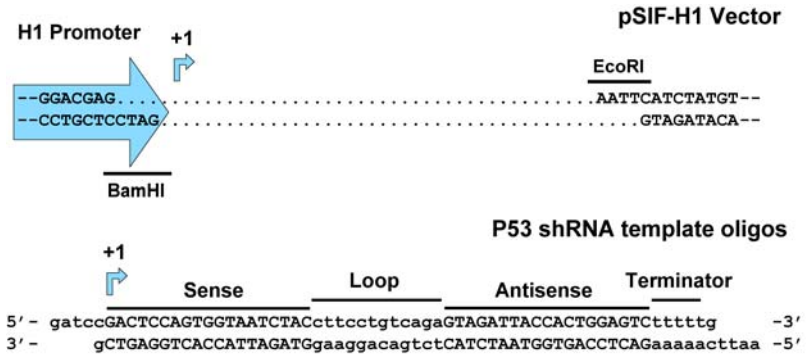


Fig. 1. Design of the single-promoter pSIF-H1 shRNA expression cassette. The dotted lines at the top of the figure indicate the position of the "stuffer fragment" that is removed during linearization by digesting the vector with BamHI/EcoRI. Your shRNA template sequence should be designed to directionally insert between the BamHI and EcoRI nucleotide overhangs (*i.e.*, sticky ends). This example shows the siRNA sequence targeting the p53 gene.

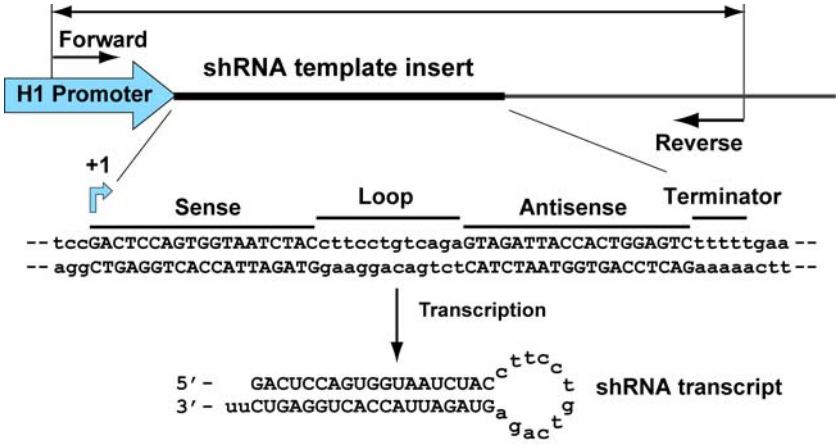


Fig. 2. Example shRNA template construct targeting the p53 gene. The nucleotides for the specific siRNA sequence targeting the p53 gene are shown in capital letters. The shRNA sense and antisense sequences flank the region coding for the loop structure. In addition, a terminator sequence for the RNA polymerase III is included after the antisense portion. The Forward and Reverse arrows refer to the PCR primers contained in this product to confirm positive clones. After transcription, a stem-loop-stem shRNA molecule is produced. This molecule is processed by the DICER enzyme to generate a double-stranded siRNA effector.

4. List of Components

Each pSIF-H1 Vector Kit provides enough plasmid for 20 ligation reactions:

- **pSIF1-H1-Puro shRNA Expression Lentivector** (Cat. # SI100C-1)
  - 50 µl pSIF1-H1-Puro vector (Non-linearized, 20 ng/µl)
  - 25 µl Luciferase Control shRNA Template Oligonucleotide Mix (20 µM each)
  - 25 µl ForwardF PCR Primer (5'-TGTCTTTGGATTTGGGAATCTTAT-3'; 10 µM)
  - 25 µl ReverseF PCR Primer (5'-ATTTATTGTATCTGTGGGAGCCTC-3'; 10 µM)

- **pSIF1-H1-copGFP shRNA Expression Lentivector** (Cat. # SI101B-1)
  - 50 µl pSIF1-H1-copGFP vector (Non-linearized, 20 ng/µl)
  - 25 µl Luciferase Control shRNA Template Oligonucleotide Mix (20 µM each)
  - 25 µl ForwardF PCR Primer (5'-TGTCTTTGGATTTGGGAATCTTAT-3'; 10 µM)
  - 25 µl ReverseF PCR Primer (5'-ATTTATTGTATCTGTGGGAGCCTC-3'; 10 µM)

The kits are shipped in dry ice and should be stored at -20°C upon receipt. Properly stored kits are stable for 12 months from the date received.

5. Additional Required Materials

For Phosphorylation and Annealing of shRNA Template Oligonucleotides
- T4 Polynucleotide Kinase and 10X reaction buffer
  (Recommended: New England BioLabs T4 Polynucleotide Kinase, 10 U/µl, Cat. # M0201S.
- rATP (Recommended: GE/Amersham, Cat. # 27-2056-01)

For Linearizing shRNA Expression Vector
- BamHI and EcoRI restriction enzymes
  (Recommended: New England BioLabs, EcoRI (20 U/µl, Cat. # R0101. BamHI (20 U/µl, Cat. #R0136S))
- Qiagen Qiaquick PCR Purification Kit. Cat No. 28104

For Ligating and Transforming shRNA Constructs
- T4 DNA Ligase and 10X ligation reaction buffer
  (Recommended: New England BioLabs, T4 DNA Ligase, 400 U/µl. Cat. # M0202S.
  Before using dilute T4 DNA-ligase 10-fold with 1X T4 DNA ligase buffer to 40 U/µl.)
- Competent *E. coli* cells (RecA$^-$)
  (Recommended: Invitrogen, OmniMAX™ 2 T1 cells, Cat. # C8540-03)
- Petri plates containing LB Agar media with 50 µg/ml Ampicillin

For Screening shRNA Inserts
- Taq DNA polymerase, and 10X reaction buffer
  (Recommended: Clontech Titanium™ Taq DNA polymerase, Cat. # 639208)
- dNTP mix

(Recommended: GE/Amersham, dNTP set, Cat. # 27-2035-01)
- PCR machine
- 3% 1X TAE Agarose gel

For Purifying shRNA Constructs after Cloning
- Plasmid purification kit
  (Recommended: QIAGEN Endotoxin-free Plasmid Kit. The following kit combinations can be used for Midi scale preparation (up to 200 μg) of endotoxin-free plasmid DNA:
  - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Maxi Kit, Cat. # 12362
  - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Buffer Set, Cat. # 19048

  Please visit the QIAGEN website to download the specialized protocol that is not contained in the user manual:
  - http://www1.qiagen.com/literature/protocols/pdf/QP15.pdf

Transfection of pSIF Constructs into Target Cells
- Transfection reagent
  (Recommended: Lipofectamine™ 2000, Invitrogen, Cat # 11668-027)

Packaging of pSIF Constructs in Pseudoviral Particles
- In order to package your pSIF shRNA constructs into VSV-G pseudotyped viral particles, you will need to purchase the pPACKF1 Lentivector Packaging Kit (Cat. # LV100A-1). The protocol for packaging and transduction of packaged pseudoviral particles is provided in the User Manual for the Lentivector Expression System.
- 293 Producer Cell Line
  (Recommended: SBI 293TN Cell Line, Cat. # LV900A-1 or ATCC 293 Cells, Cat. # CRL-11268)
- Transfection Reagent
  (Recommended: Invitrogen Lipofectamine, Cat. # 18324-111; and Plus Reagent, Cat.# 11514-015)

6. Safety Guidelines

SBI's pSIF lentivectors together with the pPACKF1 packaging plasmids comprises a third-generation FIV-based cloning vector system. The original FIV expression system was developed by Eric M. Poeschla, David J. Looney, and Flossie Wong-Staal at UCSD (Poeschla, 1998; Poeschla 2003). The feline immunodeficiency virus (FIV) was originally isolated from cat blood. Despite common close exposure of humans to FIV through contact with domestic cats (including bites, scratches, etc.), no human infection or disease has ever been associated with FIV (Poeschla, 2003). This system is designed to maximize its biosafety features including:

- Deletion in the enhancer of U3 region of 3'LTR ensures self-inactivation of lentiviral construct after transduction and integration into genomic DNA of the target cells.
- CMV promoter upstream of 5'LTR in pSIF expression vector allows efficient Tat-independent production of viral RNA, reducing the number of genes from FIV 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 a plasmid lacking packaging signals and shares 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 FIV genes (*gag, pol, rev)* will be present in the packaged recombinant expression construct, as they are expressed from a packaging plasmid lacking packaging signal. Therefore, the lentiviral particles generated are replication-incompetent.
- Pseudoviral particles will carry only the expression construct of your target gene.

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

- Wear gloves and lab coat all the time when conducting the procedure.
- Always work with pseudoviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.

All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area are to be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.

B. Protocol

1. shRNA Template Oligonucleotide Design and Synthesis

Typically, 4 or 5 target sequences in the gene of interest need to be selected and tested to identify functional siRNAs with at least 70% silencing efficiency of target mRNA. Although there is no standard rule for selecting the target mRNA binding sites for siRNA sequences, we have found the following criteria useful:

- 19-29 nt in length, usually longer oligos (25-27 nt) are more robust and give better silencing efficiencies although 19 nt oligos could be also used.
- Unique sequence with less than 70% homology with other mRNA sequences in a RefSeq database). Especially avoid homology to other non-target mRNA sequences in central portion of siRNA, flanking sequences usually tolerate mismatches (especially G:U and A:C) without reduction in silencing efficiency
- 40%-55% GC content.
- No more than 4 consecutive A's or T's.
- No more than 5 consecutive G's or C's.
- No thermodynamically stable secondary structure (< 0 Kcal/mol).
- A 5'-terminus (3-5 flanking nucleotides) on the anti-sense strand should be more AT-rich than the 3'-terminus.

The template sequences coding for the shRNA targeted to each selected target site must contain both the sense and anti-sense strand, and be designed to form a stem-loop structure when transcribed. In addition, both the top and bottom strands of the entire shRNA sequence (sense-loop-antisense-terminator) must be synthesized and annealed to make a double-stranded DNA sequence that can be cloned into the pSIF vector. The features of the oligonucleotides coding for the shRNA template sequence should include the following:

(1) The 19-29 nucleotide sense and antisense mRNA sequences. Usually longer siRNAs (25-27 nt) have better silencing efficiencies although 19-nt oligos are more commonly used. For the design of 27-nt oligos, we recommend the program available at Dr. Gregory Hannon's web site: (http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA). The program is designed to incorporate a few G-U mismatches in the sense portion of stem that will help to stabilize hairpins during propagation in bacteria.

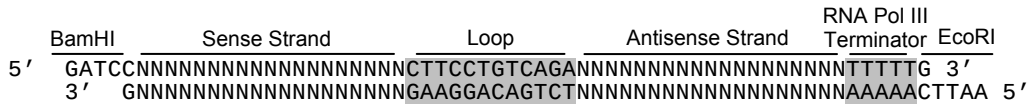
(2) A hairpin loop sequence between sense and antisense portion. The 9-nt loop sequence (5'-TTCAAGAGA-3') is most commonly used in RNA silencing experiments (Brummelkamp 2002), but we have used a 12-nt sequence (5'-CTTCCTGTCAGA-3'), which generates similar results. Loop sequences of 3 to 15 nucleotides have been used successfully by different investigators.

(3) A TTTTT terminator sequence for RNA polymerase III.

(4) A BamHI and EcoRI restriction site overhang sequences for directional cloning of annealed shRNA template oligonucleotides into the pSIF-H1 vector.

(5) Using of initiation G nucleotide in the first position of sense portion of shRNA is not necessary, as RNA polymerase III could initiate transcription from any +1 nucleotide of H1 promoter.

The top and bottom strands of the shRNA template oligonucleotides should be designed to look like the following diagram after annealing (See also Figure 1):



For each selected template sequence, two complementary oligonucleotides—the "top" strand and complementary bottom strand—need to be synthesized, phosphorylated and annealed before ligation step. A 50 μM scale reaction for oligonucleotide synthesis with regular desalting purification is sufficient for cloning into the pSIF-H1 Vectors. For the best cloning efficiency, we recommend to phosphorylate oligonucleotides using T4 polynucleotide kinase. The phosphorylation procedure is shown below in step B.1.

2. Cloning of shRNA Template Oligonucleotides into pSIF-H1 Vector

1. Linearize the pSIF vector with EcoRI/BamHI

a. Set up a 50 μl restriction digest as follows:

33.8	μl	Deionized water
5	μl	10x NEB 3 buffer
0.2	μl	100x BSA
0.5	μl	BamHI (20 U/μL, NEB)
0.5	μl	EcoRI (20 U/μL, NEB)
10	μl	pSIF vector (0.2 μg/μL)
50	μl	Total volume

b. Digest overnight at 37°C.

c. Purify linearized plasmid DNA using Qiagen Qiaquick PCR purification kit. Elute purified DNA in 30 μL EB buffer.

2. Phosphorylate and Anneal the shRNA Template Oligonucleotides

Note: This protocol was developed for regular non-phosphorylated oligos. If your oligonucleotides are already phosphorylated, dilute them to 10 μM in 1X T4 polynucleotide kinase buffer, heat at 95°C for 2 min and anneal as in steps 1.d-1.e.

a. Dissolve the shRNA template oligonucleotides in an appropriate amount of deionized water to a final concentration of 20 μM.

b. Set up 20 μl phosphorylation/annealing reactions for each experimental shRNA template and Luciferase Control Template Mix as follows:

1	μl	Top Strand shRNA template oligo (20 μM) *
1	μl	Bottom Strand shRNA template oligo (20 μM) *
2	μl	10X T4 Polynucleotide Kinase Buffer
2	μl	10 mM ATP
12	μl	Deionized water
2	μl	T4 Polynucleotide Kinase (10 U/μl)
20	μl	Total volume

* For the insert-minus control, use 2 μl deionized water in place of the top and bottom strands. For the positive control, use 1 μl of the Luciferase Control shRNA Template Mix and 1 μl deionized water.

c. Incubate the phosphorylation reaction at 37°C for 30 minutes in a thermocycler.

d. Heat the reaction mix to 95°C for 2 min in a thermocycler.

e. Turn off the thermocycler and let it cool to room temperature.

f. Use 0.5 μl of 1 μM shRNA template for the following ligation reaction.

3. Ligate the shRNA Template into Linearized pSIF-H1 Lentivector

a. Setup 10 μl ligation reactions for each phosphorylated shRNA template as follows:

1.0	μl	Linearized pSIF-H1 Vector (50 ng/μl)
0.5	μl	Phosphorylated ds shRNA template (step 1; 1μM) *
1.0	μl	10X T4 DNA Ligase Buffer
6.5	μl	Deionized water
1.0	μl	T4 DNA ligase (40 U/μl) **
10.0	μl	Total volume

* For negative control use insert-minus and for positive control use Luciferase shRNA template from step 1.

** Dilute T4 DNA ligase (400 U/μl) 10-fold to 40 U/μl with 1X T4 DNA ligase buffer if you are using New England Biolabs enzyme.

b. Incubate the ligation reaction at 16°C for 1-2 hrs.

3. Transform *E. coli* with the ligation product

a. For each experimental shRNA template, use the whole volume of ligation product for transformation.

b. Follow the manufacturer's protocol for transforming the competent cells.

c. Plate an appropriate amount of cells on LB plates with 50 μg/ml ampicillin and grow overnight at 37°C.

d. You can expect to get at least 10-fold more colonies in the experimental samples in comparison with the negative control (vector-only ligation reaction).

C. Identify clones with the target shRNA template

1. Prepare colony cultures

a. Randomly pick up 10 well-separated colonies from each plate, and grow each clone in 100 μl of LB Broth with 50 μg/ml ampicillin at 37°C for 2 hours with shaking.

b. Take 1 μl of each bacteria culture for PCR screening (see C.2) and continue to grow the culture for another 6 hours.

c. Store the bacterial culture at 4°C.

2. Screen for shRNA template inserts

a. Prepare a PCR master mix for each clone you would like to screen for the presence of a shRNA template insert as follows:

1 rxn	10 rxn	Composition
0.5 μl	5 μl	ForwardF PCR Primer (10 μM)
0.5 μl	5 μl	ReverseF PCR Primer (10 μM)
0.5 μl	5 μl	50X dNTP mix (10 mM of each)
2.5 μl	25 μl	10X PCR Reaction Buffer
19.5 μl	195 μl	Deionized water
0.5 μl	5 μl	Taq DNA Polymerase (5 U/μl)
24.0 μl	240 μl	Total volume

b. Mix the master mix very well and aliquot 24 μl into each well of a 96-well PCR plate or individual tubes.

c. Add 1 μl of each bacterial culture from C.1 into each well or tube from C.2.b. Mix.

d. Proceed with PCR using the following program:

94°C, 4 min	1 cycle
94°C, 0.5 min, then 68°C, 1 min	25 cycles
68°C, 2 min	1 cycle

e. Take 5 μl of PCR product from step d and run it on a 3% agarose/EtBr gel in 1X TAE buffer.

Clones without an insert will yield a product of 176 bp. The expected size of amplified clones with a shRNA template insert should be about 220-240 bp depending on expected length of the shRNA template insert (see Fig. 2 for details). Some clones may not yield a product. These are results of recombination during propagation in *E. coli*.

f. Confirm identity of shRNA template inserts by sequence analysis of positive PCR products using the ForwardF PCR primer.

D. Purify shRNA Lentivector Construct

a. Take 15-20 μl of each positive bacteria culture from Step C.1.c, inoculate each clone in 25 ml of LB broth media with 50 μg/ml ampicillin, and grow overnight at 37°C with shaking.

b. Purify shRNA lentivector construct plasmid DNA in Midi scale using an Endotoxin-free plasmid purification kit. (see section I.E, Additional Required Materials)

E. Transfection and Analysis of Silencing Efficiency

If you are planning to use SBI's pSIF-H1 shRNA constructs for viral delivery, we recommend first to screen the shRNA constructs generated in section D to determine their effectiveness at knocking down expression of the target gene. To rapidly screen the shRNA lentivector constructs in plasmid form, you can deliver and express them in *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) works well in our hands. 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; Roche, FuGENE 6, Cat. # 11 815 091 001). The goal of these experiments is to achieve at least 90-95% transfection efficiency of target cells, which can be measured by analysis of GFP-positive cells if you are using constructs with copGFP reporters or H2Kk-positive cells for constructs in the pSIF1-H1-H2kk vector.

For shRNA knockdown studies using transfection, it is important to optimize the selected transfection protocol and then keep the parameters constant to ensure reproducible results. Depending on what is appropriate for your target gene, the silencing efficiency of different shRNA constructs can be estimated by determining the concentration of target mRNA using RT-PCR, assessing the amount of target protein by Western blot or ELISA, or assaying for activity of the target protein. Usually shRNA constructs with 70-80% silencing efficiency are suitable for gene functional analysis studies.

Once you identify a functional shRNA construct, you can package this construct into pseudoviral particles, and efficiently transduce these shRNA constructs into target cells of your choice. For this purpose, you will need to purchase the pPACKF1 Lentivector Packaging Kit (SBI, Cat # LV100A-1) and 293TN Producer Cell Line (SBI, Cat # LV900A-1).

The pPACKF1 User Manual, "Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells" includes the procedural information for packaging the shRNA expression constructs. This user manual is also available on the SBI web site (www.systembio.com). Although you can create stable transfectants with the pSIF constructs using standard transfection and selection protocols, transduction of the lentiviral pSIF shRNA constructs using packaged pseudoviral particles is the most efficient way to express siRNA in wide range of cells, including dividing, non-dividing, and hard-to-transfect varieties.

C. Troubleshooting

A. Using the Positive Control

The Luciferase Control shRNA Template Oligonucleotide Mix is a mixture of complementary DNA strands with sticky ends (5'GATC, and 5'TTAA) to match with the BamHI and EcoRI ends on the linearized pSIF-H1 Vector. The 64-base hairpin shRNA template sequence, consisting of 26-base sense, 12-base loop, and 26-base antisense sequences, targets the wild-type Firefly Luciferase gene.

When run in parallel with your experimental annealed double-stranded shRNA oligonucleotides, Luciferase Control shRNA Template Oligonucleotide Mix serves as positive control to check if your phosphorylation and ligation reactions and transformation procedure work well. Using the protocol described in II.B, ligation with this control insert mix should provide at least 5-10 times more colonies than ligation of the vector without an insert.

The control pSIF construct with the Luciferase shRNA template can also be used to monitor the efficiency of target Luciferase mRNA silencing. A cell line with a constant expression level of Luciferase can easily be generated. The level of Luciferase expression should be reduced at least 5-fold after transfection or transduction of the pSIF-H1 Luciferase shRNA construct in the Luciferase reporter cell line.

B. Troubleshooting Specific Results

1. Getting Few or No Clones

Check design of the shRNA template
Check the sequence of the shRNA oligonucleotides to ensure that, after sense/anti-sense annealing, the ends present the 5' GATC and 5' TTAA overhangs for proper annealing with the restricted ends of linear pSIH-H1 Vector. Also, confirm that the top and bottom strand sequences are complementary to each other.

Check annealing
To ensure a high percentage (80%) of double-stranded DNA after annealing, check the concentration of shRNA template oligonucleotides using a spectrophotometer and mix equal molar amounts of each strand. To check annealing, run 5 μl of annealed insert (from step B.1.f) using a 12% polyacrylamide gel and compare the band's location with that of the original single-stranded oligonucleotides.

Confirm oligonucleotides were correctly synthesized
Verify the size of the oligonucleotides using a 12% native polyacrylamide gel.

Check quality of T4 polynucleotide kinase and T4 DNA ligase
Test the activity of your ligase and reaction buffer using a different vector and insert. Test the activity of T4 polynucleotide kinase by labeling annealed control Luciferase with ^{32}P-γATP. Replace the reagents if they show poor activity.

Ensure there are no ligation inhibitors present
EDTA and high salt can inhibit ligation reactions. Make sure that your ds shRNA template oligonucleotide concentration is 1μM and use 0.1-1 μl of this oligonucleotide in ligation reaction. Higher concentration of ds shRNA template oligonucleotide could reduce yield of shRNA lentivector construct.

Check the quality of the competent cells
Handle the competent cells gently. Many cells cannot be refrozen once thawed. The quality of the competent cells can be tested by transforming with any circular plasmid.

Check antibiotic selection
The plates used for cloning should contain 50-100 μg/ml ampicillin in the media. You can check the activity of the antibiotic by mixing wild-type *E. coli* with small numbers of *E. coli* that have been successfully transformed with any plasmid containing the Amp^R gene.

2. Too many clones without shRNA insert

Confirm pSIF vector was completely linearized.
Run a small aliquot of the EcoRI/BamHI digested vector. A single band should be observed, if not, redigest and gel purify.

Confirm activity of the EcoRI and BamHI restriction enzymes
Perform a small scale test digestion on the pSIF vector with EcoRI and BamHI separately to confirm they both are able to linearize the vector. If not, replace the enzyme.

3. No product was amplified from selected clones

Confirm activity of the Taq DNA polymerase
Test the activity of the enzyme reaction by amplifying a known sequence from any plasmid DNA. Replace the reagents if they demonstrate poor activity.

Ensure that you have not picked plasmids without shRNA template insert.
Colonies without an insert will yield a product of about 100 bp. Please also note that, due to recombination, you will not be able to amplify any product from plasmid isolated from some of the colonies. Always confirm that you have right insert by sequence analysis of PCR product or sequencing of shRNA expression cassette in purified lentivector constructs. If problematic, you may wish to consider using recombination-deficient bacterial strains such as STBL2 (Invitrogen, Cat. No. 10268-019) or SURE (Stratagene, Cat. No. 200238).

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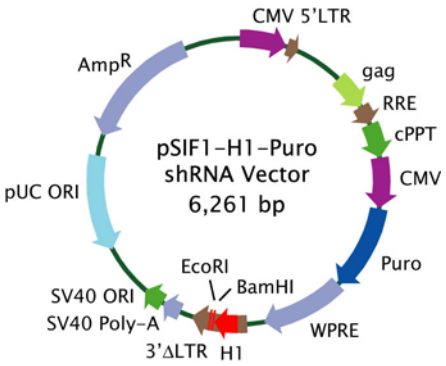
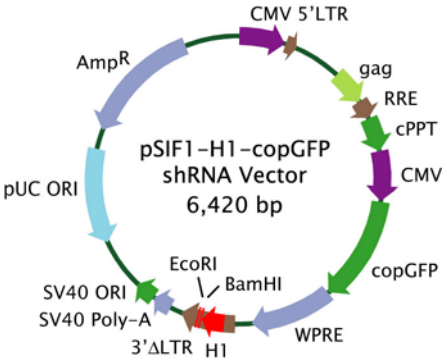
E. Appendix

A. Maps and Features for pSIF-H1 Vectors



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	1150-1391	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1394-1745	Human cytomegalovirus (CMV)--constitutive promoter for transcription of copGFP
Puro	1753-2352	Puromycin-resistant marker for selection of the transfected/transduced cells
WPRE	2359-2947	Woodchuck hepatitis virus posttranscriptional regulatory element--enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3068-3457	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
H1 RNA promoter	3098-3312	RNA polymerase III promoter for expression of siRNA insert
SV40 Poly-A	3545-3676	Transcription termination and polyadenylation
SV40 Ori	3685-3831	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4201-4874 (C)	Allows for high-copy replication in *E. coli*
AmpR	5019-5879 (C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

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



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	1150-1391	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1394-1745	Human cytomegalovirus (CMV)--constitutive promoter for transcription of copGFP
copGFP	1753-2511	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/transduced cells
WPRE	2518-3106	Woodchuck hepatitis virus posttranscriptional regulatory element--enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3227-3616	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
H1 RNA promoter	3257-3471	RNA polymerase III promoter for expression of siRNA insert
SV40 Poly-A	3704-3835	Transcription termination and polyadenylation
SV40 Ori	3844-3990	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4360-5033 (C)	Allows for high-copy replication in *E. coli*
AmpR	5178-6038 (C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

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

B. Sequences of Luciferase Control shRNA Template Oligonucleotides

sense

```
5'-GATCCGTGCGTTGTTAGTACTAATCCTATTTGTGAAGCAGATGAAATAGGGTTGGTACTAGCAACGCACTTTTTG-3'
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   3'-GCACGCAACAATCATGATTAGGATAAACACTTCGTCTACTTTATCCCAACCATGATCGTTGCGTGAAAAACTTAA-5'
```

antisense

C. Related Products

- **pPACKF1™ Lentivector Packaging Kit** (Cat. # LV100A-1)
  Unique lentiviral plasmids that produce all the necessary FIV viral proteins and the VSV-G envelope glycoprotein from vesicular stomatitis virus required to make active pseudoviral particles. The 293TN Producer Cell Line (SBI, Cat. # LV900A-1) transiently transfected with the pPACKF1 plasmids and an FIV-based expression construct produce packaged viral particles containing a lentiviral construct.

- **pSIH Single-Promoter shRNA Cloning Vectors (HIV-based)**
  - **pSIH1-H1-Puro™ shRNA Cloning and Expression Vector** (Cat. # SI500A-1)
  - **pSIH1-H1-copGFP™ shRNA Cloning and Expression Vector** (Cat. # SI501A-1)
  - **pSIH1-H1-H2Kk™ shRNA Cloning and Expression Vector** (Cat. # SI502A-1)

  These HIV-based single-promoter shRNA cloning vectors allow you to clone short-hairpin siRNA (shRNA) templates under the H1 promoter and efficiently transduce these shRNA constructs in a wide range of cells.

D. Technical Support

For more information about SBI products, to download manuals in PDF format, and to get vector map and sequence information, please use our web site:

http://www.systembio.com

For additional information or technical assistance, please call or e-mail us at:

System Biosciences (SBI)
211 South Whisman Road
Mountain View, CA 94041
Phone: (650) 968-2200
(888) 266-5066 (Toll Free)
Fax: (650) 968-2277
E-mail: tech@systembio.com

F. Licensing and Warranty Statement

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Use of the pSIF-H1 shRNA Cloning and Expression Vector (*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.

FIV Vector System

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The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242

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System Biosciences (SBI)
1616 North Shoreline Blvd.
Mountain View, CA 94043

Tel:	**888.266.5066 (Toll Free in US)**
	650.968.2200
Fax:	**650.968.2277**
E-mail:	**info@systembio.com**
Web:	**www.systembio.com**

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