Protocol

Design and Cloning of an shRNA into a Lentiviral Silencing Vector: Version A

Gustavo Tiscornia, Oded Singer, and Inder M. Verma

This protocol was adapted from "Development of Lentiviral Vectors Expressing siRNA," Chapter 3, in *Gene Transfer: Delivery and Expression of DNA and RNA* (eds. Friedmann and Rossi). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2007.

INTRODUCTION

This protocol combines the specificity of small interfering RNA (siRNA)-mediated silencing cassettes with the versatility of lentiviral vectors to stably transduce a wide range of cell types. A short hairpin RNA (shRNA) designed against a given target is cloned into a plasmid containing the pol III promoter. The design uses a 5′ forward primer upstream of the pol III promoter and a 3′ reverse primer that includes the entire shRNA sequence (i.e., sense, loop, and antisense sequences followed by five Ts), followed by 22 bases complementary to the last 22 bp upstream of the +1 transcriptional start site of the pol III promoter. An NheI-compatible restriction site is included at the 5′ end of both forward and reverse primers. A single round of PCR is used to amplify this template. The resulting DNA fragment contains an shRNA expression cassette that can be cloned into a simple cloning vector, tested, and then transferred to the lentiviral vector, or cloned into the lentiviral vector directly. This procedure uses a unique restriction site in the 3′ long terminal repeat (LTR). During integration, the 5′ LTR of the provirus is copied from the 3′ LTR, cloning the H1-driven shRNA into the 3′ LTR, resulting in duplication of the silencing cassette. This strategy maximizes the silencing power of the lentiviral vector. The combination of the lentiviral and siRNA technologies provides a powerful tool to achieve long-term down-regulation of specific target genes both in vitro and in vivo.

RELATED INFORMATION

Figure 1 presents a schematic representation of the protocol. One undesirable consequence of this procedure is that the siRNA target sequence is also present in the mRNA expressing the marker gene, resulting in somewhat lower expression of the marker. In an alternative method, **Design and Cloning of an shRNA into a Lentiviral Silencing Vector: Version B** (Tiscornia et al. 2008), the position of the silencing cassette is upstream of the marker expression cassette, thus avoiding down-regulation of the marker. Because the silencing cassette is not in the 3′ LTR, only one copy of the silencing cassette is delivered per viral particle.

A number of algorithms have been developed to predict effective siRNA sequences (e.g., http://www.ambion.com/ or http://sfold.wadsworth.org/). A database search is recommended to filter out candidate targets that are present in other genes to avoid silencing of these loci.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Advantage GC 2 polymerase mix (Clontech)
<R>2X BES-buffered saline (BBS)
<!>2.5 M $CaCl_2$ (10X stock)

Store 1.5-mL aliquots at -20°C.

Cells (293T human embryonic kidney) (Invitrogen)

Cells should be of low-passage number and should not be used after passage 20 or if growth is slow.

<R><!>Dimethyl sulfoxide (7% [v/v]) (DMSO)
Dulbecco's modified Eagle's medium (DMEM) with 2% and 10% fetal bovine serum (FBS)

Certain brands of FBS do not support efficient transfection and can result in low viral titers.

Enzyme-linked immunosorbent assay (ELISA) Kit, p24 (PerkinElmer) (optional; see Step 20)
Gels for purifying lentivector plasmid and insert
H1 promoter cloned into pGEM-T (Promega)
Hank's balanced salt solution (HBSS; Invitrogen)
<R>Phosphate-buffered saline (PBS) containing 0.001% (w/v) poly-L-lysine (Sigma-Aldrich)

Filter-sterilize and store at -20°C.

Plasmids: pMDL (Gag-Pol), pREV, and pVSV-G

For plasmid preparation, use QIAGEN plasmid maxipreps at 1 µg/µL.

Primer, 5′ forward

The 5′ forward primer must contain an XbaI site.

Primer, 3′ reverse

This primer should be designed to contain 22 nucleotides from the 3′ end of the pol III promoter, and a 5′ tail including the entire shRNA loop against the gene to be silenced, the transcriptional stop signal (T5), and XbaI site sequences.

Restriction endonucleases: NheI, SspI, XbaI
Sucrose (20% [w/v] in HBSS)

Equipment

Centrifuge
Dishes (tissue-culture, 15-cm)
Dishes (tissue-culture, six-well)
Filters (0.22- or 0.45-µm)
Incubators preset to 37°C (3% and 10% CO_2)
Microcentrifuge
PCR machine
Rotors (SW 28 and SW 55) (Beckman)
Tubes (50-mL)
Tubes (centrifuge, polyallomer, 5-mL) (Beckman)
Tubes (centrifuge, polyallomer, 30-mL) (Beckman)
Tubes (microcentrifuge)
Vortexer

METHOD

Design and Cloning of shRNAs

1. Select a target within the gene to be silenced and design primers to amplify the silencing cassette. For GFP, the target would be: GCAAGCTGACCCTGAAGTTC (Tiscornia et al. 2003). The 3′ reverse primer would be: 5′-CTGTCTAGACAAAAAGCAAGCTGACCCTGAAGTTC**TCTCTTGAA** GAACTTCAGGGTCAGCTTGC***GGGGATCTGTGGTCTCATACA***-3′, where the H1 sequence is in italic bold, XbaI (NheI compatible) is underlined, and the loop is bold underlined.

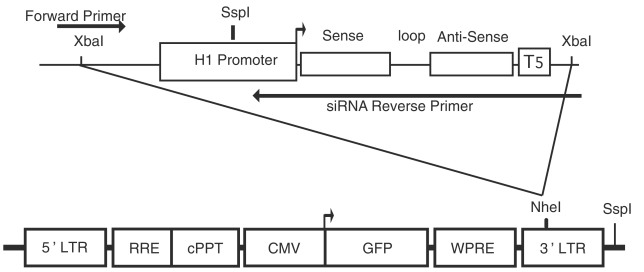

FIGURE 1. The siRNA expression cassette consists of a nucleotide sense sequence (identical to the target sequence in the mRNA to be down-regulated), followed by a 9-bp loop, an antisense sequence, and a stretch of five Ts (T5) as a pol III transcriptional termination signal downstream from an H1 promoter. After PCR amplification, this is packaged into the NheI site of a transfer vector containing all the *cis*-acting elements required for replication and packaging of RNA into viral particles, including a Rev-responsive element (RRE) to enhance nuclear export of unspliced viral genomic RNA, woodchuck hepatitis virus regulatory element (WPRE) to enhance expression of the transgene, and a central polypurine tract (cPPT) purported to increase efficiency of nuclear import of the preintegration complex. Green fluorescent protein (GFP) is used as a marker.

2. Mix 10 ng of the pGEM-T plasmid containing the H1 promoter (the template) and 10 µM (final concentration) each of the 5′ forward primer and the 3′ reverse primer, with the Advantage GC 2 polymerase mix, according to the manufacturer's instructions. Add 7% (v/v) DMSO or a similar agent to a regular *Taq* polymerase reaction to prevent hairpin formation.

   Use the GC-melt additive as 10X.

3. Amplify using the parameters listed below:

Cycle number	Denaturation	Annealing	Polymerization
First cycle	3 min at 94°C		
30 cycles	30 sec at 94°C	30 sec at 55°C	40 sec at 72°C
Last cycle			10 min at 72°C

   This results in an amplified fragment of ~400 bp that can be cloned in an A/T vector for sequencing or directly cloned in the lentivector plasmid.

4. Digest the insert with XbaI and gel-purify.

5. Digest the lentivector plasmid with NheI, gel-purify, and then dephosphorylate.

6. Validate the cloned shRNA cassettes by transfecting or transducing (as lentiviral particles) to a cell line that expresses the target gene.

   Typically, 50 ng of vector are ligated to 100 ng of insert and transformed into competent bacteria. Plasmid DNA from the resulting colonies can be screened by digestion with SspI. The parental vector should have only one SspI site, whereas the vector containing the insert will acquire an additional SspI site located in the H1 promoter. It is important to verify the integrity of the hairpin by sequencing using the H1-F primer 5′-TGGCAGGAAGATG GCTGTGA-3′, because mutations in the hairpin can significantly reduce the efficiency of down-regulation.

7. Alternatively, coexpress a tagged cDNA of the target gene together with shRNA silencing cassettes in an easily transfected cell line (e.g., 293T).

   This is useful when target mRNA is restricted to certain cell types or a specific antibody against the target is unavailable. Typically, transfect 200 ng of target cDNA plasmid plus 500-1000 ng of the plasmid containing the silencing cassette per well (six-well cluster) and harvest the cells for immunoblot analysis 48-72 h after transfection.

Preparation of Lentiviral Vectors

8. Twenty-four hours before transfection, prepare plates and cells:

   i. To increase cell adherence, precoat twelve 15-cm dishes with 10 mL of PBS containing 0.001% (w/v) poly-L-lysine. Incubate for 15 min at room temperature. Aspirate the liquid.

   ii. Immediately seed the 293T cells (from two 15-cm plates of confluent cells) to the twelve 15-cm plates in DMEM with 10% FBS.

Addition of 1% antibiotic-antimycotic solution does not interfere with transfection.

iii. Grow the cells overnight.

Make sure that the cells are 70%-80% confluent and evenly distributed at the time of transfection to optimize viral titer.

9. Transfect the plasmid mix into the cells using the $CaPO_4$ precipitation method:

   i. Aliquot the four plasmids into a 50-mL tube. For each set of twelve 15-cm dishes, use:

      270 µg of lentivector

      176 µg of pMDL (Gag-Pol)

      95 µg of pVSV-G

      68 µg of pREV

   ii. Prepare 13.5 mL of a 0.25 M solution of $CaCl_2$ from a 10X stock solution of $CaCl_2$. Add to the plasmid mix.

   iii. Add 13.5 mL of 2X BBS to the mixture from Step 9.ii. Mix gently by inversion. Incubate for 15 min at room temperature.

   iv. Add 2.25 mL of the transfection mixture from Step 9.iii dropwise to each plate. Swirl the plates gently to distribute. Incubate in a 3% CO_2 atmosphere overnight at 37°C.

10. Approximately 16-20 h after transfection, remove the media. Add 15 mL of fresh DMEM with 2% FBS to each plate. Incubate in a 10% CO_2 atmosphere overnight at 37°C.

11. Collect the supernatant from the plates. Filter through 0.22- or 0.45-µm filters. Add 15 mL of fresh medium to each plate and incubate overnight.

    Filtered supernatants can be stored for several days at 4°C.

12. Collect media from the plates and filter as in Step 11.

13. Pool collected supernatants from Steps 11 and 12. Transfer to 30-mL centrifuge tubes, using 25-29 mL per tube. Concentrate the viral particles by centrifuging in an SW 28 rotor at 19,400 rpm for 2 h at 20°C.

14. Resuspend all pellets in a total of 1 mL of HBSS. Wash tubes a second time with 1 mL of HBSS.

15. Add HBSS to the resuspended pellets and tube washing fluid to a final total volume of 3 mL.

16. Prepare a 1.5-mL cushion of 20% sucrose in HBSS in 5-mL centrifuge tubes. Layer the resuspended pellets on the sucrose cushion. Centrifuge using an SW 55 rotor at 21,000 rpm for 1.5 h at 20°C.

17. Resuspend the pellet in 100 µL of HBSS. Wash the tube with an additional 100 µL of HBSS.

18. Shake the resuspended viral preparation on a low-speed vortexer for 15-30 min.

19. Centrifuge for 10 sec to remove debris. Aliquot the cleared viral solution.

    The solution can be stored at -80°C for many months. Avoid repeated freeze-thaw cycles.

20. Titrate the viral preparations by quantitating levels of the capsid protein p24 using a p24 ELISA kit (or by biological titration if an adequate marker is contained in the lentivector).

    Titers normally range between 10^9 and 10^{10} viral particles/mL but can be lower if transfection efficiency of packaging plasmids is suboptimal.

REFERENCES

Tiscornia, G., Singer, O., Ikawa, M., and Verma, I.M. 2003. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc. Natl. Acad. Sci.* **100:** 1844–1848.

Tiscornia, G., Singer, O., and Verma, I.M. 2008. Design and cloning of an shRNA into a lentiviral silencing vector: Version B. *CSH Protocols* (this issue) doi: 10.1101/pdb.prot5010.

Cold Spring Harbor Protocols

Design and Cloning of an shRNA into a Lentiviral Silencing Vector: Version A

Gustavo Tiscornia, Oded Singer and Inder M. Verma

Cold Spring Harb Protoc; doi: 10.1101/pdb.prot5009