3 Development of Lentiviral Vectors Expressing siRNA

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ABSTRACT

This chapter describes the use of lentiviral vectors to deliver small interfering RNA (siRNA)-mediated silencing cassettes. The combination of these two technologies allows for the development of a powerful tool to achieve long-term down-regulation of specific target genes both in vitro and in vivo. It combines the specificity of RNA interference with the versatility of lentiviral vectors to stably transduce a wide range of cell types.

INTRODUCTION

RNA interference (RNAi) has recently emerged as a novel pathway that allows modulation of gene expression. The pathway has been under intense study, and details of its basic biological mechanism are described elsewhere (Denli and Hannon 2003). Briefly, long double-stranded RNA molecules are processed by the endonuclease Dicer into short 21–23-nucleotide siRNAs that are then incorporated into RNA-induced silencing complex (RISC), a multicomponent nuclease complex that selects and degrades mRNAs that are homologous to the double-stranded RNA initially delivered (Fjose et al. 2001; Hannon 2002). In mammalian systems, siRNAs can be delivered exogenously or expressed endogenously from polymerase III (pol III) promoters, resulting in sustained and specific down-regulation of target mRNAs (Elbashir et al. 2001; Brummelkamp et al. 2002; Miyagishi and Taira 2002; Oliveira and Goodell 2003). To exploit this technique, efficient siRNA delivery methods must be developed. In this chapter, we describe the design and preparation of lentiviral vectors expressing siRNA for down-regulation of specific target genes.

Overview of Lentiviral Vectors

During the past decade, gene delivery vehicles based on human immunodeficiency virus type 1 (HIV-1), the best characterized of the lentiviruses, have been developed. Lentiviral vectors derived from HIV-1 are capable of transducing a wide variety of dividing and nondividing cells, integrate stably into the host genome, and result in long-term expression of the transgene. The HIV-1 genome contains nine open reading frames (ORFs) encoding at least 15 distinct proteins involved in the infectious cycle, including structural and regulatory proteins. In addition, there are a number of *cis*-acting elements required at various stages of the viral life cycle (for review, see Trono 2002). The general strategy used to produce vector particles has been to eliminate all dispensable genes from the HIV-1 genome and separate the *cis*-acting sequences from those *trans*-acting factors that are absolutely required for viral particle production, infection, and integration.

The widely used third generation of lentiviral vectors consists of four plasmids (Fig. 1A). The transfer vector contains the transgene to be delivered in a lentiviral backbone containing all of the *cis*-acting sequences required for genomic RNA production and packaging. The packaging system involves three additional plasmids (pMDL, pRev, and pEnv) that provide the required *trans*-acting factors, namely, Gag-Pol, Rev, and an envelope protein, respectively. Gag-Pol codes for integrase, reverse transcriptase, and structural proteins. The structural proteins are required for particle production, whereas integrase and reverse transcriptase molecules are packaged into the viral particle and are required upon subsequent infection. Rev interacts with the Rev-responsive element (RRE), a sequence contained in the transfer vector, enhancing the nuclear export of unspliced viral genomic RNA and thus increasing viral titer.

Viral particles can be pseudotyped with a variety of envelope proteins. One commonly used envelope protein is the vesicular stomatitis virus protein G (VSV-G), which is incorporated into the viral membrane and confers the ability to transduce a broad range of cell types, including primary cells, stem cells, and early embryos. The transfer vector also contains the woodchuck hepatitis virus regulatory element (WPRE) that enhances expression of the transgene (Zufferey et al. 1999) and a central polypurine tract (cPPT) purported to increase efficiency of nuclear import of the preintegration complex (Zennou et al. 2000). In addition, an important safety feature is provided by a deletion in the 3′ LTR (long terminal repeat) that results in replication-defective particles. During reverse transcription, the proviral 5′ LTR is copied from the 3′ LTR, thus transferring the deletion to the 5′ LTR. The deleted 5′ LTR is transcriptionally inactive, preventing viral genomic RNA production from the integrated provirus (Fig. 1B) (Miyoshi et al. 1998). When these four plasmids are transfected into 293T human embryonic kidney cells, viral

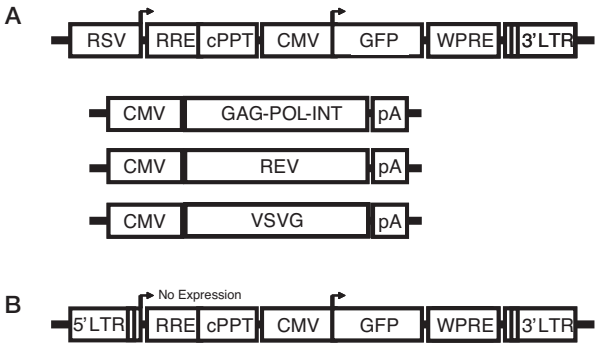

FIGURE 1. (*A*) Third-generation lentiviral vector system. The transfer vector contains all *cis*-acting elements required for replication and packaging of transfer vector RNA into viral particles. Carrying capacity is about 8 kb. The hatched box represents the self-inactivating deletion. Three helper plasmids provide all required *trans*-acting factors (see text for details). (*B*) Structure of an integrated provirus.

particles accumulate in the supernatant and high-titer viral preparations can be obtained by ultracentrifugation.

Design of Lentivectors Expressing shRNAs

A crucial breakthrough occurred with the report that siRNAs could be expressed as short hairpin RNA (shRNA) from pol III promoters cloned into plasmids (Brummelkamp et al. 2002; Miyagishi and Taira 2002). The two pol III promoters most commonly used are H1 and U6 (both human and mouse). Pol III promoters are characterized by their compact size (less than 400 bp) (Myslinski et al. 2001) and by the fact that all sequences required for promoter function are upstream of the +1 transcriptional start site. Pol III promoters have ubiquitous expression and efficiently express short RNAs (shRNAs). Thus, they are ideally suited to express shRNAs consisting of a 21–23-nucleotide sense sequence that is identical to the target sequence in the mRNA to be down-regulated, followed by a 9-bp loop and an antisense 21–23-nucleotide sequence. A stretch of five Ts provides a pol III transcriptional termination signal. The total length of the silencing cassette is about 350 bp. Thus, when this construct is expressed, a short 21–23-bp hairpin is formed; the loop is digested by Dicer and the resulting siRNA triggers degradation of the mRNA target.

Ideally, a silencing lentiviral vector would contain both a marker gene such as EGFP (enhanced green fluorescent protein) or an antibiotic resistance gene and the shRNA silencing cassette. We have designed two different versions of lentiviral silencing vectors that differ both in the position of the silencing cassette and in the cloning strategy required to construct them.

Version A (Protocol 1) involves a lentiviral vector carrying GFP as a marker and cloning the human H1-driven silencing cassette into a unique restriction site in the 3′ LTR (Tiscornia et al. 2003). As during integration, the 5′ LTR of the provirus is copied from the 3′ LTR, cloning the H1-driven shRNA into the 3′ LTR, which results in duplication of the silencing cassette. Although this strategy maximizes the silencing power of the lentiviral vector, in our experience, the main parameter determining level of silencing is the multiplicity of infection (moi). The moi required to silence a given target will depend on the levels of expression of the target mRNA, siRNA efficiency, and the transducibility of the cell type involved.

One undesirable consequence of the version-A design 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 version-B silencing lentivectors, 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. This design has been adapted to Gateway cloning technology, allowing an alternative and efficient cloning method (Protocol 2).

Design and Cloning of Lentiviral Silencing Vectors

Development and validation of an efficient lentiviral silencing vector involves the following steps: (1) selection of siRNA target sequences and design of shRNAs, (2) cloning and validation of shRNAs' effect on the target gene, and (3) cloning and testing the lentiviral silencing vector. The effectiveness of a particular siRNA is largely unpredictable and presumably reflects both mechanistic constraints of the RNAi pathway and accessibility of the target sequence within the tertiary structure of the target mRNA. A number of algorithms have been developed to predict effective siRNA sequences (Reynolds et al. 2004), and many of them are available online for free or commercial use (for example, see www.ambion.com or http://sfold.wadsworth.org). In general, the target sequence should be 21–23 bases long, but lengths of up to 28 bases have been reported (Paddison et al. 2002). Longer targets should be avoided, because longer double-stranded RNA molecules can trigger a PKR (protein kinase activated by double-stranded RNA) response

(Clemens and Elia 1997). A database search is recommended to filter out candidate targets that are present in other genes to avoid silencing of these loci. GC content should be between 40% and 55%. shRNAs to be driven by the H1 promoter can begin with any base, but the U6 promoter requires a G as its first base. shRNAs can be directed to 5′-untranslated region (5′ UTR), ORF, or 3′ UTR of the target mRNA. Strings of identical bases should be avoided. As a loop, we generally use the 9-bp sequence (TTC AAG AGA) (Brummelkamp et al. 2002).

Typically, several shRNAs must be generated and tested for every target gene. The candidate shRNAs are cloned into a simple plasmid containing only the silencing cassette and tested. Efficient silencing cassettes are then transferred to the lentiviral vector. Initial screening is best achieved by cotransfection of an shRNA-expressing plasmid and a vector-expressing tagged (myc, FLAG, etc.) cDNA of the target into 293T followed by western blot against the tag. Alternatively, provided the candidate plasmids can be efficiently transfected into a cell type of interest, the effectiveness of target down-regulation can be followed by analysis of target mRNA with quantitative reverse transcriptase–polymerase chain reaction (RT-PCR), northern blot, or analysis of target protein levels by western blot against the endogenous target.

When one or more efficient shRNA candidates have been identified, the silencing cassettes must be cloned into the lentiviral vector. High-titer viral preparations are made and should be tested by transduction of a cell line expressing the target protein, followed by measurement of target expression. The final validation of the lentiviral silencing vector against the endogenous target is crucial and should include use of a lentiviral vector lacking a silencing cassette or carrying a silencing cassette against a different target as a specificity control, because overexpression of any siRNA can cause some nonspecific down-regulation of gene expression. Several mois should be tested. Precise determination of efficiency of down-regulation of the target will require testing homogeneously transduced cell populations, which can be obtained by fluorescence-activated cell sorting (FACS) for GFP-positive cells or applying selection if an antibiotic resistance gene is used as a marker.

Protocol 1

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

This protocol describes version A for the design and cloning of an shRNA for a given target. We use a plasmid containing the poll III promoter as a template and a single round of PCR to amplify the silencing cassette. We employ a 5′ forward primer upstream of the pol III promoter and a 3′ reverse primer that includes the entire shRNA sequences (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 (such as XbaI). PCR amplification will result in a DNA fragment containing 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 (Fig. 2).

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Advantage GC-2 polymerase mix (BD)

2X BES-buffered saline (BBS) solution (50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4 <!>)

Mix 16.36 g of NaCl, 10.65 g of BES (Calbiochem 391334), and 0.21 g of Na_2HPO_4. Add double-distilled H_2O up to 900 ml. Dissolve, titrate to pH 6.95 with 1 M NaOH, and bring volume to 1 liter. Filter-sterilize and store 14-ml aliquots at 4°C.

$CaCl_2$ (2.5 M) stock solution <!>

Mix 36.75 g of $CaCl_2$ in 70 ml of double-distilled H_2O. Adjust to a final volume of 100 ml. Aliquot into 1.5-ml microcentrifuge tubes and store at –20°C.

Cells: 293T human embryonic kidney (Invitrogen)

Dimethylsulfoxide (DMSO; 7%) <!>

Dulbecco's modified Eagle's medium (DMEM)

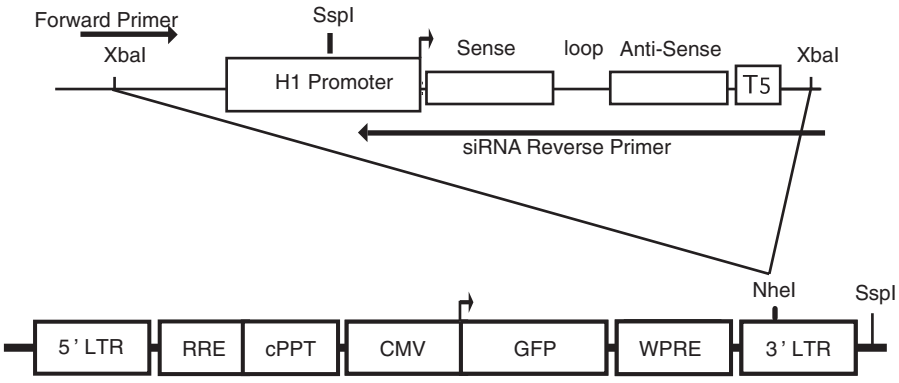

FIGURE 2. Cloning scheme for version A (see text for details).

ELISA (enzyme-linked immunosorbent assay) Kit, p24 (New England Nuclear Life Science products NEK050B) (*Optional:* See Step 15)
Fetal bovine serum (FBS)
Hank's balanced salt solution (HBSS; Invitrogen)
H1 promoter cloned into pGEM-T (Promega)
Plasmids
pMDL (Gag-Pol)
pREV
pVSV-G
For plasmid preparation, use QIAGEN plasmid maxipreps at 1 μg/μl.
Poly-L-lysine (Sigma-Aldrich P 4832)
Use 0.001% in phosphate-buffered saline (PBS). Filter-sterilize and store at –20°C.
Restriction endonucleases: NheI, SspI, XbaI
Sucrose (20% in HBSS)

Equipment

Beckman tubes (358126 and 326819)
Filters (0.22 or 0.45 μm)
Incubators, preset to 37°C (3% and 10% CO_2)
Microcentrifuge tubes
PCR machine
SW 28 and SW 55 rotors (Beckman)
Tissue-culture dishes (15 cm and six well)

METHODS

Design and Cloning of shRNAs

1. Select a target within the gene to be silenced, e.g., for GFP: GCAAGCTGACCCTGAAGTTC (Tiscornia et al. 2003).
2. Design primers to amplify the silencing cassette. As template, we use an H1 promoter cloned into pGEM-T. The 5′ forward primer must contain an XbaI site. The 3′ reverse primer contains 22 nucleotides from the 3′ end of the pol III promoter and a 5′ tail including the entire shRNA, loop, transcriptional stop signal (T5), and XbaI site sequences. For the target suggested above, design the following 3′ reverse primer: 5′CTGTCTAGACAAAAAGCAAGCTG ACCCTGAAGTTC**TCTCTTGAA**GAACTTCAGGGTCAGCTTGC***GGGGATCTGTGGTCTC ATACA***3′, where the H1 sequence is in italic bold, XbaI (NheI compatible) is underlined, the loop is bold underlined, and nucleotide +1 is in small caps.
3. With the 5′ forward primer and the 3′ reverse primer (final primer concentration is 10 μm) described above, use 10 ng of plasmid containing the H1 promoter as template to amplify the silencing cassette by PCR. We use Advantage GC-2 polymerase mix and use the GC-melt additive as 10×. It is essential to add 7% DMSO or a similar agent to a regular *Taq* polymerase reaction to prevent hairpin formation. 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

4. The result of the PCR is an amplified fragment of approximately 400 bp that can be cloned in an A/T vector for sequencing or directly cloned in the lentivector plasmid. Digest the insert with the XbaI and gel-purify. Digest the lentivector plasmid with NheI, gel-purify, and then dephosphorylate.

   Typically, 50 ng of vector is 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 following H1-F primer 5′-TGGCAGGAAGATGGCTGTGA-3′, because mutations in the hairpin can significantly reduce the efficiency because down-regulation.

5. Validate the cloned shRNA cassettes by transfecting or transducing (as lentiviral particles) to a cell line that expresses the target gene. 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 very useful when target mRNA is restricted to certain cell types or a specific antibody against the target is unavailable. Typically, we 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 hours after transfection.

Preparation of Lentiviral Vectors

6. For a 12 × 15-cm–dish lentiviral preparation: Twenty-four hours before transfection, prepare plates and cells.

   a. To increase cell adherence, precoat 12 15-cm dishes with 10 ml of poly-L-lysine, incubate 15 minutes at room temperature, and aspirate off the liquid.

   b. Immediately seed 293T cells from two confluent 15-cm plates to the 12 15-cm plates in DMEM + 10% FBS. Addition of 1% antibiotic-antimycotic solution does not interfere with transfection. Cells should be of low-passage number and should not be used after passage 20 or if growth is slow. Certain brands of FBS do not support efficient transfection and can result in low viral titers.

   c. Grow the cells overnight.

7. Make sure that the cells are 70–80% confluent and evenly distributed at the time of transfection to optimize viral titer. Transfect the plasmid mix into the cells using the $CaPO_4$ precipitation method as follows:

   a. Aliquot the four plasmids into a 50-ml tube. For a 12 × 15-cm dish, use:

      270 μg of lentivector

      176 μg of pMDL (Gag-Pol)

      95 μg of pVSV-G

      68 μg of pREV

   b. Prepare a working solution of $CaCl_2$ (13.5 ml of 0.25 M $CaCl_2$) and add to the plasmid mix. Add 13.5 ml of 2× BBS solution. Mix gently by inversion and incubate for 15 minutes at room temperature.

   c. Add the transfection mixture (spreading in drops) to each plate (2.25 ml/plate). Swirl the plates gently and incubate overnight at 37°C in a 3% CO_2 atmosphere.

8. Approximately 16–20 hours after transfection, remove media. Add 15 ml of fresh DMEM + 2% FBS to each plate and incubate overnight at 37°C in a 10% CO_2 atmosphere.

9. Collect the supernatant from the plates and 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.

10. Collect media and filter as in Step 9.

11. Pool collected supernatants from Steps 9 and 10. Transfer to Beckman tubes (358126), using 25–29 ml per tube. Concentrate viral particles by centrifuging in an SW 28 rotor at 19,400 rpm for 2 hours at 20°C.

12. Resuspend all pellets in a total of 1 ml of HBSS. Wash tubes a second time with 1 ml of HBSS.

13. Increase the combined volume from 2 to 3 ml with HBSS and layer the resuspended pellets on 1.5 ml of a 20% sucrose (in HBSS) cushion in Beckman tubes (326819). Centrifuge using an SW 55 rotor at 21,000 rpm for 1.5 hours at 20°C.

14. Resuspend the pellet in 100 μl of HBSS and wash the tube with an additional 100 μl of HBSS. Shake the resuspended viral preparation on a low-speed vortexer for 15–30 minutes. Centrifuge for 10 seconds to remove debris. Aliquot the cleared viral solution and store at –80°C. It can be stored for many months. Avoid repeated freeze-thaw cycles.

15. 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.

Protocol 2

Cloning an shRNA into a Lentiviral Silencing Vector: Version B

This protocol describes version B of the lentivector silencing system. It has been adapted to Gateway cloning technology (Invitrogen), allowing for a fast and convenient cloning procedure (see Fig. 3). Initially, an shRNA is cloned into an entry vector (pENTR/U6, Invitrogen) immediately downstream from an hU6 promoter. The silencing cassette is flanked by recombination sites from bacterophage λ (*attL1* and *attL2*). Once an effective shRNA is obtained, it can be transferred to the destination vector. The destination vector is a lentiviral vector carrying a marker (GFP or a selection marker) with a destination cassette cloned upstream of the marker (*attR1* and *attR2* flanking a *ccdB* toxic gene). Thus, the silencing cassette can be transferred from the entry vector to the destination vector in a simple LR cloning reaction.

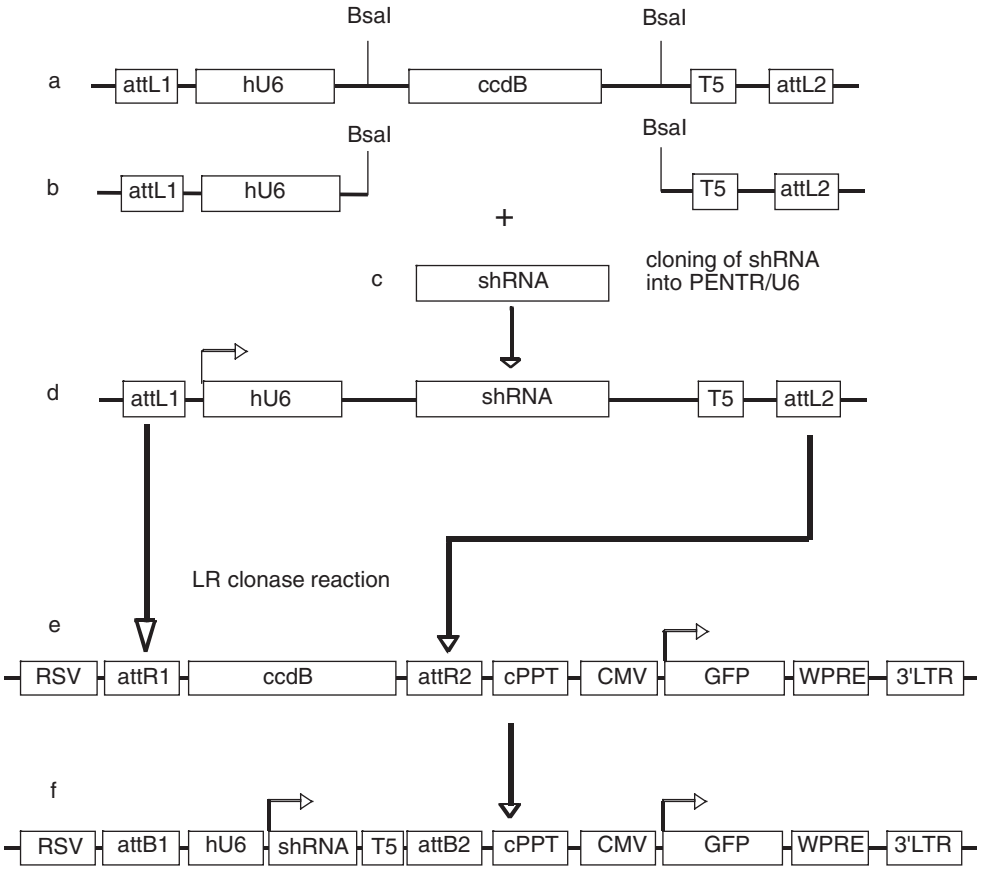

FIGURE 3. Cloning scheme for version B: (*a*) Structure of PENTR/U6. (*b*) BsaI digestion creates termini into which an shRNA duplex (*c*) can be cloned to obtain the silencing cassette (*d*). In turn, bacteriophage λ *att* recombination sites allow transfer of the silencing cassette to a lentiviral destination vector (e) to obtain the lentiviral transfer vector containing both a silencing cassette and a GFP marker (*f*).

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Agarose gel (4%)
10x Annealing buffer
  100 mM Tris-HCl (pH 8) <!>
  10 mm EDTA
  1 M NaCl
Escherichia coli DB3.1 (Invitrogen)
Kanamycin <!> and ampicillin <!> plates
5x LR buffer (Invitrogen)
Oligonucleotides; order as appropriate (200-μm concentration)
pENTR/U6 (Invitrogen)
Proteinase K <!>
Restriction endonucleases: AvaI, BsaI, ClaI, NdeI, XbaI
STBL3 (Invitrogen or equivalent recombination-deficient bacterial strain)

Equipment

PCR machine

METHOD

1. Select a target within the gene to be silenced; e.g., for GFP: GCAAGCTGACCCTGAAGTTC (Tiscornia et al. 2003).
2. Design an shRNA and clone into pENTR/U6. For a diagram of pENTR/U6, see Figure 3a. A detailed map can be downloaded from the Invitrogen Web site. pENTR/U6 has a *ccdB* toxic gene and must therefore be propagated in tolerant strains such as *E. coli* DB3.1. When this plasmid is digested with BsaI, the vector is left with the following termini:

   XXXXX 3′ 5′ TTTTTXXXXXX

   XXXXXGTGG 5′ 3′ AXXXXXXX

   To clone the shRNA, design two complementary oligonucleotides with termini compatible to those in the BsaI-digested pENTR/U6, followed by annealing and ligation as described below (Fig. 3b,c,d). For the GFP target sequence given above, the required oligonucleotides, after annealing, would look as follows:

   5′ CACC *GCAAGCTGACCCTGAAGTTC* **TTCAAGAGA** . . .

   3′ *CGTTCGACTGGGACTTCAAG* **AAGTTCTCT** . . .

   . . . GAACTTCAGGGTCAGCTTGC 3′

   . . . CTTGAAGTCCCAGTCGAACG AAAA 5′

   where the sense strand of the hairpin is in italics, the loop is in bold, and the antisense strand of the hairpin is underlined.

3. Anneal and clone according to the following procedure:

   a. Make annealing mixture:

      5 µl of P1 (50 µm final concentration)
      5 µl of P2 (50 µm final concentration)
      2 µl of 10× annealing buffer
      8 µl of H_2O

   b. Heat to 94°C for 5 minutes. Use a PCR machine to cool to 25°C at 0.1°C per second.

      Even at 50-µm final concentration, the efficiency of the reaction is only about 50%. Efficiency of annealing can be ascertained by running an aliquot of the annealed oligonucleotides (5 µl of a 1/100 dilution of the annealing mix) on a 4% agarose gel. Single-stranded oligonucleotides will run as a hairpin of approximately 30 bases, whereas the annealed product should run at its predicted size (~55 bp).

   c. Dilute annealing mix 1/1000 in H_2O and clone into the pENTR/U6 vector. Typically, 50 ng of vector are ligated to 2–3 µl of a 1/1000 dilution of the annealed product. Transform competent bacteria and select on kanamycin plates. Resulting plasmids will have the structure shown in Figure 3d.

   d. Digest plasmid DNA from colonies with NdeI-XbaI and run on a 4% agarose gel. Positive clones will contain an approximately 127-bp insert compared to 76 bp for colonies without an insert. Sequence the insert with hU6-F (GGACTATCATATGCTTACCG) and M13-R primers to check hairpin integrity.

4. Silencing cassettes can be tested as in Step 5 of Protocol 1. Once a suitable candidate is found, transfer the silencing cassette from the entry vector to the destination vector by performing an LR recombination reaction. For a typical destination vector, see Figure 3e.

   a. For LR recombination reaction, mix:

      100–300 ng of entry vector
      150 ng of destination vector
      4 µl of 5× LR buffer
      H_2O to 20 µl final volume

   b. Incubate overnight at room temperature.

   c. Add 2 µl of proteinase K. Incubate for 10 minutes at 37°C.

   d. Transform STBL3 competent bacteria (or equivalent recombination-deficient strain) with 2 µl of LR reaction mix. Plate on ampicillin plates.

      The resulting constructs will have the structure depicted in Figure 3f. The use of recombination-deficient bacteria is highly recommended, because use of other strains can result in unwanted recombination events within the plasmid.

   e. Digest plasmid DNA with ClaI-AvaI. Clones containing the silencing cassette should have an approximately 700-bp insert, whereas the unrecombined parental destination vector will show a 1.9-kb insert. Sequence positive clones with hU6-F (5′-GGA CTA TCA TAT GCT TAC CG-3′).

5. For preparation of the vector, follow Steps 6–15 in Protocol 1.

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