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RNA Interference by Short Hairpin RNAs Expressed in Vertebrate Cells

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Summary

RNA interference (RNAi) is now established as a general method to silence gene expression in a variety of organisms. Double-stranded RNA (dsRNA), when introduced to cells, interferes with the expression of homologous genes, disrupting their normal function. In mammals, transient delivery of synthetic short interfering RNAs (siRNAs), which resemble the processed form of standard double stranded RNAi triggers, is effective in silencing mammalian genes. Issues related to transfer efficiency and duration of the silencing effect, however, restrict the spectrum of the applications of siRNAs in mammals. These shortcomings of siRNAs have been solved by the cellular expression of short hairpin RNAs (shRNAs) from DNA vectors. shRNAs are indistinguishable from siRNAs in terms of efficacy and mechanism but can be produced within cells from standard mammalian expression vectors. In this way, shRNA expression makes possible the creation of continuous cell lines and transgenic animals in which suppression of a target gene is stably maintained by RNAi. As a result, the types of RNAi-based gene function analysis that can be carried out in mammals have been greatly expanded. We describe methods for the construction and transfer of stable shRNA expressing vectors suitable for generating loss of function alleles in mammalian cells in vitro or in vivo.

Key Words

RNAi; gene silencing; retrovirus; knock-outs; mammalian genetics.

1. Introduction

The recent completion of the human and mouse genomes has brought mammalian gene function analysis to the forefront of biology *(1–3)*. Until recently, the techniques available for mammalian gene function analysis were far less effective than those commonly used in model genetic organisms. This was especially true in the generation of loss-of-function mutations. Rapid methods for directed gene ablation in a number of model organisms have existed for 20 yr.

In contrast, directed gene disruption by homologous recombination or antisense or ribozyme techniques in mammalian cells, although useful, are often costly, time-consuming, ineffective, or some combination thereof.

The recent development of RNA interference (RNAi) has significantly closed the technology gap between model organisms and mammals *(4,5)*. RNAi was first described in *Caenorhabditis elegans* as a method in which exogenously supplied double-stranded RNA (dsRNA) resulted in the suppression of expression of homologous genes *(6)*. Although nonspecific effects of long dsRNA were an initial hurdle in the use of RNAi in mammals *(7,8)*, research into its mechanism yielded clues important for its eventual generalized implementation (for review, *see* **ref. 9**). In our current understanding of RNAi, dsRNA is processed by an RNAse III-like enzyme named Dicer into small approx 21 nt RNAs known as short-interfering RNAs (siRNAs) siRNAs are incorporated into RNA-induced silencing complexes and act as specificity determinants in the degradation of homologous mRNAs. The discovery that the application of Dicer product-like siRNAs were sufficient to trigger RNA interference in mammalian somatic cells revolutionized the genetics of tissue culture cells *(10)*.

Despite the triumph of siRNAs in mammalian cells, significant differences still existed between RNAi based on exogenously-supplied dsRNA in *C. elegans* and mammalian cells. Mammalian cells do not take up exogenously applied dsRNA efficiently and the persistence of the RNAi response, which is amplified in *C. elegans*, is limited to approximately six to eight cell doublings in mammals *(11)*. For this reason, several types of mammalian experiments require that dsRNA silencing triggers must be repeatedly supplied to mammalian cells with cationic lipids or via electroporation. Although this only complicates in vitro experiments, it precludes the use of siRNAs in most animal experiments.

These limitations sparked the development of stable RNAi-based silencing in mammals. Similar approaches in which long dsRNA hairpins, typically 500–1000 nt in length, are expressed from within cells have modernized RNAi analysis of gene function in lower organisms because they provide uniform delivery and duration of the silencing *(12–15)*. Largely because of the nonspecific responses to long dsRNA, these approaches are of limited use in mammals. However, basing their designs loosely on the small, hairpin-structured, noncoding microRNAs that are substrates of the RNAi pathway, several groups solved the problems related to persistence and transfer efficiency in mammalian cells by expressing short hairpin RNAs (shRNAs; **refs. *16–21***). Although somewhat variable in structure, shRNAs are expressed from mammalian promoters on DNA vectors that are introduced to cells by transfection or infection and possess double stranded stems less than 30 nt in length that serve as sub-

strates for Dicer. This permits the creation of stably silenced cell lines, which complements the use of siRNAs in tissue culture. For silencing genes in mammalian cells in vivo, shRNAs are the only viable technique *(22–25)*.

The utility of shRNAs as a genetic tool lies in the generation of cells in which the shRNA expression cassette is stable. For this reason, gene-transfer methodologies, which are inherently stable, are better platforms for the expression of shRNAs. Perhaps the best-characterized stable expression technologies for mammalian cells are systems based on retroviral integration (for a review of these methods, *see* **ref. *26***). We typically house shRNA expression cassettes on an MSCV-based retrovirus that contains a mammalian selectable drug-resistance marker. RNA polymerase III, which normally initiates and terminates small, highly structured RNA transcripts precisely *(27)*, is responsible for transcription of shRNA sequences contained immediately downstream of the human U6-snRNA promoter *(18–20)*. This promoter is active in most if not all embryonal and somatic cell types and has, to date, offered similar levels of constitutive expression in a variety of settings.

Here we present detailed protocols for the design, construction and delivery of shRNA expression vectors for use in gene silencing experiments in mammals. The protocol makes use of the pMSCV-SHAG BbsBlue vector to produce clones identical to those used in various silencing experiments (**ref. *24*** and our unpublished results). In this vector, DNA oligonucleotides encoding shRNAs are cloned into a *Bbs*I site for optimal expression by the human U6 promoter. Displacement of a *lacZα* fragment aids in the identification of shRNA-carrying clones. Once constructed, these vectors can be used to direct the production of retroviruses that silence a specific gene in a variety of host cell types. These methods can be used for gene function analysis of mammalian gene function in cultured cells or in whole animals.

2. Materials

2.1. Hairpin Oligos

1. Annealing buffer: 10 mM Tris-HCl, pH 7.5; 50 mM NaCl; 1 mM ethylenediamine tetraacetic acid (EDTA).
2. Oligo A (described in detail in **Subheading 3.1.**).
3. Oligo B (described in detail in **Subheading 3.1.**).

2.2. Polynucleotide Kinase Reaction

1. 10X Kinase buffer: 500 mM Tris-HCl, 100 mM MgCl$_2$, 1 mM EDTA, 50 mM dithiothreitol, 1 mM spermidine, pH 8.2.
2. T4-polynucleotide kinase (Roche Molecular Biochemicals, Indianapolis, IN).
3. 10 mM ATP.
4. Distilled H$_2$O.

2.3. Vector Preparation

1. pMSCV-SHAG BbsBlue vector (*see* **Fig. 1**).
2. *Bbs*I (New England Biolabs, Beverly, MA, cat. no. R0539S).
3. 10X *Bbs*I buffer: 500 m*M* NaCl, 100 m*M* Tris-HCl, 100 m*M* MgCl$_2$, 10 m*M* dithiothreitol, pH 7.9.
4. Distilled H$_2$O.
5. Phenol:chloroform:isoamyl alcohol (25:24:1).
6. Chloroform:isoamyl alcohol (24:1).

2.4. Ligation Reaction

1. 10X ligase buffer: 500 m*M* Tris-HCl, 100 m*M* MgCl$_2$, 100 m*M* dithiothreitol, 10 m*M* ATP, 250 mg/mL bovine serum albumin, pH 7.8.
2. Distilled H$_2$O.
3. DNA ligase (New England Biolabs, Beverly, MA, cat. no. M0202S).
4. Glycogen (5 mg/mL).
5. 3 *M* Sodium acetate, pH 4.8.
6. 100% Ethanol.
7. 70% Ethanol.

2.5. Transformation

1. Competent *Escherichia coli* (*see* **Note 1**).
2. LB plates containing ampicillin and X-gal (Sigma cat. no. B4452; *see* **Note 2**).

2.6. Sequencing Analysis

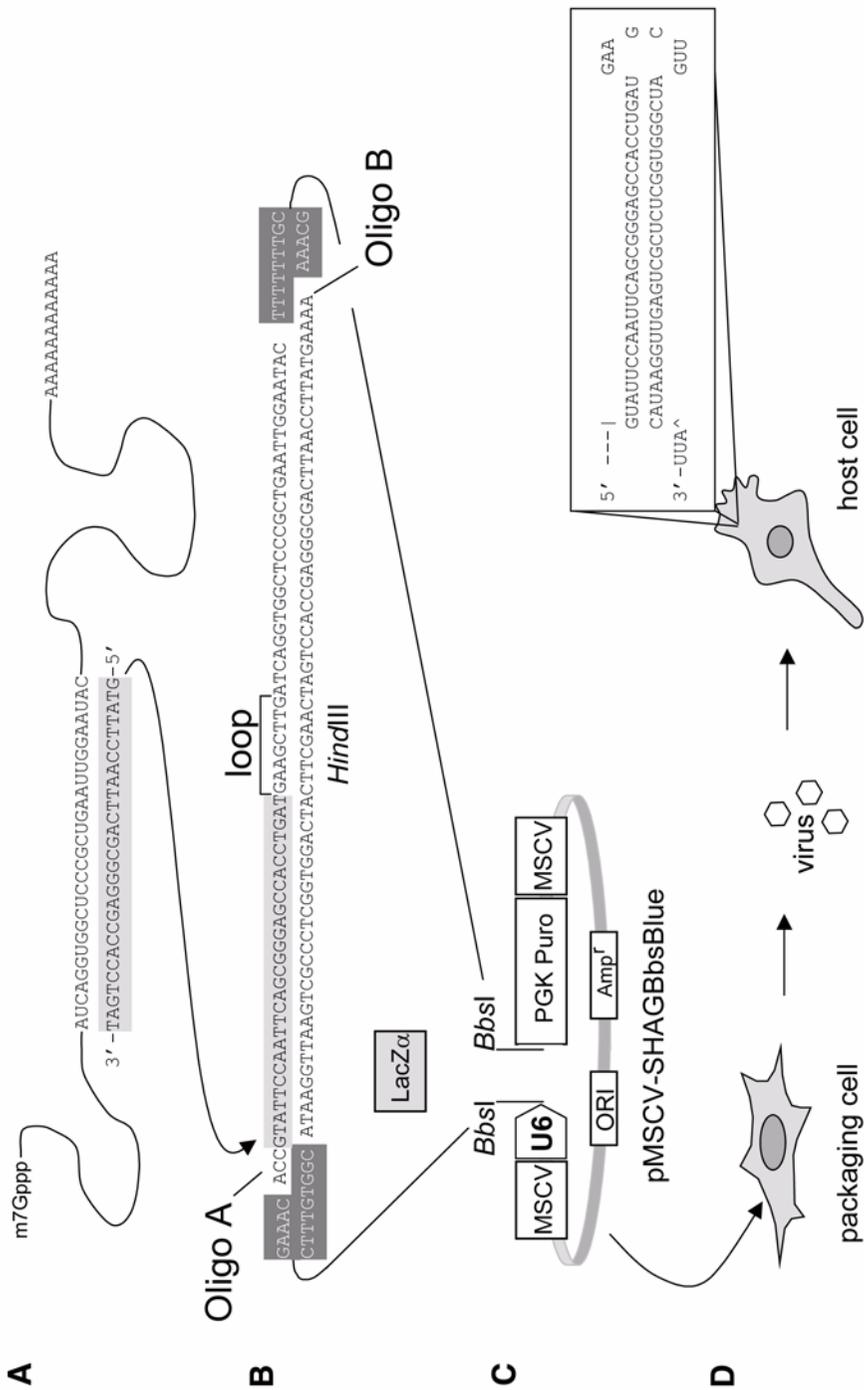1. U6 -130 primer (5'-TACAAAATACGTGACGTAGA-3').

2.7. Cell Culture

1. Retroviral packaging cell line. Various suitable retroviral packaging lines are available from the American Type Culture Collection. Methods for the use of these cells have already been published in this series *(26)*.
2. Host cells in which gene is to be silenced.
3. 10X CaCl$_2$ 2.5 *M*.

Fig. 1. (*see opposite page*) Strategy for the construction of stable silencing constructs in mammalian cells. **(A)**, A target sequence within an mRNA of interest is selected and used to design oligos that when expressed will form a short hairpin structure. **(B)**, The annealed oligos, with 5' ends shown, are ligated directly into *Bbs*I-digested pMSCV-SHAG BbsBlue **(C)**, *Bbs*I digestion of this vector releases a *lacZ*α fragment leaving ends compatible with the insertion of the designed oligo at the first transcribed base. **(D)**, The resulting clones are packaged into retroviral particles which in turn are used to infect a cell type of interest. Expression from the U6 promoter results in expression of an shRNA, which silences the cognate target via the RNAi pathway.

A

m7Gppp

AAAAAAAAAAAA

AUCAGGUGGCUCCCGCUGAUUGGAAUAC
3'-TAGTCCACCGAGGGCGACTTAACCTTATG-5'

B

Oligo A

loop

HindIII

Oligo B

GAAAC ACCGTATTCCAATTCAGGCGGAGCCACCTGATGAAGCTTGATCAGGTGGCTCCCGCTGAATTGAATAC
CTTTGTGGC ATAAGGTTAAGTCGCCCTCGGTGGACTACTTCGAACTAGTCCACCGAGGGCGACTTAACCTTATGAAA TTTTTTTGC
AAACG

C

LacZα

BbsI PGK Puro Amp^r

BbsI MSCV U6 ORI MSCV

pMSCV-SHAGBbsBlue

D

packaging cell

virus

host cell

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                                          GAA
5' ---|  GUAUUCCAAUUCAGCGGGAGCCACCUGAU      G
3'-UUA^ CAUAAGGUUAGUCGCUCGGUGGGCUA        C
                                          GUU
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4. 2X BES-buffered solution (2X BBS): 50 m*M* *N,N-bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4, Adjust to optimal pH with 1 N NaOH, room temperature (*see* **Note 3**). Sterilize by filtration through a 0.45-μm filter and store at –70°C.
5. 8 mg/mL Polybrene: hexadimethrine bromide (Sigma, H9268).
6. 1 mg/mL Puromycin dihydrochloride: cell culture-tested (Calbiochem, cat. no. 540411).

3. Methods

3.1. Oligonucleotide Design for Insertion
Into BbsI-Cut pMSCV-SHAG Vector (see Note 4)

1. Obtain the coding sequence of any gene.
2. Select any 29 base sequence within the gene that does not contain stretches of three or more consecutive adenines or three or more consecutive thymidines and ends with cytosine (*see* **Note 5**):
   5'-N1 N2 N3 .... N26 N27 N28 C-3'
   (where N1, N2, etc., is any nucleotide).
3. Determine the reverse complement of the sequence in **step 2**:
   5'- G N28' N27' N26' .....N3' N2' N1'-3'
   (where N1', N2', are the complementary bases to nucleotides N1, N2, and so on).
4. Add 5'-GAAGCTTG-3' to the 3' end of **step 3** to get:
   5'- G N28' N27' N26' .....N3' N2' N1'GAAGCTTG -3'.
5. Add the sequence in **step 2** to the 5' end of the sequence in **step 4** to get:
   5'- G N28' N27' N26' ..N3' N2' N1'GAAGCTTG N1 N2 N3 .... N26 N27 N28 C -3'.
6. Add ACC to the 5' end of the sequence in **step 6** to generate the sequence of oligonucleotide A: 5'-ACC G N28' N27' N26' ..N3' N2' N1'GAAGCTTG N1 N2 N3 .N27 N28 C -3'.
7. For oligonucleotide B, first prepare the reverse complement of the sequence in **step 6** to generate: 5'-G N28' N27' N26' ..N3' N2' N1'CAAGCTTC N1 N2 N3 .... N26 N27 N28 -3.'
8. Add AAAA to the 5' end of the sequence in **step 6** to generate oligonucleotide B: 5'-AAAA G N28' N27' N26' ..N3' N2' N1'CAAGCTTC N1 N2 N3 ... N26 N27 N28 -3'.
9. Oligonucleotides used here are obtained by standard oligonucleotide synthesis (*see* **Note 6**).

3.2. Oligonucleotide Hybridization

1. Mix 2 μL of each complementary oligonucleotide with 16 μL of annealing buffer in a 1.5-mL microfuge tube.
2. Place tube in a standard heating block at 95°C.
3. Remove the heating block from the apparatus and allow to cool to room temperature (or at least below 30°C) on the workbench. Briefly centrifuge to recover liquid. Store on ice or at 4°C until ready to use (*see* **Note 7**).

3.3. Phosphorylation of Oligonucleotides

1. To a 1.5-mL microfuge tube add: 5 μL H_2O, 2 μL of annealed oligonucleotides, 1 μL of 10X T4 polynucleotide kinase buffer, 1 μL of 1 m*M* ATP (stock is 10 m*M*), 1 μL of T4 polynucleotide kinase.
2. Incubate for 30 min at 37°C.
3 Heat-inactivate by incubation for 10 min at 70°C.

3.4. Vector Preparation

1. Digest 1 μg of pMSCV-SHAG Bbs vector in a 1.5-mL microfuge tube in a reaction with 2 μL of 10X buffer, 5 units of *Bbs*I, and distilled H_2O to 20 mL total for 60 min (*see* **Note 8**).
2. Heat inactivate the enzyme by incubation for 10 min at 70°C .
3. Remove protein from the reaction by addition of an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1). Briefly vortex and centrifuge at 14,000*g* for 2 min.
4. Recover the aqueous phase to a new tube and extract with one volume of chloroform:isoamyl alcohol (24:1). Briefly vortex and centrifuge at 14,000*g* for 2 min.
5. Recover the aqueous phase to a new tube and adjust to 0.3 *M* sodium acetate by addition of 1/10 vol of 3 *M* sodium acetate, pH 4.8. Precipitate the vector by adding 1 μL of glycogen, 2.5 vol of ice-cold 100% ethanol, and incubating at 0°C for 10 min. Centrifuge at 14,000*g* for 10 min to recover the vector DNA.
6. Wash the vector pellet with 100 μL of 70% ethanol, dry briefly and resuspend in 10 μL of H_2O.

3.5. Insertion of Oligonucleotides Into the Expression Vector

1. In a 1.5-mL microfuge tube, combine 14 μL of H_2O, 2 μL of 10X ligase buffer, 1 μL of prepared vector, and 2 μL of the phosphorylated oligonucleotides.
2. Add 1 μL of DNA ligase.
3. Incubate at 14°C overnight.
4. Use 2 μL to transform standard competent cells (>10^7 colonies/μg).
5. Plate on LB plates containing ampicillin and X-gal.
6. Incubate at 37°C overnight.
7. Pick white colonies to prepare template for DNA sequence analysis (*see* **Note 9**).

3.6. Sequencing of Hairpin Clones

1. Prepare template DNA.
2. Analyze the DNA sequence using the U6-130 primer.
3. Confirm that the sequence is correct by comparing it to oligonucleotide A designed in **Subheading 3.1.**, **step 7**.
4. Make large-scale preparations of clones that yield correct sequence. Various commercially available kits are ideal for production of transfection-ready DNA.

3.7. Transient Production of Silencing Retroviruses in Packaging Cell Lines After Transfection (see Note 10)

1. Production, day 1: Plate exponentially growing packaging cells at a density of 1×10^6 cells per 10-cm culture dish.
2. Production, day 2: Inspect each plate from day 1 to ensure that each has cells at 50–80% confluency.
3. Production, day 2: 1–2 h before transfection, remove the media from the previous day and replace with fresh medium.
4. Production, day 2: Prepare the transfection mixture: Also you may want to incubate your solutions at room temperature or 37°C before mixing to standardize the precipitate characteristics. For each 10-cm plate to be transfected, add 15 μg of plasmid DNA and distilled H_2O to a total volume of 900 μL in a polypropylene tube (Falcon 2063).
5. Production, day 2: Add 100 μL of $CaCl_2$ and mix.
6. Production, day 2: Add 1 mL of 2X BBS while bubbling the solution vigorously for approx 10 s by forcing air with an automatic pipettor through a pipet positioned at the bottom of the tube.
7. Production, day 2: Immediately (within approx 1–2 min) add dropwise (one drop every other second) the transfection mixture to the cell monolayer while rocking the plate. Briefly rock the plates back and forth and side to side to evenly distribute DNA/$CaPO_4$ particles and place plates in 37°C incubator.
8. Production, day 3: Replace the medium with 10–15 mL of fresh, prewarmed medium.
9. Production, day 3: For virus production move the plates to a 32°C incubator 2 d before infection (*see* **Note 11**).
10. Production, day 5: Carefully remove the retrovirus-containing media and filter this through a 0.45-μm syringe filter. If infection is planned for that day the viral filtrate should be stored at +4°C. Longer term storage should be at –80°C.

3.8. Introduction of Silencing Retroviruses Into Host Cell Lines

1. Infection, day 1: Plate exponentially growing host cells at a density that will allow them to proliferate for at least 3 d. Be sure to have at least one plate as an uninfected control.
2. Infection, day 2: For each 10 cm plate to be infected prepare an infection mixture containing 5 mL of host cell media, 5 mL of viral supernatant from **Subheading 3.7.**, **step 10**, and 10 μL 8 mg/mL polybrene (*see* **Note 12**).
3. Infection, day 2: Remove the media from host cell and replace with infection mix.
4. Infection, day 2: Incubate for 8–24 h at 32°C.
5. Infection, day 3: Carefully replace the medium with the normal host cell medium and return the cells to the 37°C incubator.
6. Infection, day 5: Add an appropriate dilution of puromycin to the plates (*see* **Note 13**).

7. Infection, day 7: Inspect uninfected control plates for extensive cell death. Although infection rates vary depending on cell type, infected plates should have surviving cells on the second day of selection.

8. Expand the surviving cell cultures and test for silencing of the gene of interest and resulting phenotype.

4. Notes

1. Commercially available competent cells that yield >10^7 colonies/μg are ideal for this purpose. Any of a variety of strains that carry the *lacZ*(M15) mutation should be appropriate for blue–white screening. Such strains include JM109, XL-1 Blue, and DH5α. Despite the presence of tandemly repeated sequences, we find that hairpin constructs are stable in most commonly used bacterial strains.

2. To premade LB plate containing appropriate antibiotics, spread 40 μL of a stock solution of X-gal (20 mg/mL in dimethylformamide) and 4 μL of a stock solution of IPTG (200 mg/mL) on the surface of the plate. These volumes can be scaled up and added to media immediately before pouring.

3. The pH (pH 6.95–7.10) of the 2X BBS solution is critical. When preparing 2X BBS buffer, pH should be checked against a *bona fide* reference stock when available. Alternatively, several test batches can be produced at once that differ from each other by 0.05 pH units, for example, pH 6.90, 6.95, 7.00, 7.05, and 7.10. Each test batch can be examined for transfection efficiency using vectors that express easily scored markers, such as β-galactosidase or green fluorescent protein. Store the optimal solutions at –70°C in 50-mL aliquots.

4. The design of shRNA constructs is relatively flexible. Double stranded stems between 18 and 29 nt in length are approximately equivalent in efficacy *(28)*. Either strand of the stem structure should be complementary to the sense strand of the targeted mRNA. It does not seem to matter whether it is the 5' stem strand or the 3' stem strand *(16–21)*. The loop sequences are unimportant. Various sequences between 3 and 9 nt in length work well, but longer loops appear to be deleterious *(28)*. At present, RNAi target sequences within mRNAs are poorly defined. The somewhat imprecise published guidelines with which to select hairpin target sites suggest a target sequence near the 5' end of the gene with a GC content of approx 50%. Many target sites that do not share these criteria are highly effective including several cases in which the 3' end of the gene was the best choice *(29,30)*. The protocol shown here in **Subheading 3.1.** has been incorporated into an online design tool for short hairpin construct design: http://katahdin. cshl.org:9331/RNAi/ . Several other similar sites exist: http://jura.wi.mit.edu/bioc /siRNA/, http://www.dharmacon.com/, http://www.ambion.com/techlib/misc/ siRNA_finder.html.

5. The oligonucleotide must end in a C so that RNA polymerase III, which initiates at a G in the U6 promoter, will initiate precisely at the first base of the antisense strand. Runs of A's or T's will cause premature termination by RNA polymerase III.

6. Oligonucleotides can be obtained from any commercial supplier. A 0.05-μmol synthesis scale is sufficient. Resuspend the oligonucleotides at 100 μ*M* before annealing.

7. A thermal cycler can also be used to anneal oligonucleotides. Add 10-μL aliquots of mixed oligonucleotides into polymerase chain reaction tubes (500-μL size). Place the tubes in a thermal cycler and set up a program to perform the following profile: step 1: heat to 95°C and remain at 95°C for 2 min, step 2: ramp cool to 25°C over a period of 10 min, step 3: proceed to a storage temperature of 4°C.

8. The completeness of digestion can be monitored by agarose gel electrophoresis. Although not generally necessary, digested vector can also be purified following agarose gel electrophoresis.

9. White colonies result from the displacement of the *lacZα* gene, which drastically reduces the vector background in the cloning reaction. The inclusion of the *Hin*dIII site in the loop of the hairpin allows for rapid identification of clones containing a hairpin. Digestion of successful hairpin constructs with *Hin*dIII produces a 570-bp band. A few colonies for each hairpin construct should be sequenced.

10. Retroviruses produced by these methods are potentially hazardous! Caution should be exercised in their production and use. Although a powerful technique for gene transfer, appropriate NIH and other regional guidelines should be followed to ensure the safety of those working in the laboratory.

11. Retroviruses are more stable at 32°C than 37°C. Although incubation at 32°C during production and infection is not necessary, it will improve titers.

12. The volume of virus will depend on several factors including the titer of virus, the susceptibility of the strain to infection, and so on. A good starting point is to infect the host cells using a 0.5X dilution of the retroviral media supernatant collected in **Subheading 3.7.**, **step 10**.

13. Reverse transcription and integration of viral genomes generally take place within the first 24–36 h. Although cells can be assayed for the phenotype of interest at this time, selection of infected cells with puromycin greatly improves the penetrance of most phenotypes. For each host cell line, the minimum puromycin concentration required to kill uninfected cells should be determined in titration experiments before infection. One mg/mL final concentration is a good starting point for titration. It should be noted that puromycin typically kills most cells after only 2-d exposure.

Acknowledgments

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