Double-Promoter pFIV-H1/U6 siRNA Cloning and Expression Vectors

(Cat. # SI110A-1; SI111A-1)

User Manual

Store kit at -20°C on receipt

(Ver. 2-041015)

Contents

I. Introduction and Background

A. Overview

This manual provides details and information necessary to clone an siRNA template into the double-promoter pFIV-H1/U6 siRNA Cloning and Expression Vectors (pFIV-H1/U6-Puro™ and pFIV-H1/U6-copGFP™ Vectors). Specifically, it provides critical instructions on designing and synthesizing siRNA templates, cloning the siRNA templates into the pFIV-H1/U6 Vectors, and confirming successful cloning. This manual does not include information on packaging pFIV-H1/U6 Vector constructs into pseudoviral particles or transducing your target cells of choice with these particles. This information is available in the user manual provided with the pFIV Lentiviral Vector Packaging Kit from SBI (Cat. # LV100A-1), 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.

B. siRNA Technology

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, siRNA templates can be cloned into an siRNA expression vector—such as SBI's pFIV™ Series of siRNA Cloning and Expression Vectors—and expressed in the cells of choice. 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.

Two approaches have been developed for *in vivo* expression of siRNAs 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 siRNA sequence with a fold-back stem-loop structure (also known as a "hairpin") is expressed from a single promoter. 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).

C. FIV Lentiviral siRNA Expression System

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 lentiviral siRNA constructs in plasmid form into the cells with low-to-medium efficiency using conventional transfection protocols. However, by packaging the lentiviral siRNA vector construct in viral 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 lentiviral siRNA expression system consists of three main components:
(1) The lentiviral expression vector
(2) The lentiviral packaging vector
(3) A packaging cell line

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. To produce a high titer of viral particles, transiently co-transfect packaging cells (*e.g.*, HEK 293 cells) with the expression and packaging vectors.

The most popular lentiviral expression system is HIV based (Abbas-Terki 2002, Qin 2003, Wiznerowicz 2003). In spite of improved biosafety features, third generation HIV cloning vectors still pose a slight biohazard risk due to possible recombination with endogenous viral sequences to form a self-replicating HIV virus. SBI's novel pFIV siRNA Vectors address these issues since they are derived from feline immunodeficiency virus (FIV; Poeschla, 2003; for Safety Guidelines when working with these vectors, see section G). In addition, the pFIV Vectors developed at SBI are self-inactivating as a result of a deletion in the U3 region of 3'-ΔLTR (see Appendix for Vector Features). Upon integration into the genome, the 5' LTR promoter is inactivated, which prevents formation of replication-competent viral particles.

When expressed, the hybrid CMV/FIV 5' LTR drives high level transcription of the viral construct and produces a transcript that contains all the necessary functional elements (*i.e.*, Psi, RRE, and cPPT) for efficient packaging. When this construct is expressed in HEK 293 cells that also express viral coat proteins (*i.e.*, a packaging cell line), the pFIV transcripts are efficiently packaged into pseudoviral particles. After isolation, these pseudoviral particles containing the RNA version of the pFIV expression cassette can be efficiently transduced into any mammalian target cells. Following transduction into the target cells, this expression cassette is reverse transcribed and integrated into the genome of the target cell. The pFIV-H1/U6 Vectors also contain a bacterial origin of replication and ampicillin resistance (Amp^R) gene for propagation and selection in *E. coli*. The pFIV-H1/U6-Puro Vector (Cat. # SI110A-1) contains a puromycin resistance gene, under the control of a constitutive CMV promoter and a WPRE regulatory element, to enable selection of target cells stably expressing the siRNA template. The pFIV-H1/U6-copGFP Vector (Cat. # SI111A-1) contains a copGFP reporter gene under the CMV promoter and WPRE element. CopGFP is a novel fluorescent protein, derived from copepod plankton (Panalina sp.), which is similar to EGFP but has a brighter color.

D. Double-Promoter pFIV siRNA Vectors

The unique double-promoter lentiviral pFIV-H1/U6 siRNA Cloning Vectors contain opposing modified RNA polymerase III promoters (H1 and U6) that flank the siRNA template (Fig. 1). The pFIV-H1/U6 Vectors already have terminator sequences (T_5) just upstream of the transcriptional start sites (see Figs. 1 and 2). After transcription, the resulting ds siRNA product has the same structure as "natural" double-stranded siRNA and does not require the "dicer" processing step—as is the case with short-hairpin RNA (also known as shRNA). The

double-promoter pFIV-H1/U6 Vectors also provide higher stability of siRNA template inserts during propagation in *E. coli* because it does not contain a hairpin structure that is often removed during bacterial replication. This increased stability is very critical for construction of representative high complexity siRNA libraries. In addition, shorter siRNA sequences are less costly to produce and are easier to clone. These advantages make the double-promoter pFIV-H1/U6 siRNA Vectors highly suitable for siRNA library construction, as well as convenient for cloning and expressing single siRNA sequences.

The pFIV-H1/U6 Cloning Vectors are provided in a ready-to-ligate linearized form that has been digested with BbsI, and purified to remove the stuffer fragment. The linearized vector contains two unique 5' overhangs to facilitate directional cloning with minimal self-ligation background (Fig. 1). Two PCR primers that flank the H1 and U6 promoter regions are included to provide a simple way to screen plasmid clones for the presence of siRNA inserts (Fig. 2).

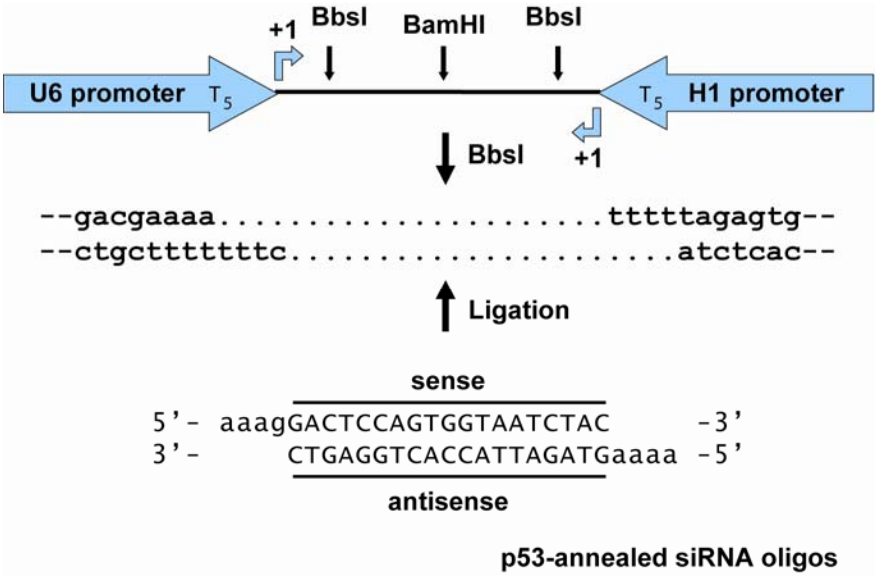

Fig. 1. Design of double-promoter siRNA cassette and siRNA template oligos. The dotted lines in the middle of the figure indicate the position of the "stuffer fragment" that was removed by digesting the vector with BbsI. A BamHI site, which is useful in screening (see Fig. 2), is located in the stuffer fragment. The transcriptional start site is indicated by the arrows denoted +1. The T_5 transcription terminators are also indicated. On the bottom of the figure, an example of the sequence for the p53 siRNA template oligonucleotides is shown.

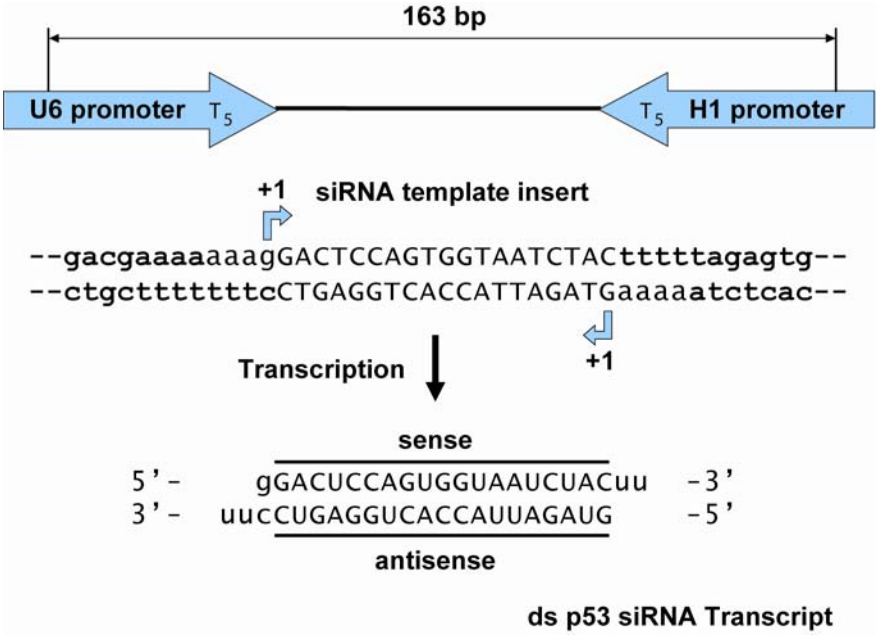

Fig. 2. Transcription of the p53 example construct from a double-promoter pFIV-H1/U6 Vector. Transcription of the p53 siRNA template starts at the indicated arrows and occurs simultaneously from both the H1 and U6 promoters. The structure of the p53 siRNA molecule is shown. Also shown is the location of the H1 and U6 PCR primers. Amplification with these primers spans the cloning site and produces a 163 bp PCR product for the p53 siRNA sequence shown. The size of the product is similar for both positive and negative clones because of the similarity in sizes of the insert and the stuffer fragment. When screening for inserts, the absence of the BamHI site (see Fig. 1) indicates a positive clone.

E. List of Components

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

- **pFIV-H1/U6-Puro™ siRNA Cloning and Expression Vector** (Cat. # SI110A-1)
  - 50 µl pFIV-H1/U6-Puro™ vector (linearized, 20 ng/µl)
  - 25 µl Luciferase Control siRNA Oligonucleotide (annealed; 100 ng/µl)
  - 25 µl H1 PCR primer (5'-CTGGGAAATCACCATAAACGTGAA-3'; 10 µM)
  - 25 µl U6 PCR primer (5'-GCTTACCGTAACTTGAAAGTATTTCG-3'; 10 µM)

- **pFIV-H1/U6-copGFP™ siRNA Cloning and Expression Vector** (Cat. # SI111A-1)
  - 50 µl pFIV-H1/U6-copGFP™ vector (linearized, 20 ng/µl)
  - 25 µl Luciferase Control siRNA Oligonucleotide (annealed; 100 ng/µl)
  - 25 µl H1 PCR primer (5'-CTGGGAAATCACCATAAACGTGAA-3'; 10 µM)
  - 25 µl U6 PCR primer (5'-GCTTACCGTAACTTGAAAGTATTTCG-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.

F. Additional Required Materials

For Annealing siRNA Oligonucleotides
- 2X DNA Annealing Buffer
  (20mM Tris, pH 7.8; 100mM NaCl; 0.2mM EDTA)

For Phosphorylation of Annealed siRNA Oligonucleotides
- T4 Polynucleotide Kinase
  (Recommended: New England BioLabs T4 Polynucleotide Kinase, Cat. # M0201S)

For Ligating and Transforming siRNA Vector Construct
- T4 DNA Ligase and ligation reaction buffer
  (Recommended: New England BioLabs T4 DNA Ligase, Cat. # M0202S. Dilute to 5 U/µl with the provided 1X reaction buffer just before use)
- Competent *E. coli* cells ($RecA^-$)
  (Recommended: BDB Clontech Fusion-Blue™ competent cells, Cat. # 636700)
- Petri plates containing LB Agar media with 50 µg/ml Ampicillin

For Screening siRNA Inserts
- Taq DNA polymerase, reaction buffer, and dNTP mix
  (Recommended: BDB Clontech Titanium™ Taq DNA polymerase, Cat. # 639208)
- PCR machine
- BamHI enzyme (New England BioLabs, Cat. # R0136M)
- 2-3% 1X TAE Agarose gel

For Purifying siRNA Vectors after Cloning

- Plasmid purification kit
  (Recommended: QIAGEN Endotoxin-free Plasmid Kit. The following kit combinations can be used for Midi scale preparation of endotoxin-free 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

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 pFIV-H1/U6 Vectors for cloning and expressing siRNA were developed at SBI. These vectors have 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 pFIV-H1/U6 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.

II. Protocol

A. siRNA Oligonucleotide Design and Synthesis

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

- 19-29 nt in length (usually longer oligos (25-27 nt) have slightly better silencing efficiencies although 19 nt oligos are more commonly used).
- Unique sequence (< 70% Homology with other sequences in a RefSeq database).
- 40%-50% 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 on the anti-sense strand that is more AT-rich than the 3'-terminus.

For each selected sequence, two complementary siRNA oligonucleotides (a sense strand and an anti-sense strand) need to be synthesized, then annealed before the phosphorylation and ligation steps. Below are guidelines for synthesis of the siRNA template oligonucleotides:

(1) A 50 nmol scale reaction for DNA oligonucleotide synthesis with regular desalting purification is sufficient for cloning into the pFIV Vectors.

(2) For the best cloning efficiency, we recommend using phosphorylated oligonucleotides which can typically be ordered from the supplier. Alternatively, you can phosphorylate the oligonucleotides after synthesis using T4 polynucleotide kinase. The phosphorylation procedure is shown below in step B.2.

(3) In addition to the sense or anti-sense siRNA sequence the oligonucleotide needs to include a 4-base sequence at the 5' end of each oligonucleotide (AAAG on sense strand and AAAA on the anti-sense strand). These sequences form "sticky-ends" that facilitate ligation with the BbsI-digested vector. The annealed sequences should have a double-stranded siRNA structure as shown in the following diagram (See also Fig. 1).

Sense (top)

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5′- AAAGNNNNNNNNNNNNNNNNNNN      -3′
3′-      NNNNNNNNNNNNNNNNNNNAAAA -5′
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Anti-sense (bottom)

B. Cloning of siRNA Template into pFIV-H1/U6 Vector

1. Anneal siRNA Oligonucleotides

a. Dissolve the siRNA oligonucleotides in an appropriate amount of deionized water to a final concentration of 1 μg/μl.

b. Prepare the ds siRNA oligonucleotide as follows:

2.5	μl	Sense strand siRNA oligonucleotide
2.5	μl	Anti-sense strand siRNA oligonucleotide
25.0	μl	2X Annealing Buffer
20.0	μl	Deionized water
50.0	μl	Total volume

c. Heat the mixture to 95°C for 5 min in a thermocycler or heating block.

d. Turn off the thermocycler or heating block and let it cool to room temperature.

e. The annealed oligonucleotide (100 ng/μl) is ready for phosphorylation and ligation steps. Store the annealed oligonucleotides at -20°C until use.

2. Phosphorylate the Template siRNA

Note: If your oligonucleotides are already phosphorylated, dilute them to 10 ng/μl in 1X Annealing Buffer, skip this phosphorylation step, and proceed to ligation in step 3. However, you must phosphorylate the Luciferase Control Oligonucleotide before continuing with step 3. For the insert-minus control, you may either follow step 2 or use 1 μl Annealing Buffer in step 3.

a. Set up 10 μl phosphorylation reactions for each experimental siRNA template as follows:

1	μl	Annealed ds siRNA template oligos (100 ng/μl) *
1	μl	10X T4 Polynucleotide Kinase Buffer
1	μl	10 mM ATP
6	μl	Deionized water
1	μl	T4 Polynucleotide Kinase (10 U/μl)
10	μl	Total volume

* For the insert-minus control, use 1 μl 1X Annealing Buffer
For the positive control, use 1 μl of the Luciferase Control Oligonucleotide.

b. Incubate the phosphorylation reaction at 37°C for 30 minutes. To stop the reaction, heat at 70°C for 10 min.

c. Use 1 μl (10 ng) for the following ligation reaction.

3. Ligate the Template siRNA

a. Set up 10 μl ligation reactions for each phosphorylated siRNA template as follows:

2.5	μl	Linearized pFIV-H1/U6 Vector (20 ng/μl)
1.0	μl	Phosphorylated ds siRNA template (step 2; 10 ng/μl) **
1.0	μl	10X T4 DNA Ligase Buffer
4.5	μl	Deionized water
1.0	μl	T4 DNA ligase (5 U/μl)
10.0	μl	Total volume

** For controls, use insert-minus and positive control from step 2.

b. Incubate the ligation reaction at 16°C for 2-4 hrs.

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

a. For each experimental siRNA 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.

C. Identify clones with the target siRNA 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 100 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 siRNA template inserts

There are two options for insert screening. The first is screening using the H1 and U6 PCR primers provided in the kit followed by digestion of the PCR product by BamHI which is only present in negative clones. Alternatively, you can simply use your anti-sense strand siRNA oligo and U6 PCR primer (or sense strand siRNA oligo and H1 PCR primer) to amplify only positive clones.

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

1 rxn	10 rxn	Composition
0.5 µl	5 µl	H1 PCR primer (10 µM)
0.5 µl	5 µl	U6 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 (approx. 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 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, 3 min	1 cycle

e. Dilute BamHI enzyme to 5 U/µl in 1X BamHI buffer.

f. Take 15 µl of PCR product from step d and add 1 µl of diluted BamHI from step e. Mix.

g. Incubate at 37°C for 30 min.

h. Take 5 µl of the digestion reaction and run it on a 2-3% agarose/EtBr gel in 1X TAE buffer.

PCR product from clones without an insert will be digested, resulting in two bands at 81 and 91 bp. Product from positive clones will not be digested, and the resulting band is 163 bp for 19 bp siRNA templates (see Fig. 2).

Grow a positive clone in an appropriate amount of LB-Amp Broth, and purify the plasmid construct using an endotoxin-free plasmid purification kit (see Section I.F).

2A. Screen for siRNA template inserts (Alternative Protocol)

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

1 rxn	10 rxn	Composition
0.5 µl	5 µl	U6 PCR primer (10 µM)*
0.5 µl	5 µl	Anti-sense strand siRNA oligonucleotide (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 (approx. 5 U/µl)
24.0 µl	240 µl	Total volume

* ***Optional*****:** To confirm the insert in the reverse direction, replace the first two components with the H1 PCR primer and the sense strand siRNA oligonucleotide.

b. Mix the master mix very well and aliquot 24 µl into each well of 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, 3 min	1 cycle

e. Take 5 µl of PCR product from step d and run it on a 2-3% agarose/EtBr gel in 1X TAE buffer.

Only those clones with a correct insert will amplify. For 19 bp siRNA templates, the expected size of amplified siRNA inserts should be 87 bp if using the U6 PCR primer and anti-sense strand siRNA oligo. If you are using the H1 PCR primer and sense strand oligo, the product should be 95 bp.

Grow a positive clone in an appropriate amount of LB-Amp Broth, and purify the plasmid construct using an endotoxin-free plasmid purification kit (see Section I.F).

D. Transfection and Analysis of Silencing Efficiency

If you are planning to use SBI's pFIV siRNA vectors for viral delivery, first screen the siRNA constructs generated in section C to determine their effectiveness at knocking down expression of the target gene of interest. To rapidly screen the lentiviral siRNA 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) 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, BDB Clontech's CLONfectin™, Cat. # 631301).

For siRNA 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 siRNA 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 siRNA constructs with 70-80% silencing efficiency are suitable for gene functional analysis studies.

Once you identify a functional siRNA construct, you can package this construct into FIV pseudoviral particles, and efficiently transduce it into any target cells of choice. For this purpose, you will need to purchase the pFIV-PACK™ Lentiviral Vector Packaging Kit from SBI (Cat. # LV100A-1) and HEK 293T cells (ATCC, Cat. # CRL-11268).

The pFIV-PACK User Manual includes the procedural information for packaging the viral vector. This user manual is also available on the SBI web site (www.systembio.com). Although you can create stable transfectants with the pFIV construct using standard transfection and selection protocols, transduction of the lentiviral pFIV siRNA construct 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.

III. Troubleshooting

A. Using the Positive Control

The Luciferase Control siRNA Oligonucleotide is a double stranded DNA fragment with sticky ends (5'AAAG, and 5'AAAA) to match with the BbsI-digested ends on the linearized pFIV-H1/U6 vector. The 19-base siRNA template sequence targets the Luciferase gene.

When run in parallel with your experimental annealed double-stranded siRNA oligonucleotides, Luciferase Control siRNA Oligonucleotide 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 should provide 2-10 times more colonies than ligation of the vector without an insert.

The control pFIV construct with the Luciferase siRNA 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 pFIV-H1 Luciferase siRNA construct in the cell.

B. Troubleshooting Specific Results

1. Getting Few or No Clones

Check design of the siRNA template
Check the sequence of the siRNA oligonucleotides to ensure that, after sense/anti-sense annealing, the ends present the 5' AAAG and 5' AAAA overhangs for proper annealing with the restricted ends of linear pFIV-H1/U6 Vector. Also, confirm that the sense and anti-sense strands sequences reverse complement each other.

Check sense/anti-sense annealing
To ensure a high percentage (80%) of double-stranded DNA after annealing, check the concentration of siRNA oligonucleotides using a spectrophotometer and mix equal molar amounts of each strand. For optimal annealing, turn off the thermocycler after denaturation and let the tubes cool down to room temperature. Evaluate 5 μl of annealed insert (from step II.B.1.e) 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 double strand oligonucleotide concentration is only 100 ng/µl and that you dilute it at least 10-fold before adding it to the ligation reaction.

Check the quality of the competent cells
Handle the competent cells gently. Many cells can not be refrozen once thawed. The quality of the competent cell 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. 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.

Confirm pairing of the oligo sequence and PCR primer
If you used the alternative insert screening protocol, confirm that the sense strand oligonucleotide is paired with the H1 PCR primer, and the anti-sense strand sequence with the U6 PCR primer.

3. None of the PCR products were digested with BamHI enzyme

Confirm activity of the BamHI enzyme
Test the activity of the enzyme reaction by digesting a DNA sequence known to have a BamHI restriction site. Replace the reagents if they demonstrate poor activity. Alternatively, use the alternative insert screening protocol on page 13.

IV. References

General references:

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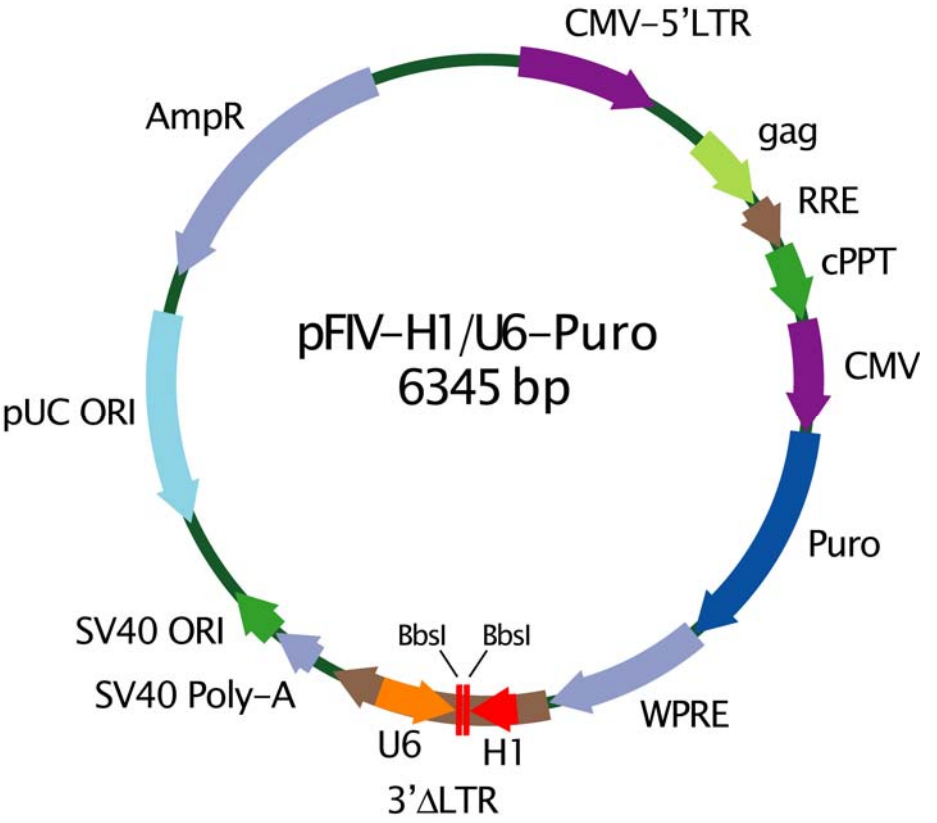
V. Appendix

A. Maps and Features for pFIV-H1/U6 Vectors

pFIV-H1/U6-Puro Map

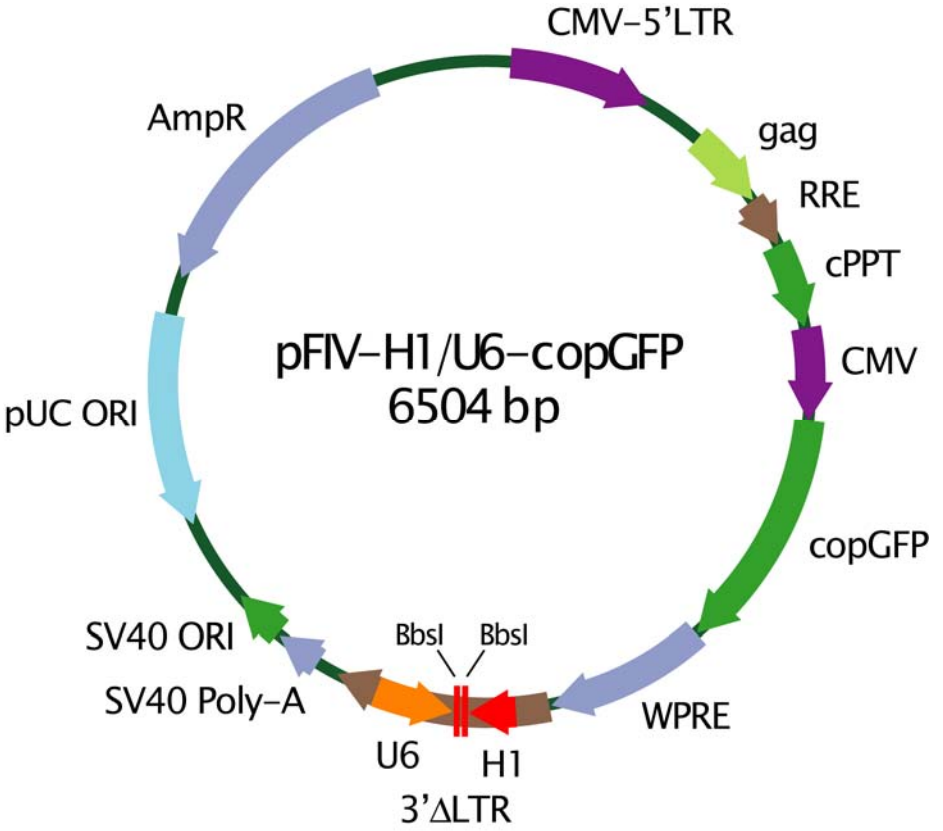


pFIV-H1/U6-Puro Features

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 purine pyrimidine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1394-1745	Constitutive promoter for transcription of Puro gene
Puro	1753-2352	Puromycin-resistant marker for selection of the transfected/transduced cells
WPRE	2359-2887	Posttranscriptional regulatory element which enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	2993-3541	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	3051-3141	RNA polymerase III promoter for expression of siRNA insert
U6 RNA promoter	3194-3436(C)	RNA polymerase III promoter for expression of siRNA insert
SV40 Poly-A	3629-3760	Transcription termination and polyadenylation
SV40 Ori	3769-3915	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4285-4958(C)	Allows for high-copy replication in *E. coli*
AmpR	5103-5963(C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

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

pFIV-H1/U6-copGFP Map



pFIV-H1/U6-copGFP Features

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 purine pyrimidine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1394-1745	Constitutive promoter for transcription of copGFP gene
CopGFP	1753-2511	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/ transduced cells
WPRE	2518-3046	Posttranscriptional regulatory element which enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3152-3700	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	3210-3300	RNA polymerase III promoter for expression of siRNA insert
U6 RNA promoter	3353-3595(C)	RNA polymerase III promoter for expression of siRNA insert
SV40 Poly-A	3788-3919	Transcription termination and polyadenylation
SV40 Ori	3928-4074	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4444-5117(C)	Allows for high-copy replication in *E. coli*
AmpR	5262-6122(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 Fluorescent Protein

The pFIV-H1/U6-copGFP 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 FIV constructs into cells.

C. Related Products

- **Single-Promoter pFIV-H1 siRNA Cloning Vectors**
  - **pFIV-H1-Puro™ siRNA Cloning and Expression Vector** (Cat. # SI100B-1)
  - **pFIV-H1-copGFP™ siRNA Cloning and Expression Vector** (Cat. # SI101A-1)

  These FIV-based single-promoter siRNA cloning vectors allow you to clone short-hairpin siRNA (shRNA) templates under the H1 promoter and efficiently transduce these siRNA constructs in a wide range of cells. It is biologically safer than similar siRNA expression vectors that are based on HIV.

- **pFIV-PACK™ Lentiviral Vector Packaging Kit** (Cat. # LV100A-1)
  A unique lentiviral vector that produces all the necessary FIV viral proteins and the VSV-G envelop glycoprotein from vesicular stomatitis virus required to make active pseudoviral particles. 293T cells (ATCC, Cat. # CRL-11268) transiently transfected with the pFIV-PACK and one of the pFIV siRNA Vectors produce packaged viral particles containing a pFIV siRNA Vector.

- **Packaged Transduction Control pFIV-copGFP Reporter Vector** (Cat. # LV200A-1)

D. Useful Oligonucleotide Conversion Factors

- The average molecular weight of a nucleotide is 330
  N bases of single-stranded DNA = 330 X N
  N bases of double-stranded DNA = 660 X N

- 1 μM = 1 μmol/L = 1 nmol/ml = 1 pmol/μl

- Mass concentration to molar concentration:
  μg/μl = μM X molecular weight ÷ 10^6

Sample calculation for a 20 mer:
Calculate the mass concentration for 100 nmol of a 20 base single-stranded oligonucleotide in 500 μl of water:

Molecular weight for a single-stranded 20 mer:
20 x 330 = **6600**

Molar concentration for 100 nmol in 500 μl:
100 nmol in 500 μl = 200 nmol/ml = **200 μM**

Mass concentration for a 200μM solution:
200 μM X 6600 ÷ 10^6 = **1.32 μg/μl**

E. Technical Support

For more information about SBI products, to download manuals in PDF format, and to get vector map and sequence information, please visit 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: info@systembio.com

VI. Licensing and Warranty Statement

Limited Use License

Use of the pFIV-H1/U6 siRNA 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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CMV Promoter

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

CopGFP Marker

The product pFIV-H1/U6-copGFP siRNA Cloning and Expression Vector contains a proprietary nucleic acid coding for a proprietary fluorescent protein(s) intended to be used for research purposes only. Any use of the proprietary nucleic acids other than for research use is strictly prohibited. USE IN ANY

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