pGreenPuro™ shRNA Cloning and Expression Lentivector

(Cat. #s SI505A-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. 081124)

Contents

I. Introduction and Background

A. Purpose of this Manual

This manual provides details and information necessary to clone an shRNA template into the pGreenPuro™ shRNA Cloning and Expression Vector. Specifically, it provides critical instructions on designing and synthesizing shRNA templates, cloning the shRNA templates into the H1 expression cassette of the vector, and verifying final vector constructs. This manual does not include information on packaging the pGreenPuro™ 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.

B. 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

SBI offers a third generation of the most popular HIV-1 based lentivector expression system, consisting of three main components:

(1) The lentiviral expression vector (*e.g.*, pGreenPuro™, pSIH1-H1-Puro™)

(2) The lentiviral packaging plasmids (*e.g.*, pPACKH1™ 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.

C. pGreenPuro™ shRNA Expression Lentivector

The pGreenPuro™ expression vector is an improved third generation of HIV-based expression lentivector developed for gene therapy applications (Sodroski, J.G., 1997, 1999, Federico, 2003, Heiser, 2004, Machida, 2003). See section F for safety guidelines. The pGreenPuro™ vector (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 a wide range of cell lines.
- CMV promoter promotes high level of expression of both copGFP (fluorescent reporter) and puromycin-N-acetyl transferase (drug selectable marker) in the same vector for detection and selection of either transduced or transfected cells.
- Hybrid RSV-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 pGreenPuro™ plasmid in 293 producer cells.
- The pUC origin for high copy replication and maintenance of the plasmid in *E.coli* cells.
- The ampicilin-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 pGreenPuro™ Vector (Cat. # SI505A-1) contains a puromycin resistance gene to enable drug selection of target cells stably expressing the siRNA, and a copGFP gene. The 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. The open reading frames of puromycin resistance and copGFP genes are connected by a T2A sequence and are transcribed from the CMV promoter as a bicistronic transcript. The two proteins are then separated through translational cleavage at the T2A site.

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).

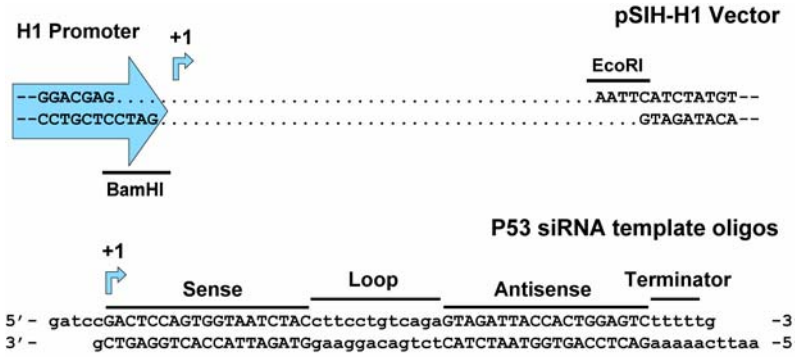


Fig. 1. Design of the single-promoter pPurGreen™ shRNA expression cassette. The dotted lines at the top of the figure indicate the position of the "stuffer fragment" that is removed during linearization by digesting the vector with BamHI/EcoRI. Your shRNA template sequence should be designed to directionally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

The pGreenPuro™ Vector is 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 pGreenPuro™ vector needs to be linearized by restriction digest with BamHI and EcoRI, and purified to remove the stuffer fragment. When linearized, the 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).

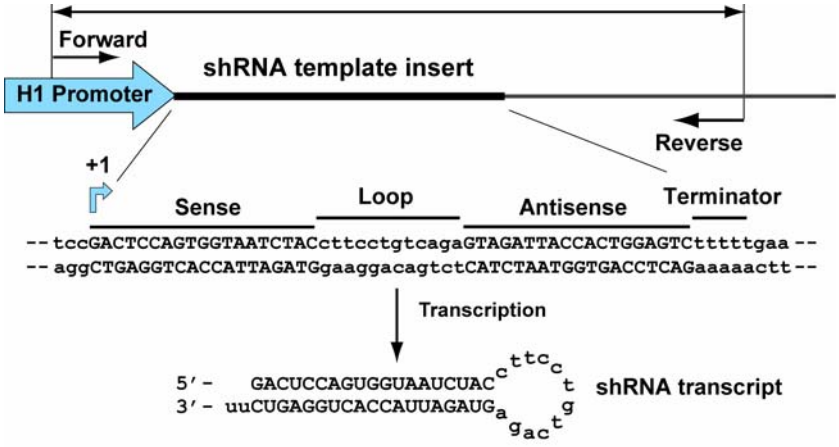


Fig. 2. Example shRNA template construct targeting the p53 gene. The nucleotides for the specific siRNA sequence targeting the p53 gene are shown in capital letters. The shRNA sense and antisense sequences flank the region coding for the loop structure. In addition, a terminator sequence for the RNA 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.

D. List of Components

Each pGreenPuro™ Vector Kit provides enough plasmid for 20 ligation reactions:

50 µl	pGreenPuro™ vector (non-linearized, 0.2 µg/µl)
25 µl	Luciferase Control shRNA Template Oligonucleotide Mix (20 µM each)

The kit is 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.

E. Additional Required Materials

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.
- Competent *E. coli* cells (RecA$^-$) (Recommended: Invitrogen, Max Efficiency Stbl2™ cells, Cat.# 10268-019)
- 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
- ForwardH PCR Primer (5'-AATGTCTTTGGATTTGGGAATCTTAT-3'; 10 µM
- ReverseH PCR Primer (5'-TGGTCTAACCAGAGAGACCCAGTA-3'; 10 µM)

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 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

Transfection of pGreenPuro™ Construct into Target Cells

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

Packaging of pGreenPuro™ Constructs in Pseudoviral Particles

- pPACKH1 Lentivector Packaging Kit (SBI, Cat # LV500A-1)
- 293TN Producer Cell Line (SBI, Cat # LV900A-1; or ATCC, 293T/17, Cat # CRL-11268)
- Lipofectamine™ Transfection Reagent (Invitrogen, Cat # 18324-111)
- Plus™ Reagent (Invitrogen, Cat # 11514-015)

F. Safety Guidelines

SBI's pGreenPuro™ lentivectors together with the pPACKH1 packaging plasmids comprises a third-generation HIV-1-based cloning vector system. These lentivectors are based on the vectors developed for gene therapy applications by Dr. J. G. Sodroski (US patent #5,665,577 and # 5,981,276). 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.
- RSV promoter upstream of 5'LTR in pGreenPuro™ expression vector allows efficient Tat-independent production of viral RNA, reducing the number of genes from HIV-1 that are used in this system.
- Number of HIV-1 viral genes necessary for packaging, replication and transduction is reduced to three (gag, rev and pol), and these genes are expressed from different plasmids lacking packaging signals and significant homology to the pGreenPuro™ expression vector, VSV-G expression vector, or each other to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (*gag, pol, rev)* will be present in the packaged viral genome, as they are expressed from packaging plasmids lacking packaging signal--therefore, the lentiviral particles generated are replication-incompetent.
- Pseudoviral particles will carry only the expression construct of your target gene.
- The lentiviral particles produced in this system are pseudotyped with envelope G glycoprotein from Vesicular Stomatitis Virus.

Despite the above safety features, use of HIV-based vectors falls within NIH Biosafety Level 2 criteria. 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 follows standard microbiological practices, which include:

- Wear gloves and lab coat all the time when conducting the procedure;
- 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 are to be placed in a durable, leakproof container and closed for transport from the laboratory.

II. Protocol

A. shRNA Template Oligonucleotide Design and Synthesis

Typically, 3 - 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 pGreenPuro™ 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.

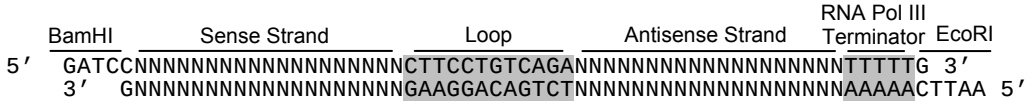
(2) A hairpin loop sequence between sense and antisense portion. The 9-nt loop sequence (5'-TTCAAGAGA-3') is most commonly used in RNA silencing experiments (Brummelkamp 2002), but we have used a 12-nt sequence (5'-CTTCCTGTCAGA-3'), which generates similar results. Loop sequences of 3 to 15 nucleotides 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 pGreenPuro™ 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, and annealed before ligation step. A 50 μM scale reaction for oligonucleotide synthesis with regular desalting purification is sufficient for cloning into the pGreenPuro™-H1 Vectors. Phosphorylation of the oligonucleotides using T4 polynucleotide kinase is not necessary.

B. Cloning of shRNA Template Oligonucleotides into pGreenPuro™ Vector

1. Linearize the pGreenPuro™ 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	pGreenPuro™ 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. Anneal the shRNA Template Oligonucleotides

Note: This protocol was developed for regular non-phosphorylated oligos.

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 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) *
18	µl	10 mM Tris-HCl, pH 8.5
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. Heat the reaction mix to 95°C for 2 min in a thermocycler.

d. Turn off the thermocycler and let it cool to room temperature over 20 min.

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

3. Ligate the shRNA Template into Linearized pGreenPuro™ Lentivector

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

1.0	µl	Linearized pGreenPuro™ Vector (50 ng/µl)
0.5	µl	Annealed double-stranded 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** overnight.

4. 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 could expect to get at least 10-fold more colonies in experimental samples in comparison with 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	ForwardH PCR Primer (10 μM)
0.5	μl	5	μl	ReverseH 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 105 bp. The expected size of amplified clones with a shRNA template insert should be about 150-170 bp depending on expected length of the shRNA template insert (see Figure 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 ForwardH 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 pGreenPuro™ 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.

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 pPACKH1 Lentivector Packaging Kit (SBI, Cat. # LV500A-1) and 293TN Producer Cell Line (SBI, Cat. # LV900A-1).

The pPACKH1 User Manual "Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells" includes the procedural information for packaging the shRNA lentivector constructs. This user manual is also available on the SBI web site (www.systembio.com). Although you can create stable transfectants with the pGreenPuro™ constructs using standard transfection and selection protocols, transduction of the lentiviral pGreenPuro™ 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.

F. Selection of Stably Infected Cells:

Because infected cells stably express copGFP and puromycin, as well as the shRNA cloned into the pGreenPuro™ vector, they can be selected either for GFP positive cells by FACS or for puromycin resistance cells by puromycin treatment. Since puromycin resistance can vary from cell type to cell type, we recommend you generate a killing curve of your target cells with different concentrations of puromycin in a 96-well plate, and then use the lowest concentration that can kill your target cells the next day after the addition of puromycin for selecting your infected cells.

III. 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 pGreenPuro™ 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 pGreenPuro™ 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 pGreenPuro™ 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 pGreenPuro™ 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 DNA ligase
Test the activity of your ligase and reaction buffer using a different vector and insert. 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 pGreenPuro™ 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 pGreenPuro™ 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 (even when using recA$^-$ bacteria), 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 saran expression cassette in purified lentivector constructs.

IV. References

General references:

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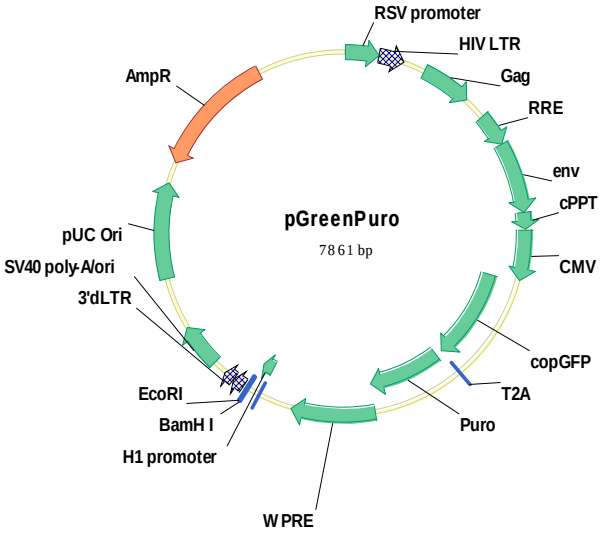
V. Appendix

A. Map and Features for pGreenPuro™ Vector



Feature	Location*	Function
RSV/5'LTR	7-413	Hybrid RSV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	566-920	Packaging signal
RRE	1076-1309	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1806-1923	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1929-2278	Human cytomegalovirus (CMV)--constitutive promoter for transcription of copGFP-T2A-puro
copGFP	2293-3048	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/transduced cells
T2A	3049-3102	*Thosea asigna* virus 2A translational cleavage site containing 18 amino acid residues. Cleavage occurs via a co-translational ribosome skipping mechanism between the C-terminal Glycin and Prolin residues, leaving 17 residues attached to the end of copGFP and 1 residue to the start of the puromycin resistance marker
Puro	3103-3702	Puromycin-resistant marker for selection of the ransfected/transduced cells
WPRE	3703-4291	Woodchuck hepatitis virus posttranscriptional regulatory element--enhances the stability of the viral transcripts
3' ΔLTR(ΔU3)	4631-4813	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	4526-4616	RNA polymerase III promoter for expression of siRNA insert
SV40 Poly-A	4911-5219	Transcription termination and polyadenylation
SV40 Ori	4911-5219	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	5584-6252	Allows for high-copy replication in *E. coli*
AmpR	6397-7257 (C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

B. Sequences of the Luciferase Control shRNA Template Oligonucleotide

sense

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

antisense

C. Related Products

- **pPACKH1™ Lentivector Packaging Kit** (Cat. # LV500A-1)
  Unique lentiviral plasmids that produce all the necessary HIV viral proteins and the VSV-G envelope glycoprotein from vesicular stomatitis virus required to make active pseudoviral particles. 293TN Producer Cell Line (SBI, Cat. # LV900A-1; or ATCC, Cat. # CRL-11268) transiently transfected with the pPACKH1 plasmids and an HIV-based lentiviral construct produce packaged viral particles containing a lentiviral construct.

- **pSIF Single-Promoter shRNA Cloning Vectors (FIV-based)**
  - **pSIF1-H1-Puro™ shRNA Cloning and Expression Vector** (Cat. # SI100C-1)
  - **pSIF1-H1-copGFP™ shRNA Cloning and Expression Vector** (Cat. # SI101B-1)

  These FIV-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. They are biologically safer than similar shRNA expression vectors that are based on HIV.

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)
1616 North Shoreline Blvd.
Mountain View, CA 94043
Phone: (650) 968-2200
(888) 266-5066 (Toll Free)
Fax: (650) 968-2277
E-mail: tech@systembio.com

VI. Licensing and Warranty Statement

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Use of the pGreenPuro™-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.

HIV 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

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

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